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1 **Female mating preferences for outbred versus inbred males are conditional**
2 **upon the female's own inbreeding status**

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13

14 **ABSTRACT**

15 Inbreeding occurs when relatives mate with each other, and it often has detrimental effects for

16 the fitness of the resulting offspring. It is an important issue in ecology and evolutionary biology

17 with profound implications for genetic variation and the evolution of mating systems and

18 reproductive strategies. Inbreeding may shape mate choice through the avoidance of outbred,

19 related individuals, in order to prevent inbreeding, or through the avoidance of inbred, unrelated

20 individuals that have been produced through inbreeding. Although the former has been studied

21 extensively, little is known about mating preferences based on the inbreeding status of potential

22 partners. It is also unclear whether these mating preferences are influenced by the inbreeding

23 status of the choosing sex. Here, we examine female mating preferences for outbred and inbred

24 males using dichotomous choice tests in the burying beetle *Nicrophorus vespilloides*. We show
25 that these mating preferences are conditional upon the female's own inbreeding status: inbred
26 females preferentially mate with outbred males, whereas outbred females do not show such a
27 preference. Our findings suggest that inbred males suffer reduced mating success only when
28 interacting with inbred females. In species where this is the case, the fitness costs of inbreeding
29 with respect to male mating success will therefore depend on the frequency of inbred females
30 relative to outbred females, which is determined by the rate of inbreeding in the population.

31

32 Keywords: burying beetle, direct benefits, inbreeding, mate choice, mating success, *Nicrophorus*
33 *vespilloides*, sexual selection

34 **INTRODUCTION**

35 Inbreeding refers to the mating between close relatives and is often associated with a reduction in
36 the fitness of any resulting offspring, known as inbreeding depression (Charlesworth &
37 Charlesworth, 1987). These fitness costs are due to a general loss of heterozygosity, which
38 increases the likelihood that recessive, deleterious alleles are expressed (Charlesworth &
39 Charlesworth, 1987). Given its detrimental effects on the fitness of inbred offspring, inbreeding
40 may influence mate choice, which is the outcome of interactions between males and females,
41 with females usually being the choosing sex and males the competing sex (Andersson, 1994).

42 Inbreeding can affect mating patterns at two distinct levels. Firstly, the costs of inbreeding
43 may lead to mating preferences for unrelated over related individuals. Active mate choice is a
44 key mechanism for inbreeding avoidance, whereby individuals avoid mating with relatives to
45 reduce the risk of producing inbred offspring (Frommen & Bakker, 2006; Gerlach & Lysiak,
46 2006; Hansson et al., 2007). Secondly, females might avoid mating with inbred, unrelated
47 partners if outbred, unrelated partners are of higher quality (Ilmonen et al., 2009). Hence,
48 inbreeding may shape mate choice through the avoidance of related individuals in order to
49 prevent inbreeding and/or through the avoidance of low-quality inbred individuals produced as a
50 consequence of inbreeding.

51 Inbreeding avoidance by active mate choice has been studied extensively across a wide range
52 of taxa (Pusey & Wolf, 1996; Tregenza & Wedell, 2000; Szulkin et al., 2013). This work has
53 focused on when and why animals may avoid, tolerate, or in some cases prefer to mate with their
54 relatives (Kokko & Ots, 2006; Szulkin et al., 2013). Relatively little is known about whether and
55 when inbred individuals might be less preferred as potential mates, although a growing number
56 of empirical studies in mammals, birds, fishes, and insects have shown that outbred partners are

57 typically preferred over inbred ones (Ilmonen et al., 2009; Bolund et al., 2010; Zajitschek &
58 Brooks, 2010; Okada et al., 2011; Pölkki et al., 2012; McKee et al., 2014; Ala-Honkola et al.,
59 2015; but also see Drayton et al., 2010; Michalczyk et al., 2010). Theoretical work suggests that
60 these preferences for outbred males are unlikely to be driven by indirect (genetic) benefits,
61 because homozygosity is not heritable (Reinhold, 2002; Lehmann et al., 2007; but see Neff and
62 Pitcher, 2008; Nietlisbach et al., 2015). A more likely explanation is that inbreeding reduces
63 overall male quality and condition such that females gain fewer direct benefits from mating with
64 an inbred male (Fox et al., 2012). Direct benefits that may be affected by inbreeding include
65 sperm number and quality, nuptial gift size, and parental care ability (Fox et al., 2012).

