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1 **Copulation duration, but not paternity share, potentially mediates**
2 **inbreeding avoidance in *Drosophila montana***

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13

14 **Abstract**

15 Studying the incidence of inbreeding avoidance is important for understanding the evolution of
16 mating systems, especially in the context of mate choice for genetic compatibility. We
17 investigated whether inbreeding avoidance mechanisms have evolved in the malt fly, *Drosophila*
18 *montana*, by measuring mating latency (a measure of male attractiveness), copulation duration,
19 days to remating, offspring production and the proportion of offspring sired by the first (P₁) and

20 second (P_2) male to mate in full-sibling and unrelated pairs. SNP markers were used for paternity
21 analysis and for calculating pairwise relatedness values (genotype sharing) between mating pairs.
22 We found 18 % inbreeding depression in egg-to-adult viability, suggesting that mating with close
23 relatives is costly. Copulation duration was shorter between previously mated females and their
24 brothers than with unrelated males. Based on an earlier study, shorter copulation is likely to
25 decrease the number of inbred progeny by decreasing female remating time. However, shorter
26 copulations did not lead to lower paternity (P_2) of full-sibling males. Progeny production of
27 double-mated females was lower when the 2nd male was a full-sibling as compared to an
28 unrelated male, but we could not distinguish between inbreeding depression and lower female
29 reproductive effort after mating with a relative. Relatedness estimates based on 34 SNPs did not
30 detect any quantitative effect of relatedness variation on copulation duration and progeny
31 production. We suggest that inbreeding depression has been strong enough to select for
32 inbreeding avoidance mechanisms in our Finnish *D. montana* population.

33 **Keywords:**

34 SNP genotyping, inbreeding depression, Bayesian statistics, beta-binomial distribution

35

36 **Introduction**

37 Even though mating with a close relative often leads to a decrease in fitness known as inbreeding
38 depression (Charlesworth and Willis 2009), inbreeding avoidance does not always occur. One
39 potential explanation for this is that kin selected benefits accrue when you help your relatives to
40 mate (Parker 1979; Kokko and Ots 2006; Puurtinen 2011). The optimal level of inbreeding that
41 maximizes inclusive fitness depends on the strength of inbreeding depression (Puurtinen 2011)
42 and on the costs of inbreeding avoidance *versus* the benefits of mating with kin (Kokko and Ots
43 2006).

44

45 Strong inbreeding depression should increase the likelihood of the evolution of inbreeding
46 avoidance. For example, sand lizards, *Lacerta agilis* (Olsson et al. 1996a; Olsson et al. 1996b),
47 cockroaches, *Blattella germanica* (Lihoreau et al. 2007; Lihoreau et al. 2008), the least killifish,
48 *Heterandria formosa* (Ala-Honkola et al. 2009; Ala-Honkola et al. 2010) and *Silene latifolia*
49 plants (Teixeira et al. 2009) all suffer from strong inbreeding depression and avoid inbreeding. In
50 these species, the probability of mating with a relative is high, which selects for the evolution of
51 inbreeding avoidance mechanisms. Likewise, in line with current theory, inbreeding preference
52 has been found in a cichlid fish (*Pelvicachromis taeniatus*) that does not suffer from inbreeding
53 depression (Thunken et al. 2007), but also in species or populations that do suffer from
54 inbreeding depression, such as the cestode *Schistocephalus solidus* (Schjørring and Jäger 2007)
55 and an Australian population of the fruit fly *Drosophila melanogaster* (Robinson et al. 2009;
56 Robinson et al. 2012b). Selection for an optimal level of inbreeding probably occurs more
57 commonly than we currently appreciate.

58

59 The mechanisms of inbreeding avoidance or preference vary between species and can occur
60 before, during or after copulation (Pusey and Wolf 1996; Tregenza and Wedell 2000). For
61 example, female sticklebacks, *Gasterosteus aculeatus*, and female cockroaches, *Blattella*
62 *germanica*, show a pre-copulatory preference toward unrelated mates (Frommen and Bakker
63 2006; Lihoreau et al. 2007). In pea aphids, *Acyrtosiphon pisum* and *D. subobscura* flies
64 inbreeding avoidance is manifested during copulation (Huang and Caillaud 2012; Lizé et al.
65 2014) and in the adzuki bean beetle, *Callosobruchus chinensis*, inbreeding avoidance is mediated
66 through female remating behaviour (Harano and Katsuki 2012). Examples of post-copulatory
67 inbreeding avoidance are differential sperm storage in the red jungle fowl, *Gallus gallus* (Pizzari
68 et al. 2004), in the crickets *Gryllus bimaculatus* (Bretman et al. 2009) and *Teleogryllus oceanicus*
69 (Tuni et al. 2013) and the effects of ovarian fluid on sperm velocity based on male relatedness in
70 guppies, *Poecilia reticulata* (Gasparini and Pilastro 2011).

