Does the response of *D. melanogaster* males to intrasexual competitors influence sexual isolation?

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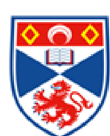

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1 SUMMARY

2 The strength of reproductive isolation between diverging populations may
3 depend on the social interactions experienced by individuals. We used partially
4 isolated populations of fruit flies, *Drosophila melanogaster*, and showed that
5 whether males had previously interacted with homopopulation or
6 heteropopulation male partners did not affect the strength of pre- or postmating
7 sexual isolation. Thus, although male sexual traits are highly labile, this flexibility
8 does not seem to affect the strength of sexual isolation in this system.

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ABSTRACT

The evolutionary consequences of phenotypic plasticity are debated. For example, reproductive barriers between incipient species can depend on the social environment, but most evidence for this comes from studies focussing on the effects of experiencing heterospecific individuals of the opposite sex. In *Drosophila melanogaster*, males are well known to invest strategically in ejaculate components and show different courtship behaviour when reared in the presence of male competitors. It is unknown whether such plasticity in response to same-sex social experience influences sexual isolation, so we tested this using African and cosmopolitan lines which show partial sexual isolation. Males were housed in social isolation, with homopopulation, or with heteropopulation male partners. We then measured their mating success, latency, and duration, their paternity share, and female re-mating success. Isolated males copulated for a shorter duration than males housed with any male partners. However, we found no difference in any measure between homo- or heteropopulation treatments. Our findings suggest that the male intrasexual competitive social environment does not strongly influence sexual isolation in *D. melanogaster*, and that plastic effects on reproductive isolation may be influenced more strongly by the experience of social isolation, than by the composition of individuals within different social environments.

32 # INTRODUCTION

33 The role of phenotypic plasticity in evolution is intensely debated. Organisms can
34 adjust phenotypic traits within a generation, but whether and how this plasticity
35 impacts longer term evolutionary change is less clear (Price et al. 2003; West-
36 Eberhard 2005; Ghalambor et al. 2007; Scoville and Pfrender 2010; Parsons et
37 al. 2016; Schmid and Guillaume 2017; Bailey et al. 2018). One factor to which
38 animals show considerable phenotypic plasticity is the social environment.
39 Socially-mediated plasticity can allow individuals to cope with variation in
40 demography and social interactions within generations, but whether it influences
41 evolutionary processes across generations is poorly understood and challenging
42 to study empirically. One route by which socially-mediated plasticity could affect
43 trait evolution or speciation dynamics is if the expression of traits involved in
44 mate recognition and choice are sensitive to the social environment (Rodríguez
45 et al. 2013). Theory suggests that evolutionary effects of socially-mediated
46 plasticity might accelerate or decelerate the evolution of reproductive isolation,
47 depending on whether individuals encounter conspecifics or heterospecifics, the
48 fitness consequences of the encounters, and the genetics of plasticity (Servedio et
49 al. 2009; Servedio and Dukas 2013). For instance, a recent study has found that
50 bird songs diverged faster in songbird species with innate song than in species
51 with socially learnt songs (Freeman et al. 2017), suggesting that socially mediated
52 phenotypic plasticity can slow down evolution of traits involved in reproductive
53 isolation.

54 Although reproductive barriers between species have usually been
55 assumed to be relatively canalised traits, theoretical and empirical data both

56 challenge this view (Irwin and Price 1999; Servedio and Noor 2003; Servedio et
57 al. 2009; Verzijden et al. 2012; Kawecki 2013; Verzijden et al. 2013; Servedio and
58 Bürger 2014; Yeh and Servedio 2015). The social environment experienced by
59 individuals has repeatedly been found to influence traits with roles in
60 reproductive isolation, such as mating rates (e.g., Billeter et al. 2012), sexual
61 signals (e.g., Krupp et al. 2008; Groot et al. 2010), mating preferences (e.g., Bailey
62 and Zuk 2009; Danchin et al. 2018), courtship behaviour (e.g., Lehtonen et al.
63 2016), aggressive behaviour (e.g., Carazo et al. 2014), and ejaculate allocation
64 (e.g., Wigby et al. 2009). Female preferences can be modified according to
65 experience with heterospecifics (Li et al. 2018), and it is well established that
66 post-mating pre-zygotic reproductive interactions evolve rapidly and are an early
67 acting component of reproductive isolation (Alipaz et al. 2001; Manier et al. 2013;
68 Jennings et al. 2014; Turissini et al. 2018). But is the response to male social
69 environment tuned to the identity of the competitor? Is it population-specific?
70 And does it influence pre- and post-mating reproductive success?