66 Traditionally, studies on female mate choice have focused only on the inbreeding status of
67 males, giving outbred females a choice between outbred and inbred males. Nevertheless, the
68 females' own inbreeding status might also influence their mating preferences. If low-quality
69 females cannot afford the costs of being choosy (Hunt et al., 2005; Cotton et al., 2006; Burley &
70 Foster, 2006; Ilmonen et al., 2009; McKee et al., 2014), we might expect a stronger mating bias
71 towards outbred males by outbred (high-quality) females than by inbred (low-quality) females.
72 On the other hand, if the benefits gained from being choosy are inversely related to female
73 quality, inbred females should have a stronger preference for outbred males in order to
74 compensate for their own shortcomings (Ilmonen et al., 2009). It is important to better
75 understand how inbreeding affects female choosiness, as this could have important implications
76 for sexual selection dynamics.

77 In this study, we used the burying beetle *Nicrophorus vespilloides* to test whether females
78 preferentially mate with outbred over inbred males and whether female choosiness is influenced
79 by the female's own inbreeding status. Matthey and Smiseth (2015a) found no evidence for

80 inbreeding avoidance in this species despite severe inbreeding depression in the offspring
81 (Mattey et al., 2013; Pilakouta et al., 2015a; Pilakouta & Smiseth, 2016; Pilakouta et al., 2016)
82 and heavy investment by both sexes in parental care (Smiseth & Moore, 2004; Smiseth et al.,
83 2005). Nevertheless, it is possible that females exhibit mating preferences based on the
84 inbreeding status rather than the relatedness of potential partners. To test this, we conducted
85 dichotomous choice tests during which we recorded the copulation rate of an outbred or inbred
86 female presented with two potential mates, one outbred and one inbred. We predicted that
87 females would avoid mating with inbred males, because they are low-quality mates (Mattey et
88 al., 2013). We also expected that outbred and inbred females would differ in their choosiness, but
89 we did not have an *a priori* prediction about the direction of this effect.

90

91 **METHODS**

92 *Beetle Husbandry*

93 We used virgin beetles from an outbred laboratory population maintained at the University of
94 Edinburgh. The beetles used in this study comprised of second- and third-generation beetles from
95 lines originally collected in Edinburgh, UK. They were housed individually in transparent plastic
96 containers (12 × 8 × 2 cm) filled with moist soil and kept at 22 °C and a 16:8 h light:dark cycle.

97 All non-breeding adults were fed small pieces of raw organic beef twice a week.

98

99 *Generating Outbred and Inbred Beetles*

100 In the first part of our experiment, we generated outbred and inbred males and females for use in
101 the mate choice trials. To produce outbred individuals, we paired outbred beetles ($N = 25$) that
102 had no common ancestors for at least two generations. To produce inbred individuals, we paired

103 outbred beetles ($N = 25$) that were full siblings. Each pair ($N = 50$) was placed in a transparent
104 plastic container ($17 \times 12 \times 6$ cm) filled with 1 cm of moist soil. Burying beetles use carcasses of
105 small vertebrates as a breeding resource, so we provided each of these pairs with a freshly
106 thawed mouse carcass (Livefoods Direct Ltd, Sheffield, UK). We did not disturb them until the
107 larvae started dispersing from the carcass, which occurs approximately five days after hatching.
108 At the dispersal stage, we placed five larvae from each brood into individual containers ($12 \times 8 \times$
109 2 cm) filled with moist soil. The inbred and outbred offspring eclosed as adults about 20 days
110 later, at which point they were sexed based on differences in the terminal segments of the
111 abdomen (Trumbo, 1996). We only used one female and two males from each family. We also
112 recorded the body size of all individuals by measuring their pronotum width using a digital
113 caliper with a precision of 0.01 mm (Bartlett & Ashworth, 1988).