71

72 Studying the incidence of inbreeding avoidance is therefore important for our understanding of
73 the evolution of mating systems especially in the context of mate choice for genetic
74 compatibility. In this study, our aim was to investigate whether inbreeding avoidance
75 mechanisms have evolved in the boreal malt fly, *Drosophila montana*. The mating system of *D.*
76 *montana* is dominated by male courtship song and associated female preferences. The song,
77 produced by wing vibration, is obligatory for successful mating (Liimatainen et al. 1992). Female
78 *D. montana* from Finland have been shown to prefer males that produce a courtship song with a
79 high carrier frequency (short sound pulses with many sound cycles each (Ritchie et al. 1998))

80 both in the field (Aspi and Hoikkala 1995) and in the laboratory (Ritchie et al. 1998). Male song
81 presumably indicates male quality, as the frequency of a male's song correlates with the egg-to-
82 adult viability of his progeny (Hoikkala et al. 1998) and is condition dependent (Hoikkala et al.
83 2008). Cuticular hydrocarbons can also influence mate choice in *D. montana* (Veltsos et al. 2012)
84 and have been implicated as a potential cue for inbreeding avoidance in insects (Thomas and
85 Simmons 2011). However, what makes *D. montana* an extremely interesting species to study
86 inbreeding avoidance is that courtship song frequency shows inbreeding depression (Aspi 2000)
87 and therefore females that mate with close relatives would produce unattractive male offspring.
88 Because females are polyandrous in nature (Aspi and Lankinen 1992) and in the laboratory (Aspi
89 1992), we anticipated that possible inbreeding avoidance mechanisms may be manifested before,
90 during, or after copulation. In many *Drosophila* species copulation duration seems to be under
91 male control (Kaul and Parsons 1965; Parsons and Kaul 1966; Macbean and Parsons 1967;
92 Jagadeeshan and Singh 2006) but in contrast to this, *D. montana* females make a substantial
93 contribution towards shortening the duration of copulation by kicking the males at the end of the
94 copulation (Mazzi et al. 2009). When female resistance attempts were suppressed, males
95 persisted in copula far longer than they managed to in unmanipulated matings (Mazzi et al.
96 2009).

97 We manipulated relatedness in potential mating partners derived from a recent wild collection
98 and measured possible inbreeding avoidance in mating latency (a typical measure of male
99 attractiveness in *Drosophila*, see Ala-Honkola et al. (2013), Barth et al. (1997) and Ritchie et al.
100 (1999)), copulation duration, days to remating, offspring production and the proportion of

101 offspring sired by the first (P_1) and second (P_2) male to mate. In addition, we quantified the level
102 of inbreeding depression in egg-to-adult viability in our study population.

103 **Methods**

104 **Fly population**

105 Experimental flies were descendants of flies that were collected from riparian habitats in Oulanka
106 (Finland) in the summer of 2008. Once in the laboratory, isofemale lines were established for
107 each wild-caught female in half-pint bottles on Lakovaara malt medium (Lakovaara 1969) until a
108 large number of F3s were available. From each isofemale line ($N = 20$), 20 F3 males and 20 F3
109 females (800 total flies) were then combined in a $25 \times 25 \times 60$ cm wooden population cage with a
110 Plexiglas top and eight available food bottles for feeding, oviposition and larval rearing and bred
111 in overlapping generations in constant light and temperature (18°C). Constant light is necessary
112 to prevent flies from undergoing reproductive diapause (Lumme 1978).