71 Here we evaluate the impact of socially-mediated plasticity on sexual
72 isolation between diverging populations of *D. melanogaster*, focusing on male
73 responses to the presence of potential sexual competitors in their social
74 environment. In *Drosophila*, many plastic responses of male reproductive traits
75 are adaptive responses to the perceived likelihood of intrasexual competition. For
76 example, males may produce more competitive behaviours or ejaculates, which
77 increase sperm competition success when they experience rivals during
78 development (Bretman et al. 2009), as predicted under classic models of strategic
79 investment in sperm competition (Parker 1970; Parker and Pizzari 2010).

80 Moreover, young male flies court each other in the first day after eclosion (Gailey
81 et al. 1982), which is thought to potentially contribute to courtship learning
82 (Griffith 2014). Interestingly, the genetic makeup of other males encountered in
83 the social environment may influence the expression of both pre- and postmating
84 reproductive traits (reviewed in Bretman et al. 2011; Schneider et al. 2012;
85 Griffith 2014; Schneider et al. 2017). For instance, the expression of key cuticular
86 pheromones and male mating behaviour are affected by the genetic composition
87 of male social partners (Kent et al. 2008; Krupp et al. 2008). The degree of
88 familiarity and genetic relatedness among males impacts female reproduction
89 and female lifespan, in that males exposed to familiar or related males seem to be
90 less harmful to females (Carazo et al. 2014; Hollis et al. 2015; Le Page et al. 2017).
91 Such an effect is presumably mediated by the ejaculate transferred to females,
92 which is known to be highly flexible. Males adjust the transfer of sperm and
93 seminal fluid proteins when they are exposed to rivals (e.g., Bretman et al. 2009;
94 Wigby et al. 2009) presumably as part of a flexible strategic investment strategy
95 influenced by the likelihood of sperm competition and mating opportunities.
96 Despite what is known about flexibility in male *D. melanogaster* ejaculate
97 characteristics, relatively little is known about how this might translate to
98 flexibility in sexual isolation.

99 In this study, we take advantage of African and cosmopolitan populations
100 of *D. melanogaster*, which show incomplete sexual isolation at both the pre- and
101 postmating stages (Hollocher et al. 1997; Alipaz et al. 2001). We used multiple
102 lines from these two populations to test if plastic responses of males to intrasexual
103 competitors influences the strength of sexual isolation in the early stages of

104 evolutionary divergence. We manipulated the male social environment by
105 housing focal males in social isolation, or with either five homo- or five
106 heteropopulation males for five days. We assessed the effects of this treatment on
107 premating isolation by measuring mating latency, mating success, and mating
108 duration with heteropopulation females. To assess effects on postmating
109 isolation, we measured remating rates of the females with second males and,
110 when copulations occurred, we quantified the focal males' paternity share. We
111 test several predictions about how the male social environment may influence
112 sexual isolation. First, *D. melanogaster* males are known to plastically increase
113 mating duration or ejaculate components in the presence of other males (e.g.,
114 Bretman et al. 2009). If male only perceive homopopulation males as sexual
115 competitors, then we would expect that males exposed to heteropopulation males
116 would show a similar strength of sexual isolation as previously isolated males.
117 Second, *D. melanogaster* males can plastically modify the expression of cuticular
118 pheromones according to the genetic composition of the other group members
119 (Kent et al. 2008; Krupp et al. 2008). If such a plastic response allow males to
120 better match the pheromones profiles of their male social partners, then males
121 exposed to heteropopulation male social partners would show a lower strength of
122 sexual isolation than males previously exposed to homopopulation males. Such
123 effects can influence both the premating (e.g., mating success) and/or the
124 postmating (e.g., mating duration, sperm precedence) episodes of selection.

125 MATERIAL AND METHODS

126 **Line establishment**

127 We used six lines of *D. melanogaster* (3 African, 3 cosmopolitan, Table S1) into
128 which we inserted markers allowing parentage scoring. We backcrossed two
129 dominant fluorescent markers, a green fluorescent protein (GFP) and a red
130 fluorescent protein (RFP) into these strains.