114

115 *Dichotomous Choice Tests*

116 Adult beetles become sexually mature around 10 days after eclosion. For our mate choice trials,
117 we only used virgin beetles aged between 10 and 20 days after eclosion to minimize variation in
118 male and female age and prevent variation due to previous mating experience. Each trial
119 consisted of a single outbred or inbred female that was given a choice between an outbred and an
120 inbred male. This design simulates a situation where a female encounters multiple males on a
121 carcass in the wild (i.e., simultaneous mate choice). In half of the trials, we used an outbred
122 female ($N = 15$) and in the other half we used an inbred female ($N = 15$). The two males used in a
123 given pair were size-matched based on their pronotum width (difference < 0.10 mm) to exclude
124 differences in female mating preferences due to male size. We always used unrelated individuals
125 in each trial.

126 Mate choice trials took place in a transparent container ($17 \times 12 \times 6$ cm) filled with 0.5 cm of
127 moist soil and a freshly thawed mouse carcass (Livefoods Direct Ltd, Sheffield, UK) of a
128 standardized size (27–30 g). We first tethered each male by tying one end of a string of dental
129 floss around the male's pronotum and taping the other end to the side of the box. The two males
130 were tethered to opposite sides of the box to prevent competition between the two males, which
131 otherwise would restrict the female's ability to choose between them (Otronen, 1988). We tied
132 the string such that there was about 3 cm of give to ensure that we did not limit the males' ability
133 to mount and mate with the female (Mattey & Smiseth, 2015a). Both males could reach the
134 carcass, which was placed in the middle of the box, but they could not come in direct contact
135 with each other. We alternated between trials whether it was the outbred or inbred male that was
136 tethered on the side close to the front versus the back of the carcass (Mattey & Smiseth, 2015a).

137 At the start of the trial, we placed the female at the center of the carcass such that she was
138 equidistant from the two males. We recorded the time when the female first came into contact
139 with the outbred and the inbred male and the number of copulations she had with each male over
140 the next 45 minutes. Successful copulations occurred when the male inserted his aedeagus
141 (intromittent organ) into the female's vagina (House et al., 2008). Given that each copulation
142 typically lasts about 90 s and females do not have a refractory period (House et al., 2008), it was
143 possible for females to mate repeatedly with the same male or both males. All outbred and inbred
144 females mated at least once over the course of the 45-minute mate choice trial. All trials ($N = 30$)
145 were successful, and they were all included in the analyses described below.

146

147 *Data Analysis*

148 A female might show a preference for the outbred male either by only mating with that male or
149 by mating with the outbred male more times than she mates with the inbred male. To examine
150 the former scenario, we used a generalised linear model (GLM) where the response variable
151 indicated whether a female mated with only one male or both males during the dichotomous
152 choice test. Our two explanatory variables were female inbreeding status (outbred or inbred) and
153 female pronotum width. This model was fitted using a binomial error distribution with a
154 complementary log-log link function. To examine the latter scenario, we first tested for a
155 negative correlation between the number of times the female copulated with the outbred and
156 inbred male in a given trial, which would indicate that mating with one male reduced the
157 likelihood of mating with the other male. After confirming the absence of such a correlation
158 (Spearman's rank test: $\rho = 0.063$, $P = 0.74$), we tested whether females copulated more
159 frequently with the outbred or the inbred male, using a generalised linear mixed model (GLMM)
160 with a Poisson error distribution ('glmer' function in the 'lme4' package). The starting model
161 included the following factors: male inbreeding status (outbred or inbred), female inbreeding
162 status (outbred or inbred), female pronotum width, the male's position relative to the carcass
163 (front or back), and whether that male was the first the female interacted with (yes or no).
164 Female identity was added as a random effect to account for the non-independence between the
165 observations on the two males in the same trial. Decisions about which variables to include in the
166 final model were based on AIC model selection criteria in order to obtain the minimal adequate
167 model. As a result of model simplification, our final model included the following factors: male
168 inbreeding status, female inbreeding status, and the interaction between male and female
169 inbreeding status. Statistical results for factors dropped from the final model (i.e., female
170 pronotum width, the male's position relative to the carcass, and whether he was the first male the

171 female interacted with) are the values from the full model prior to being removed. This model
172 was fitted using maximum likelihood methods. Lastly, we used a two-sample t-test to compare
173 the total number of copulations by outbred and inbred females, as a measure of female mating
174 activity or eagerness to mate. We used R version 3.3.1 (R Core Team, 2013) for all analyses.