113 Families for this experiment were created by placing pairs of randomly selected virgin females
114 and virgin males into plastic fly vials ($d = 20$ mm) containing malt medium and a few grains of
115 live yeast. Malt medium consists of 8 % malt extract, 6 % corn meal, 2 % yeast, 1 % agar and 0.5
116 % propionic acid added to water. In addition, 1.4 % of 10 % methyl-4-hydroxybenzoate solution
117 (dissolved in 96 % ethanol) was added to the medium. Each pair was transferred to a new vial
118 every four days to avoid larval crowding. Virgin females and males for the experiment were
119 collected under CO_2 anesthetization and kept in single sex vials until they matured (about 3
120 weeks).

121 **Experimental design**

122 P_1 and P_2 were quantified in two separate experiments as in Ala-Honkola et al. (2011). The P_1
123 experiment was designed to measure the effect of relatedness on sperm defense, or P_1 , but from
124 this experiment, we also gained data allowing us to analyse the effect of first male relatedness on
125 mating latency, copulation duration in the first and the second mating, female egg production
126 after single mating, egg-to-adult viability and female remating interval (females had a possibility
127 to remate either two or three days after the first mating). The P_2 experiment was designed to
128 measure the effect of relatedness on sperm offense, or P_2 , and it also allowed us to investigate the
129 effect of second male relatedness on female remating interval, copulation duration of previously
130 mated females and the number of progeny produced after remating (about 70 % of the offspring
131 are sired by the second male to mate (Aspi and Lankinen 1992)).

132 In each experiment, a focal pair of males (a ‘test’ male and a ‘standard competitor’ male) was
133 mated to two females: to the test male’s full-sibling and to an unrelated female (Table 1). In the
134 P_1 experiment, the test male mates first as we are interested if his relatedness to the two females
135 affects the traits of interest, and the competitor male mates second. In the P_2 experiment, the
136 standard competitor male mates first and the test male mates second. The use of a standard
137 competitor male removes the influence of male \times male interactions on P_1 and P_2 , thus enhancing
138 our ability to detect any male \times female interactions (Bjork et al. 2007) through pairwise
139 comparisons of paternity success and other traits of interest in related and unrelated females.
140 Similarly, to remove variation in P_1 and P_2 attributable to possible virgin male effects (Bjork et
141 al. 2007), all test and competitor males were initially mated to non-experimental virgin females
142 one day before their first experimental mating.

143 Both experiments were balanced for mating order (i.e., whether the test male's first mating was
144 with a sibling or a non-sibling female) by randomly assigning half of the males from each family
145 to each mating order. In both the P1 and the P2 experiment, we used four randomly selected
146 males and females from each of 25 different families and assigned the females randomly to
147 "sibling" and "unrelated" mating roles. Thus the initial sample size was $N = 100$ for both the P1
148 and P2 experiments. All males were 23-29 d old and all females 25-29 d old (typical reproductive
149 age for slowly maturing *D. montana*) at their own first experimental mating day.

150 On the first experimental mating day, the first females and the first males were individually
151 paired in vials to mate (see Table 1). For all pairs, we recorded the time when flies were paired in
152 a vial, the start of copulation, and the end of copulation. Following copulation, each male was
153 moved to an individual vial until his second mating (see below). Females were provided the
154 opportunity to mate with the second male 2 and 3 days after their first mating by aspirating the
155 second male into the female's vial in the morning and providing a 3 h opportunity to interact.
156 Again, after copulation, the male was moved into his own vial until his second test mating.

157 Four days after their first test copulation, the first males were mated to their second test female
158 (see Table 1). After copulating, the male was removed from the vial and stored in 70 % ethanol.
159 Second females were provided a 3 h opportunity to mate with the second male on days 2 and 3
160 after their first mating. After copulation, second males were removed from the second female's
161 vial and stored in 70 % ethanol. Although the remating interval for second males was variable
162 due to variation in remating latency among females, this experimental schedule enabled at least 3
163 days remating interval for all second males.

164 We quantified inbreeding depression in egg-to-adult viability in the P1 experiment for all eggs
165 laid during the two-day interval between the first mating and the initial exposure to the second
166 male. After remating, females were transferred to fresh vials daily for three days. P_1 and P_2 were
167 estimated from the first 30 offspring produced. If more than 30 offspring were produced from day
168 1 vials, we randomly chose 30 of these for genotyping. In case less than 30 offspring were
169 produced from the day 1 vial, we added randomly chosen flies from day 2 vials, and if needed,
170 from the day 3 vial, to get 30 offspring in total.