131 In the first generation, we pooled 5 virgin wild-type females with 5 males
132 from the marker lines (parental individuals), replicated three times per line. We
133 then grouped five of the resulting F_1 virgin females with five males of the
134 corresponding wild-type line, replicated three times per line. We continued
135 backcrossing for 10 generations by sampling the females carrying the marker, i.e.
136 expressing the green or red fluorescent protein. In every generation, we crossed
137 females from the backcross to males from the wild-type line, to allow
138 recombination. Because the first generation did not yield any offspring in some
139 lines, we made the reverse cross (i.e., female from marker line × wild-type male)
140 in the first generation, but used wild-type females subsequently (F_2 or F_3), so that
141 the mitochondrial DNA was correctly introgressed into all newly established
142 lines.

143 To create homozygous lines for the introgressed marker, we made $F_{10} \times F_{10}$
144 crosses within each line and selected homozygous individuals by eye based on the
145 intensity of the fluorescent signal using a fluorescence microscope (Tritech
146 Research, Inc). Hence, the backcross breeding program yielded 12 newly
147 established lines (2 markers × 2 populations × 3 lines), with genetic backgrounds
148 from African or cosmopolitan populations and stable expressions of GFP or RFP
149 (Figure S1). These lines are expected to share more than 99.9% of their genome

150 with the initial wild-type lines (Hartl and Clark 1997), and to contain on average
151 10cM DNA segments from the marker line on each side of the locus of the
152 introgressed markers (Hospital 2001).

153 During the backcrossing, we assayed the fitness of individuals carrying the
154 markers using two tests. First, we sampled 566 and 870 F_4 offspring in the GFP
155 and RFP backcross respectively, and counted the number of offspring carrying
156 the markers of interest vs. wild-type offspring. We tested for viability effects of
157 the markers by calculating heterogeneity and pooled G tests. There were no
158 significant deviations (see supplementary information). Second, in the fifth
159 generation, we sampled males that did and did not carry markers, and tested their
160 reproductive success in a competitive mating situation. We grouped two males
161 (one of each type) with two wild-type females in vials for 10 days, which we
162 replicated 20 times per marker, and assessed the status of 40 resulting offspring
163 per replicate. The observed proportion of offspring expressing the markers were
164 tested against an expectation of 0.25 using G tests. There was significant
165 heterogeneity but for the GFP marker, no overall difference from expectations.
166 For the RFP marker there was again significant heterogeneity but individual
167 comparisons were inconsistent in direction, so there was no consistent evidence
168 for an excess of wild type, as would be expected if the marker was less competitive
169 in these assays (see supplementary information).

170 **Experimental design**

171 *Rearing and social environment manipulation.* All flies were maintained at 23°C
172 on a 12:12 light:dark cycle and we standardised stock densities to 12 males and 12
173 females per vial (25 x 95mm, Scientific Laboratory Supplies) for two generations

174 before sampling flies used in experiments. To manipulate the social environment
175 of males we raised virgin focal males for five days either in isolation, or with five
176 homo- or five heteropopulation virgin male partners in small vials (15 x 95mm,
177 Sarstedt) containing food and yeast (Figure 1). The five male partners were from
178 the same line, which either matched the line of the focal male (i.e.,
179 homopopulation treatment), or the line of the female (i.e., heteropopulation
180 treatment) (see below for line combinations). Focal males carried markers (GFP
181 or RFP), and male partners were wild-type.

182 *Line combinations for reproductive isolation tests*. We crossed African and
183 cosmopolitan *D. melanogaster* populations as follows: Chipata1.1 × IT-IV-69;
184 LZV3.4 × FIN-I-15-17; Zim30 × Canton-S. Each cross was performed in both
185 directions (i.e., ♀ cosmopolitan × ♂ African, and ♀ African × ♂ cosmopolitan).
186 However, we observed few copulations in ♀ LZV3.4 × ♂ FIN-I-15-17 and ♀ Zim30
187 × ♂ Canton-S, confirming that these African females discriminate strongly
188 against Cosmopolitan males (Hollocher et al. 1997), so we discarded these two
189 crosses from subsequent analyses. Our initial sample size was 18 replicates per
190 treatment and cross (i.e., 324 samples). However, we lost replicates over course
191 of the experiment due to unsuccessful 1st or 2nd mating trials, fly death, or
192 handling mistakes (see Figure 2 and Table S2 for final sample sizes).

