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

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Abstract

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

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

Keywords:

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

Introduction

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

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

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))

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

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

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

Methods

Fly population

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

Families for this experiment were created by placing pairs of randomly selected virgin females and virgin males into plastic fly vials ($d = 20$ mm) containing malt medium and a few grains of live yeast. Malt medium consists of 8 % malt extract, 6 % corn meal, 2 % yeast, 1 % agar and 0.5 % propionic acid added to water. In addition, 1.4 % of 10 % methyl-4-hydroxybenzoate solution (dissolved in 96 % ethanol) was added to the medium. Each pair was transferred to a new vial every four days to avoid larval crowding. Virgin females and males for the experiment were collected under CO₂ anesthetization and kept in single sex vials until they matured (about 3 weeks).

Experimental design

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

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

Both experiments were balanced for mating 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 males from each family to each mating order. In both the P1 and the P2 experiment, we used four randomly selected males and females from each of 25 different families and assigned the females randomly to "sibling" and "unrelated" mating roles. Thus the initial sample size was $N = 100$ for both the P1 and P2 experiments. All males were 23-29 d old and all females 25-29 d old (typical reproductive age for slowly maturing *D. montana*) at their own first experimental mating day.

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

Four days after their first test copulation, the first males were mated to their second test female (see Table 1). After copulating, the male was removed from the vial and stored in 70 % ethanol. Second females were provided a 3 h opportunity to mate with the second male on days 2 and 3 after their first mating. After copulation, second males were removed from the second female's vial and stored in 70 % ethanol. Although the remating interval for second males was variable due to variation in remating latency among females, this experimental schedule enabled at least 3 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. P₁ and P₂ 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.

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

SNP markers

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

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

Paternity tests and relatedness analysis

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

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

given male) were modelled as fixed factors. We performed model validations by examining the homogeneity and independence of errors.

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

Results

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

572

573

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

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

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586

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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