171 We only analysed data when mating was successful with both the full-sibling and unrelated
172 female. In the P1 experiment, final $N = 64$ for mating latency, copulation duration with the first
173 male and number of eggs laid after the first mating. Females producing 0 eggs (12 cases) were
174 included in the analysis of number of eggs laid after the first mating in order not to exclude
175 females that might choose not to lay eggs after mating with a brother (excluding these cases does
176 not change the conclusions). However, for egg-to-adult viability analysis, we only included males
177 both mates of which produced some offspring prior to remating (*i.e.*, viability values of 0 were
178 excluded) to exclude unsuccessful sperm transfers leading to final $N = 36$. For copulation
179 duration with the 2nd male, $N = 57$.

180 In the P2 experiment, we analysed copulation duration only when both females mated to the test
181 male ($N = 49$). Similarly, data were analysed only for progeny production if both females
182 produced progeny (to exclude unsuccessful sperm transfers ($N = 45$)).

183 For the paternity analysis, we included test males when both mates produced offspring prior to
184 remating (ensuring that the first mating was successful) and at least 30 offspring after remating.

185 The first 30 offspring and the potential parents of these families were genotyped (i.e., potential
186 offspring of 20 test males from the P1 experiment and 18 test males from the P2 experiment).
187 From the P₁ analysis, males were excluded if their P₁ was 1, because that is symptomatic of an
188 unsuccessful second copulation (two such cases, N final = 18). From the analysis P₂, males were
189 excluded if P₂ = 0 (symptomatic of an unsuccessful second copulation, 2 cases, N final = 16).

190 **SNP markers**

191 We used a subset of the genetic markers described in Veltsos *et al.* (in prep). Information on the
192 markers is provided in Electronic supplementary material (ESM) Files 1 & 2.

193 DNA was extracted from whole flies that had been stored in 70 % ethanol using standard
194 methods by Kbiosciences (Herts, UK). SNP genotyping was performed with PCR-based KASPTM
195 genotyping assay by Kbiosciences (Herts, UK).

196 **Paternity tests and relatedness analysis**

197 The SNP markers were analysed in Cervus v3.0.3 (Kalinowski et al. 2007). Fewer markers were
198 typed in the offspring compared to the parents (17 compared to 49). For the offspring, after allele
199 frequency analysis, only the markers with estimated null allele frequencies smaller than 0.10
200 were retained (14 markers). For the parents, we used only the markers that were in Hardy
201 Weinberg (HW) equilibrium or had estimated null allele frequencies below 0.05 (34 markers).

202 For parentage analysis, a simulation was run in Cervus with simulated offspring set to 10,000,
203 proportion sampled 1, minimum number of typed loci 7 and the remaining parameters at the
204 default settings. Paternity analysis was then performed to identify the most likely father of each
205 offspring.

206 Relatedness between the parents was analysed in the Demerelate v0.8 (Kraemer& Gerlach 2013)
207 package in R v3.03 (R DevelopmentCore Team 2014). We used the M_{xy} (genotype sharing)
208 estimate of relatedness, as described in (Blouin et al. 1996). Other parameters of the Demerelate
209 command are not relevant to our study because they concern analysis of multiple populations.
210 The full command was `Demerelate(parentData, value="Mxy", file.output=FALSE,`
211 `object=TRUE, pairs=10, iteration = 100, Fis=FALSE, p.correct=TRUE)`.

212 **Statistical analyses**

213 We analysed the effect of relatedness on mating latency (\log_{10} -transformed due to
214 heteroscedasticity in residuals), copulation duration and progeny production with linear mixed
215 models, using the library nlme (Pinheiro *et al.* 2013) in R 3.0.2 (R Development Core Team
216 2013). The number of eggs produced was analysed with a generalized linear mixed model
217 (GLMM) with negative binomial distribution using the glmmADMB package (Fournier et al.
218 2012), see also (Skaug *et al.* 2013).

219 Male family was fitted as a random factor in the models, which means that we imposed
220 correlation structure in the family level, because there was not enough replication per family for a
221 nested random factor (male nested within male family). This is because the number of males per
222 family varies from 1-4, but was most often 1. As the design is paired, we have at least two
223 observations per family (those from the same male).