193 *Premating isolation*. To test premating isolation among lines and evaluate the
194 effect of male social environment manipulation upon it, we exposed focal males
195 to virgin heteropopulation females (i.e., 1 day old) in small vials containing food.
196 We first sampled all males—without anaesthetisation—and then distinguished

197 focal males from male partners by momentarily exposing flies to epifluorescence
198 illumination using a fluorescence compound microscope. We observed all male-
199 female pairs for 2h, and recorded mating success, mating duration, and room
200 temperature. Importantly, pair formation and mating observation were done by
201 two different experimenters to ensure that the data were recorded blind with
202 regards to the fly lines and the treatment. We then kept females in isolation for
203 five days. Note that we confirmed at this stage that focal males were homozygotes
204 for the marker by verifying that all offspring produced during these five days
205 expressed the marker.

206 *Postmating isolation.* To assess postmating isolation, we exposed females from
207 the procedure above that had been isolated for 5 days after their first mating to a
208 second male which came from the same line as the female. We observed the pair
209 for 2h, and recorded mating success, mating duration, and room temperature.
210 Again, mating observations were blinded. We kept the twice-mated female in
211 isolation for a further five days and counted all resulting offspring and scored the
212 marker, allowing quantification of offspring sired by focal males.

213 **Data analysis**

214 We measured pre-mating isolation using three response variables (mating
215 success, mating latency, and mating duration), and post-mating isolation using
216 two (re-mating success, and paternity share). We tested whether these responses
217 were influenced by the male social environment (isolated, homopopulation male
218 partners, heteropopulation male partners), by line, and by a male social
219 environment × line interaction. We included room temperature as a covariate in
220 all data analyses. We used binary nominal logistic regressions for mating success

221 and re-mating success, ANCOVAs for mating latency and mating duration, and a
222 Binomial GLM with logit link function for paternity share. Note that when we
223 found a significant interaction effect, we tested for male social environment effect
224 within each line. Similarly, when we found significant male social environment
225 effects, we ran post-hoc pair-wise comparisons to determine which treatment
226 explained the overall effect. All statistical analyses were carried out in JMP (SAS
227 Institute Inc., Cary, NC, USA).

228 # RESULTS

229 The male social environment did not affect mating success or mating latency
230 (Table 1, Figure 2A and 2B). The significant interaction observed for mating
231 success × line suggested that social environment affected mating success
232 differently among lines. However, we did not find significant social environment
233 effects on mating success in follow-up analyses conducted within each line (all
234 P>0.05; binary nominal logistic regressions accounting for multiple testing) so
235 any effect was weak. The only significant effect of social environment we found
236 was on mating duration (Table 1, Figure 2C). Post-hoc analyses showed that
237 males previously raised in isolation copulated for a shorter duration than males
238 raised with either homopopulation male partners (2.04 ± 0.81 min [0.43-3.65],
239 t=2.5, df=144, P=0.013; mean duration difference ± SE [lower and upper
240 confidence limit]; posthoc Student's t test), or with heteropopulation male
241 partners (1.72 ± 0.80 min [0.13-3.31], t=2.1, df=144, P=0.033). Males raised with
242 homo- or heteropopulation male partners did not significantly differ in
243 copulation duration (0.32 ± 0.79 min [-1.24—1.87], t=0.4, df=144, P=0.688)
244 (Figure 2C). We did not find significant effects of male social environment on
245 either measure of postmating isolation, female re-mating success and paternity
246 share (Table 1, Figure 2D and 2E). Note that the exclusion of the two outliers on
247 male paternity share (>0.75) does not qualitatively change the statistical
248 outcomes (all NS).