175

176 *Ethical Note*

177 Our study adheres to the ASAB/ABS Guidelines for the Use of Animals in Research, the legal
178 requirements of the UK, as well as all institutional guidelines at The University of Edinburgh.
179 None of the procedures used in this study had the potential to cause pain or distress to the
180 beetles.

181

182 **RESULTS**

183 We found that female mating preferences for inbred versus outbred males were conditional upon
184 the female's own inbreeding status (Fig. 1). Inbred females copulated more with outbred males,
185 while outbred females showed no preference between outbred and inbred males (GLMM: male
186 inbreeding status: LR $\chi_1^2=5.47$, $P=0.02$; female inbreeding status: LR $\chi_1^2=0.87$, $P=0.35$,
187 interaction: LR $\chi_1^2=9.01$, $P<0.01$). These mating preferences were not influenced by female
188 pronotum width (GLMM: LR $\chi_1^2=0.15$, $P=0.70$), whether the male was tethered to the side
189 closest to the front or back of the carcass (GLMM: LR $\chi_1^2=0.54$, $P=0.46$), or which male the
190 female interacted with first (GLMM: LR $\chi_1^2=0.34$, $P=0.56$). Inbred females were also less likely
191 to mate with both males during the dichotomous choice test (GLM: $\chi_1^2=0.432$, $P=0.038$; Fig. 2).
192 Female pronotum width did not influence the likelihood of mating with both males (GLM:
193 $\chi_1^2=0.42$, $P=0.52$). Lastly, we found that outbred and inbred females were equally eager to mate,

194 as measured by the number of total copulations they had over the course of the 45-minute choice
195 test (two-sample t-test: $t_{28}=1.38$, $P=0.17$).

196

197 **DISCUSSION**

198 We found that female mating preferences for outbred versus inbred males were conditional upon
199 the female's own inbreeding status: inbred females preferred outbred males over inbred males,
200 whereas outbred females did not show a preference between outbred and inbred males. Inbred
201 females not only copulated with the outbred male more often than with the inbred male (Fig. 1)
202 but also the majority of inbred females mated only with the outbred male (Fig. 2). Our results
203 highlight the potential importance of male inbreeding status as a factor influencing female choice
204 and demonstrate that this effect may depend on the female's own inbreeding status. Below, we
205 discuss possible explanations for our findings and their wider implications for female mate
206 choice and male mating success in other species.

207 We found that inbred females showed a mating preference for outbred males over inbred
208 males, whereas outbred females showed no such preference. Given that choosiness is thought to
209 be costly (Pomiankowski, 1987), our results suggest that inbred females may be prepared to pay
210 the costs of being choosy in order to gain higher marginal benefits (Mazzi et al., 2004; Bolund et
211 al., 2010). Theoretical models predict only small indirect (genetic) benefits to mating with
212 outbred over inbred males (Reinhold, 2002; Lehmann et al., 2007), because mating with an
213 unrelated partner restores offspring heterozygosity regardless of whether that partner is inbred or
214 outbred. Thus, the observed preference of inbred females for outbred males is more likely due to
215 direct benefits (Fox et al., 2012).

216 One direct benefit that plays a role in mate choice in many species is parental care (Johnstone
217 et al., 1996; Møller & Jennions, 2001). In burying beetles, males often assist the female in
218 providing care to the offspring, by removing any fur or feathers from the carcass, applying
219 antimicrobials to prevent bacterial and fungal growth, protecting the brood from predators and
220 conspecifics, and provisioning the larvae with pre-digested carrion (Eggert et al., 1998; Rozen et
221 al., 2008; Walling et al., 2008; Arce et al., 2012). However, it is unlikely that the observed
222 preference of inbred *N. vespilloides* females for outbred males is driven by a direct benefit of
223 paternal care. This is because the opportunity for female choice is restricted by male-male
224 competition over ownership of the carcass. Vertebrate carcasses suitable for breeding are
225 relatively scarce in the wild, so it is common for multiple male and female burying beetles to
226 arrive on a carcass at the same time, resulting in fierce intrasexual competition (Otronen, 1988).
227 Thus, if the female's preferred mate is defeated by another male and driven away from the
228 carcass, he will not provide any care for the resulting offspring.