224 Relatedness (sibling or nonsibling), mating order of the male (“sibling female first, nonsibling
225 second” or “nonsibling female first, sibling second”) and female order (first or second mate of a

226 given male) were modelled as fixed factors. We performed model validations by examining the
227 homogeneity and independence of errors.
228
229 Because of overdispersion (the overdispersion parameter varied from 3.2 to 4.7) in binomially
230 distributed data (egg-to-adult viability, P_1 and P_2), we could not use GLMM with a binomial
231 distribution and a logit link function with sample sizes as weights (a method recommended by
232 Engqvist (2013) for analysing paternity data). We solved the overdispersion problem by using
233 beta-binomial distribution that allows for more dispersion in binomial data. Parameters were
234 estimated in a Bayesian framework using JAGS (Plummer 2003) and the R2jags package (Su &
235 Yajima 2014) as suggested by Zuur et al. (2013). As above, male family was fitted as a random
236 factor, relatedness, mating order of the male and female order were entered as fixed factors. We
237 used a logit link function with sample sizes as weights. Three chains, each with 50000 iterations
238 were used in the MCMC process with a burn-in of 4000 iterations and a thinning rate of 45.
239 Therefore, 3336 iterations were used for each posterior distribution. We used diffuse normal
240 priors for all regression parameters and half-Cauchy(25) priors for variance parameters (see Zuur
241 et al. (2013) for explanation of half-Cauchy(25) distribution). Mixing of chains was good in all
242 analyses. We assessed the goodness of fit of the models using the Bayesian p-values and
243 performed model validations by examining the homogeneity and independence of errors.
244 The standardized coefficient of inbreeding depression, δ , for egg-to-adult viability was counted
245 by dividing the difference in mean trait values between outbred and inbred individuals by the
246 mean trait value of outbred individuals (Lande and Schemske 1985).

247 **Results**

248 Our population was genetically variable and the relatedness treatments differed from each other
249 genetically. Genotype sharing, as expected, showed greater relatedness of full-sibling females to
250 the test males than unrelated females; paired $t = -9.97$, $p < 0.001$, $df = 37$. Relatedness of the test
251 male to full-siblings was $0.84 (0.06)$, mean (\pm SD), and to unrelated females $0.73 (0.06)$, Fig. S1
252 in ESM. The relatedness of standard competitor males to females was similar to that of unrelated
253 males to females, as expected (data not shown).

254 We did not find inbreeding avoidance in the behavior of virgin females or in male behavior
255 towards virgins. Specifically, in the P1 experiment, mating latency, first and second copulation
256 duration and egg production after the first mating were not affected by whether the first mate of a
257 female was a full-sibling or an unrelated male (Table 2; all full models are presented in Table S1
258 in ESM).

259 There was 18 % inbreeding depression in egg-to-adult survival of progeny produced after the first
260 mating as measured in the P1 experiment (shown by the 95 % credible interval that does not
261 include 0; Table 2; Fig. 1; Table S2 in ESM). However, we decided not to correct our P_1 or P_2
262 values for the lower survival of the inbred offspring of full-sibling matings because there was no
263 postcopulatory inbreeding avoidance in either P_1 or P_2 in the uncorrected data (see Table 2; Table
264 S2 and Fig. S2 & S3 in ESM). Correction would increase the P_1 and P_2 values in full-sibling
265 treatments and does not change the conclusions of no inbreeding avoidance. Secondly, applying
266 correction factors from separate single mating to P_1 or P_2 values may not be accurate (Droge-
267 Young et al. 2012).

268 The P2 experiment suggests a behavioral mechanism of inbreeding avoidance: on average,
269 copulations of previously mated females with full-sibling males were 30 s (10 %) shorter than
270 those with unrelated males (Table 2; Fig. 2; Table S3 in ESM). Also, these females produced
271 fewer offspring after remating with a full-sibling male (Table 2; Fig. 3; Table S3 in ESM).
272 However, there was no correlation between female's 2nd copulation duration and offspring
273 production after remating ($t = 0.12$, $df = 96$, $p = 0.91$), suggesting that shorter copulations do not
274 directly result in reduced offspring production.