249 ## DISCUSSION

250 The strength of sexual isolation between animal species can depend on whether
251 individuals have previously experienced heterospecific individuals of the
252 opposite sex (e.g., Magurran and Ramnarine 2004; Fincke et al. 2007; Dukas
253 2008; Kujtan and Dukas 2009). Here we test if male experience of other males
254 could also influence isolation. For example, strategic allocation of courtship effort
255 or ejaculate components could influence sexual isolation, both in terms of mating
256 success and post mating fertilisation success. However, we found that the male
257 social environment had little influence on sexual isolation between African and
258 cosmopolitan *D. melanogaster* populations. Whether males experienced homo-
259 or heteropopulation males did not affect the strength of sexual isolation despite
260 examining both pre- and postmating reproductive barriers. The only significant
261 difference we found was on mating duration. Previously isolated males copulated
262 for a shorter duration than males that had (any) social partners. Thus, despite the
263 fact that many pre- and postmating reproductive traits are known to depend on
264 the male social environment in *D. melanogaster*, our findings suggests that
265 plastic responses in these traits might have limited effects on sexual isolation.

266 If males can alter their reproductive strategy due to the likelihood of sperm
267 competition intensity, how phylogenetically related must males encountered in
268 the social environment be for focal individuals to perceive them as sexual
269 competitors? In this study examining intraspecific, but population-level,
270 variation in social experience, we found that males showed similarly longer
271 mating durations in response to the presence of either homo- or heteropopulation
272 males, suggesting that focal males perceived both as sexual competitors. In a

previous study examining interspecific variation in social experience, we found that *D. melanogaster* and *D. simulans* males produce longer courtship songs after being raised with other males, regardless of whether social partners were *D. melanogaster* or *D. simulans* (Marie-Orleach et al. 2019). In contrast, Bretman et al (2017) found that *D. melanogaster* males increase their mating duration in response to the presence of heterospecific males, but not as might be predicted based on genetic distances between species. Responses to other species may be related to phenotypes rather than genetic distance per se. *Drosophila melanogaster* males increase their mating duration in response to the presence of *D. simulans* and *D. pseudoobscura* males (though not to the same extent as to the presence of *D. melanogaster* males), but not of the closely related *D. yakuba* or *D. virilis* males. More surprisingly, such a response to the risk of sperm competition is also seen in monandrous populations of *Drosophila subobscura* (Fisher et al. 2013), and such responses may have evolved in the context of direct male-male competition rather than (or alongside) sperm competition to maximise strategic investment (Lize et al. 2014). Altogether, our data and these previous findings suggest that plasticity mediated by male competition may be a general response to interactions with other males, but not in a manner that is generally predicted by phylogenetic distance, suggesting such plasticity may not be instrumental in influencing subtle levels of sexual isolation .

Our results suggest that phenotypic plasticity mediated by the male social environment is unlikely to play a role in accelerating population divergence, which is important in light of current debates about how socially mediated phenotypic plasticity affects trait evolution and speciation processes (Price et al.

297 2003; West-Eberhard 2005; Ghalambor et al. 2007; Scoville and Pfrender 2010;
298 Parsons et al. 2016; Schmid and Guillaume 2017; Bailey et al. 2018). In contrast,
299 it is clear that male-male competition itself is a strong agent of selection, and
300 likely responsible for rapid evolutionary change in multiple phenotypes. For
301 instance, accelerated evolutionary rates are observed in gonadal and genital traits
302 (e.g., Civetta and Singh 1998), and in ejaculate proteins (Swanson et al. 2001).
303 Similarly, closely related species are often found to have higher levels of
304 diversification in sperm traits and in genital morphology (e.g., Pitnick et al.
305 2003), as well as in sperm precedence traits (e.g., Manier et al. 2013). Our study
306 suggests that this accelerated evolutionary rate of male traits is not reflected in
307 species-specific plasticity in their expression, or that any such plasticity in traits
308 is not effective in influencing sexual isolation.

309 Our data confirm that previously isolated *D. melanogaster* males engage
310 in shorter copulations than males previously housed with (any) types of social
311 partners do. This is consistent with previous studies showing that males respond
312 to the risk of sperm competition by copulating for longer. This is usually thought
313 to increase the number of sperm transferred and offspring sired (Bretman et al.
314 2009; Garbaczewska et al. 2013). However, in our study, this effect did not
315 translate to subsequent increases in offspring production, as we did not find that
316 the social environment influenced paternity share. This discrepancy is surprising.
317 Perhaps any influence is relatively subtle and not detected in our experiment.
318 Because paternity share can only be assessed on the subset of females that re-
319 mate, our sample size decreased over the course of the experiment. Nevertheless,
320 our findings indicate that there are no large effect of the male social environment

321 on postmating sexual isolation despite our observation of increased copulation
322 duration when reared in the presence of rivals.