229 Instead, it is more likely that the mating preferences we observed were driven by another
230 type of direct benefits, such as sperm number or quality. Inbred males tend to transfer less sperm
231 during copulations and their sperm is less motile and has more abnormalities, leading to lower
232 fertilisation success (Zajitschek et al., 2009; Fitzpatrick & Evans, 2014; Ala-Honkola et al.,
233 2013). Inbred and outbred females may also produce eggs of different quality, which may be
234 differentially affected by low- versus high-quality sperm produced by inbred and outbred males,
235 respectively. We suggest that the lower sperm quality of inbred males might be more detrimental
236 to fertilisation success if the female is also inbred, but to our knowledge, this has not yet been
237 tested. We encourage future research to investigate whether there is an interaction between male
238 and female inbreeding status on fertilisation success.

239 Although the avoidance of inbred males by inbred females might have evolved in direct
240 response to inbreeding, another possibility is that it reflects a general response to an overall
241 decline in condition due to inbreeding depression. Inbreeding is relatively uncommon in most
242 species, so it seems unlikely that the mating preferences we observed evolved in the specific
243 context of inbreeding (Mattey & Smiseth, 2015b; Pilakouta et al., 2015a). Instead, these mating
244 preferences may be mediated through pre-existing mechanisms that evolved to serve an adaptive
245 function in a different context. For example, females might have evolved general mating
246 preferences for high-quality males, which may be conditional upon their own quality. All
247 populations are potentially at risk of inbreeding in the future, given increasing habitat loss and
248 other human-induced disturbances that increase the chances of inbreeding (Andersen et al.,
249 2004). Whenever species with no prior history of inbreeding depression become subject to
250 inbreeding, the associated fitness costs may be mediated through pre-existing mechanisms that
251 evolved outside this context (Mattey & Smiseth, 2015b; Pilakouta et al., 2015a).

252 The fact that inbred females preferentially mated with outbred males suggests that females
253 responded to a cue that differentiated inbred and outbred males, such as cuticular hydrocarbons
254 (CHCs) or other chemical cues (Howard & Blomquist, 2005). In insects, CHCs are often used to
255 discriminate between relatives and non-relatives (Howard & Blomquist, 2005; Tsutsui, 2004;
256 Weddle et al., 2013). More specifically, in burying beetles, CHCs are used for partner
257 recognition based on information about sex and breeding status (Müller et al., 2003; Steiger et
258 al., 2007), as well as for parent-offspring discrimination (Smiseth et al., 2010). Females might
259 have been under selection to differentiate between males based on their CHC profiles
260 specifically as a mechanism to avoid mating with inbred males, or as a more general mechanism
261 to avoid mating with males that are in poor condition. Our suggestion that female burying beetles

262 use CHCs to discriminate between outbred and inbred males is in line with a recent study in the
263 butterfly *Bicyclus anynana* showing that inbreeding reduces the production of a male sex
264 pheromone, thereby allowing females to discriminate between males based on their inbreeding
265 status (van Bergen et al., 2013). Similarly, there is evidence that female discrimination between
266 outbred and inbred males in mealworm beetles is odour-based (Pölkki et al., 2012). Given that
267 there is a genetic basis to CHCs (Ferveur, 2005; Dronnet et al., 2006; Foley et al., 2007) and that
268 traits with a genetic basis are prone to inbreeding (van Bergen et al., 2013), CHCs are a plausible
269 mechanism for discrimination between outbred and inbred individuals in *N. vespilloides* and
270 many other insects.