275 Flies did not vary their remating interval based on the relatedness of their 1st mate in the P1 ($\chi^2 =$
276 0.87 , $df = 1$, $p = 0.35$) or their 2nd mate in the P2 experiment ($\chi^2 = 0$, $df = 1$, $p = 1$), see Table 3.
277 However, this was a crude estimate of remating interval as we only tested remating over two days
278 (48 and 72 hours after the first mating). The overall remating propensity of females did not differ
279 between treatments (data not shown).

280 **Comparing relatedness index (M_{xy}) with known treatment effects**

281 We wanted to examine whether our pairwise relatedness estimates (genotype sharing) based on
282 34 SNPs would give additional information as our two level factor; full-sibling or unrelated. As
283 we found that copulations between previously mated females and unrelated males were longer
284 and females produced more offspring after mating with unrelated males, we expected to see a
285 negative correlation between male-female relatedness and copulation duration and between male-
286 female relatedness and offspring production. However, neither of these correlations was
287 significant (for copulation duration; $t = -0.72$, $df = 34$, $p = 0.47$; Fig. S4 in ESM and for
288 offspring production; $t = -1.26$, $df = 34$, $p = 0.22$; Fig. S5 in ESM).

289

290 **Discussion**

291 We found that our Finnish study population of *D. montana* suffered from 18 % inbreeding
292 depression in egg-to-adult viability after one generation of full-sibling mating. Male courtship
293 song frequency, which is sexually selected, also shows inbreeding depression (Aspi 2000)
294 suggesting that inbreeding is indeed costly in this species. Inbreeding depression seems to have
295 been strong enough to select for inbreeding avoidance mechanisms as copulations between
296 previously mated females and their full-brothers were about 10 % shorter than those with
297 unrelated males. In addition, females produced fewer offspring when their second mate was a
298 full-brother as compared to an unrelated male. However, we did not see any post-copulatory
299 inbreeding avoidance in terms of paternity bias towards unrelated males in the P1 or P2
300 experiments. Also, virgin females did not behave any differently toward their brothers than
301 toward unrelated males as mating latency, female egg production or remating interval did not
302 differ between those two treatments. Similarly, the relatedness of the first mate did not affect
303 copulation duration.

304

305 Copulations between previously mated females and their full-brothers were about 10 % (30 s)
306 shorter than those with unrelated males. Longer copulations extend female refractoriness to
307 remating, which benefits the male (Mazzi et al. 2009). Shorter copulations with full-brothers are
308 therefore likely to reduce remating interval of the mated females and reduce the production of
309 inbred offspring, since paternity share was not affected by the relatedness of a male. We cannot

310 be certain about which sex is avoiding inbreeding by shortening copulations as both males and
311 females influence copulation duration in *D. montana* (Mazzi et al. 2009). Females would benefit
312 from shorter copulations by producing fewer inbred offspring whereas males would benefit from
313 saving ejaculatory resources for future copulations.

314
315 Remating interval has been shown to shorten for females mated with related males in seed
316 beetles, *Callosopbruchus chinensis* (Harano and Katsuki 2012) and green-veined white
317 butterflies, *Pieris napi* (Välimäki et al. 2011) suggesting that it is a common way to avoid
318 inbreeding. In our study, virgin females did not directly avoid inbreeding, which may be a way to
319 guarantee reproduction in case no better mate is encountered. It is also possible that females
320 require experience in order to judge whether or not the male is related (see Tan et al. 2012 for the
321 effect of mating history on inbreeding likelihood in *D. melanogaster*).

322
323 There are three plausible explanations for the decreased offspring production in the P2
324 experiment in a situation when the second mate is a full-brother compared to an unrelated male. It
325 could indicate lower female reproductive effort after mating with an incompatible male
326 (behavioral inbreeding avoidance). Second, it could be a manifestation of inbreeding depression
327 in egg-to-adult viability. If fewer adults eclose from eggs fertilized by a brother compared to
328 those fertilized by unrelated males, as suggested by inbreeding depression in egg-to-adult
329 survival after single mating, then inbreeding depression could explain the lower number of
330 offspring. We did not try to correct for inbreeding depression in offspring production because
331 viabilities in single vs double matings can be very different even for a single male (Droge-Young
332 et al. 2012). Unfortunately, our data does not allow us to distinguish between these two

333 possibilities because we did not count the number of eggs laid after remating. The third
334 possibility is that the shorter copulations between previously mated females and their full-
335 brothers would lead to decreased offspring production if less sperm or seminal fluid proteins are
336 transferred in shorter copulations. This seems to be the least likely explanation, however, because
337 there was no correlation between offspring production after remating and 2nd copulation duration
338 in the P2 experiment. Similarly, Mazzi et al. (2009) did not find a correlation between copulation
339 duration and offspring production after single matings.

340

341 Relatedness estimates based on genotype sharing (M_{xy}) confirmed that brothers were more
342 related to their sisters than to unrelated females. However, M_{xy} estimates did not detect a further
343 effect of relatedness on copulation duration and progeny production (there was no correlation
344 between these traits and relatedness values) that we found between the full-sibling and unrelated
345 mate treatments. That may be because relatedness estimators have very large variances and some
346 simulations suggest that over 100 SNPs are needed for even moderate confidence around
347 pairwise estimates (Blouin 2003; Glaubitz et al. 2003). We decided to use M_{xy} because it does not
348 require population allele frequencies to be known. Relatedness values (r) can become biased if
349 the reference sample is the same one that is used for estimating relatedness and the proportion of
350 closely related individuals is high (Wang 2014), both of which are true in our case.

351

352 Previous inbreeding avoidance studies performed with *Drosophila* flies have mainly used *D.*
353 *melanogaster* as a model and the results vary extensively. Post-copulatory inbreeding avoidance

354 was found by Mack et al. (2002) but not by Ala-Honkola et al. (2011). Pre-copulatory preference
355 for related males has been found in two studies (Loyau et al. 2012; Robinson et al. 2012b) but no
356 effect of relatedness for pre-copulatory behavior was found in another two studies (Ala-Honkola
357 et al. 2011; Tan et al. 2012). Lizé et al. (2014) found that males copulated longer with unrelated
358 females, but only when their gut microbiota was removed. Lizé et al. (2014) also studied
359 monandrous *D. subobscura* and polyandrous *D. bifasciata* and showed that *D. subobscura* males
360 copulated longer with unrelated females whereas *D. bifasciata* males did not avoid inbreeding.
361 They suggested that monandrous species would be more likely to exhibit kin recognition than
362 polyandrous species because of higher inbreeding costs (Lizé et al. 2014). However, our study
363 shows that polyandrous *D. montana* also exhibits kin recognition.

364
365 Tan et al. (2012) found a similar magnitude of inbreeding depression in egg-to-adult viability (18
366 %) as we did in the current study, but found no inbreeding avoidance suggesting that factors
367 other than the amount of inbreeding depression are also important for the evolution of inbreeding
368 avoidance. The probability of mating with relatives is a potential factor affecting the evolution of
369 inbreeding avoidance that differs between *D. montana* and *D. melanogaster* as population sizes
370 of these species are likely to be radically different. Intense collection efforts for *D. montana* often
371 produce only dozens of individuals at most (Anneli Hoikkala, personal communication) whereas
372 *D. melanogaster* populations often consist of thousands of flies (Kusakabe et al. 2000; Shapiro et
373 al. 2007) suggesting that encounters between close relatives are more likely in *D. montana* and
374 recognizing relatives can be beneficial. Intriguingly, some recent studies of wild *D. melanogaster*
375 have suggested that there is assortative mating between relatives (Robinson et al. 2012a) and this
376 may reflect active mate choice of relatives (Robinson et al. 2012b).

377
378 To conclude, our study population of *D. montana* suffered from 18 % inbreeding depression in
379 egg-to-adult viability after one generation of full-sibling mating. Inbreeding depression seems to
380 have been strong enough to select for inbreeding avoidance mechanisms as copulations between
381 previously mated females and their full-brothers were about half a minute (10%) shorter than
382 those with unrelated males. The shorter copulations are likely to decrease female remating time
383 and lead to fewer inbred progeny.

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388 **Appendices**

389 Electronic Supplementary Material (ESM).

390 **Ethical standards**

391 The experiments performed comply with the current laws of UK, in which they were performed.

392 **Conflict of interest**

393 The authors have no conflicts of interest to declare.

394

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554 **Figure Legends**

555

556 **Fig. 1** Boxplots of egg-to-adult viabilities after a single mating with a full-sibling or an unrelated
557 male in the P1 experiment.