271 We believe that our findings could have important implications for male mating success in
272 the wild. Earlier work has shown that inbred males often suffer reduced mating success (Joron &
273 Brakefield, 2003; van Oosterhout et al., 2003; Mariette et al., 2006; Ala-Honkola et al., 2009;
274 Enders & Nunney, 2010; Ketola & Kotiaho, 2010). Here, we demonstrate that inbred females
275 avoid mating with inbred males while outbred females do not. This suggests that inbred males
276 suffer reduced mating success only when interacting with inbred females. We therefore propose
277 that in species where female inbreeding status influences mate choice for outbred versus inbred
278 males, the fitness costs of inbreeding with respect to male mating success may be frequency-
279 dependent. In populations with high rates of inbreeding, a larger proportion of breeding females
280 will be inbred, and we would expect inbred males to experience lower mating success than in
281 populations with low rates of inbreeding. Such social effects on inbreeding depression in male
282 mating success may be widespread, but their occurrence is still largely unexplored. We
283 encourage future research to further investigate this issue, as it could have important implications
284 for the rate and direction of sexual selection in populations that are subject to inbreeding. For

285 example, under a scenario where inbred females are choosier than outbred females, directional
286 selection on male sexual traits will be stronger when inbreeding rates are high than when they are
287 low.

288 In summary, we have shown that a female's mating bias for an outbred versus an inbred male
289 depends on her own inbreeding status. This is the first example of a species where inbred
290 females discriminate against inbred males while outbred females show no preference between
291 inbred and outbred males. Our findings suggest that inbred females may gain more direct
292 benefits from mating with an outbred male than outbred females do. Lastly, in species where
293 female inbreeding status influences mate choice for outbred versus inbred males, the fitness costs
294 of inbreeding with respect to male mating success may depend on the frequency of inbred
295 females relative to outbred females and thus the rate of inbreeding in the population.

296

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- 456

457 **FIGURE LEGENDS**

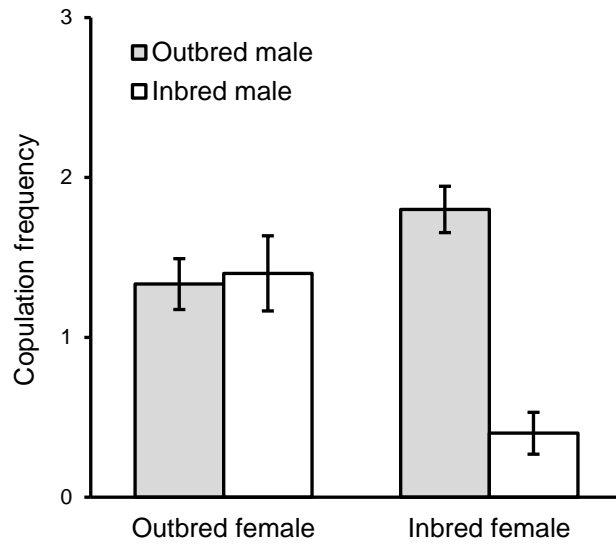
458

459 **Fig. 1** Mean (\pm SE) number of times an outbred or inbred female mated with the outbred male
460 (grey) and the inbred male (white) during a 45-minute mate choice trial.

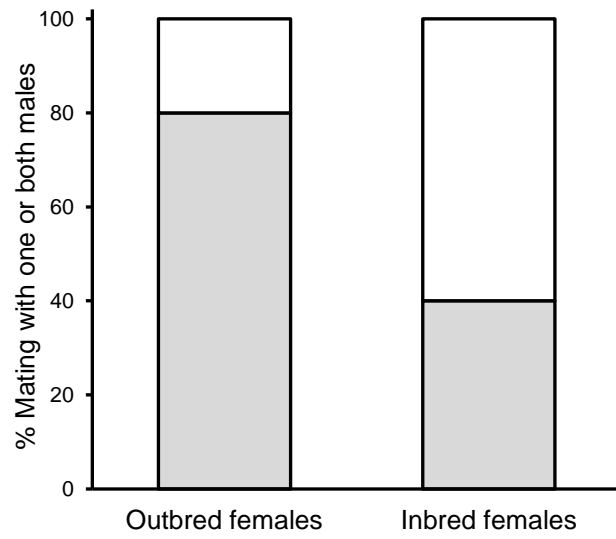
461

462 **Fig. 2** Percentage of outbred and inbred females that mated with only one of the two males
463 (white) or both males (grey) over the course of the 45-minute mate choice trials.

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