558 **Fig. 2** Boxplots of copulation durations with unrelated females and full-siblings in these females'
559 second mating in the P2 experiment.

560 **Fig. 3** Boxplots of progeny production of females that remated with unrelated or full-sibling
561 males in the P2 experiment (uncorrected for low viability of inbred offspring).

562

563 **Tables**

564 Table 1. Experimental design. A focal pair of males (a ‘test’ male and a ‘standard competitor’ male) was mated to the test male’s
 565 full-sibling and to an unrelated female. Both females were unrelated to the standard competitor male. This design allows pairwise
 566 comparison of the effect of relatedness on the measured traits within a male. For the P1 experiment, the test male mated to both
 567 females first, and for the P2 experiment, the test male mated to both females second. Both experiments were balanced for mating
 568 order (i.e., whether the test male’s first mating was with a sibling or a non-sibling female) by randomly assigning half of the
 569 males to each mating order. F_1 = female 1 in a quartet of flies, F_2 = female 2 in a quartet of flies. In half of the cases F_1 was the
 570 test male’s full-sibling while F_2 was unrelated and in half of the cases F_1 was unrelated to the test male and F_2 was his full-
 571 sibling.

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
P1 EXPERIMENT	$F_1 + M_{\text{Test}}$		$F_1 + M_{\text{Competitor}}$ 1 st remating possibility	$F_1 + M_{\text{Competitor}}$ 2 nd remating possibility	$F_2 + M_{\text{Test}}$		$F_2 + M_{\text{Competitor}}$ 1 st remating possibility	$F_2 + M_{\text{Competitor}}$ 2 nd remating possibility
P2 EXPERIMENT	$F_1 + M_{\text{Competitor}}$		$F_1 + M_{\text{Test}}$ 1 st remating possibility	$F_1 + M_{\text{Test}}$ 2 nd remating possibility	$F_2 + M_{\text{Competitor}}$		$F_2 + M_{\text{Test}}$ 1 st remating possibility	$F_2 + M_{\text{Test}}$ 2 nd remating possibility

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574 Table 2. Means, standard deviations, and the significance of the factor relatedness in full linear mixed effects models or GLMMs
 575 comparing P₁, P₂, and other measured reproductive behaviors in the full-sibling and the unrelated pair treatments. Note that p-
 576 values are not available for Bayesian analyses but 95 % credible intervals are presented. See full models in ESM Tables S1-S3.

Trait	Mean (SD)	Mean (SD)	N	Test statistic	p
	Sibling	Unrelated			
P1 EXPERIMENT					
Mating latency (min)	38.7 (46.1)	38.4 (46.1)	64	t = 14.3	0.99
Copulation duration with the first male (test male) (s)	255 (57.3)	269 (60.5)	64	t = -1.36	0.18
Number of eggs laid before remating	33.3 (22.2)	30.0 (22.4)	64	z = 0.65	0.52
Copulation duration with the second male* (s)	260 (67.9)	277 (68.3)	57	t = -1.27	0.21
Egg-to-adult viability after single mating	0.50 (0.23)	0.61 (0.23)	36	95 % credible interval: -0.86 to -0.07	Significant effect
P ₁	0.31 (0.26)	0.34 (0.24)	18	95 % credible interval:	Effect NS

				-0.68 to 0.51	
P2 EXPERIMENT					
P ₂	0.62 (0.23)	0.68 (0.20)	16	95 % credible interval: -0.83 to 0.41	Effect NS
Copulation duration with the second male (test male) (s)	260 (69.3)	289 (66.4)	49	t = -2.21	0.030
Offspring produced during 3 days after remating (uncorrected for low viability of inbred offspring)	51.4 (25.4)	65.7 (25.6)	45	t = -2.74	0.0079

577 *refers to whether the first male was a sibling or an unrelated male.

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587 Table 3. Number of females that remated in the 1st or 2nd remating opportunity (48 vs 72 hours after the first mating) in each
588 experiment and relatedness treatment.

		Number remating	
		1 st remating day	2 nd remating day
P1 EXPERIMENT	1 st male full-sib	43	14
	1 st male unrelated	48	9
P2 EXPERIMENT	2 nd male full-sib	40	9
	2 nd male unrelated	39	10

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