323 Any phenotypic plasticity mediated by the male social environment is not likely
324 to accentuate the population divergence seen here, and such plasticity seems to
325 be relatively broadly tuned to the identity of interacting partners. Additional
326 experiments investigating more diverse components of the social environment,
327 and pre- and postmating sexual isolation, at different stages of evolutionary
328 divergence, are required to fully address how the social environment affects
329 speciation processes in general.

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FIGURE CAPTIONS

Figure 1. Experimental set-up used to manipulate male social environment and assess pre- and postmating isolation. First, males were sampled from lines of either African or cosmopolitan populations. Second, we experimentally manipulated the social environment experienced by focal males by raising them for five days either in isolation, or with five homo- or heteropopulation male partners. Third, we exposed focal males for 2h to a virgin heteropopulation female, and scored mating success and mating duration. Fourth, females had a second mating opportunity with a homopopulation male, and we scored mating success and mating duration, as well as the resulting paternity share. Fly colour denotes fly population.

Figure 2. The effects of male social environment on pre- and postmating sexual isolation. We manipulated the social environment of focal males, and then measured mating success (A), mating latency (B) and mating duration (C) with heteropopulation females. Females were then exposed to a second male, and we measured female re-mating success (D) and focal male's paternity share (E). Stars and ns stand for significant and non-significant pair-wise differences, respectively. In panels B, C, and D, all datapoints are shown jittered, thick black bars indicate standard errors, and the white gap between them the means for each comparison. . Sample sizes are indicated under brackets. See results for statistics.

520 TABLES

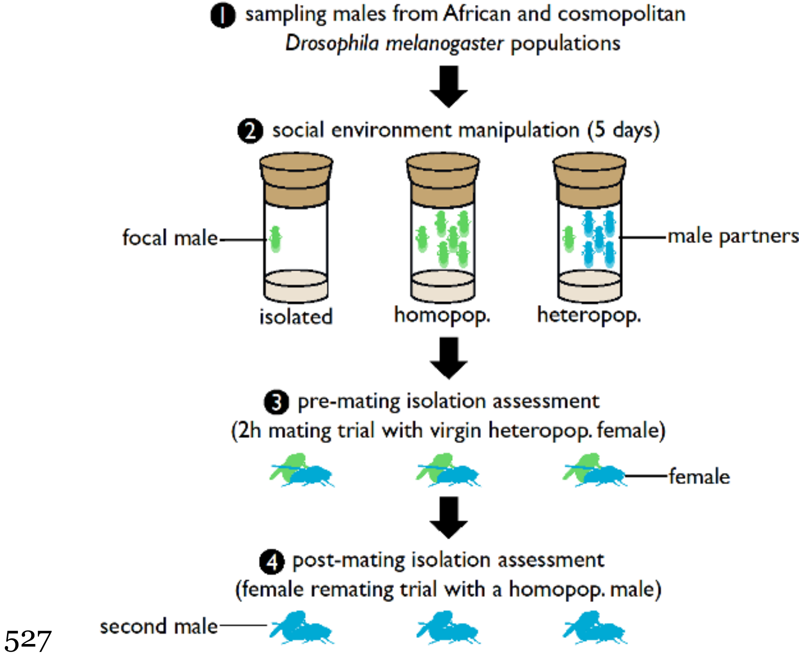
521 **Table 1. The effect of male social environment, line, social environment × line social environment, and temperature**
522 **on measures of premating isolation (mating success, mating latency, mating duration) and postmating isolation**
523 **(re-mating success, paternity share).** See methods for details.

		mating success		mating latency		mating duration		re-mating success		paternity share	
	df	χ^2	*P*	*F* ratio	*P*	*F* ratio	*P*	χ^2	*P*	χ^2	*P*
social environment	2	0.0	1.000	0.2	0.847	3.6	0.030	3.5	0.1701	0.4	0.838
line	3	53.1	<0.001	18.0	<0.001	33.2	<0.001	16.3	0.001	0.1	0.995
social environment × line	6	13.5	0.035	1.1	0.355	1.8	0.103	4.0	0.671	5.5	0.486
temperature	1	0.3	0.597	0.8	0.364	2.7	0.105	0.7	0.672	0.0	0.841

524

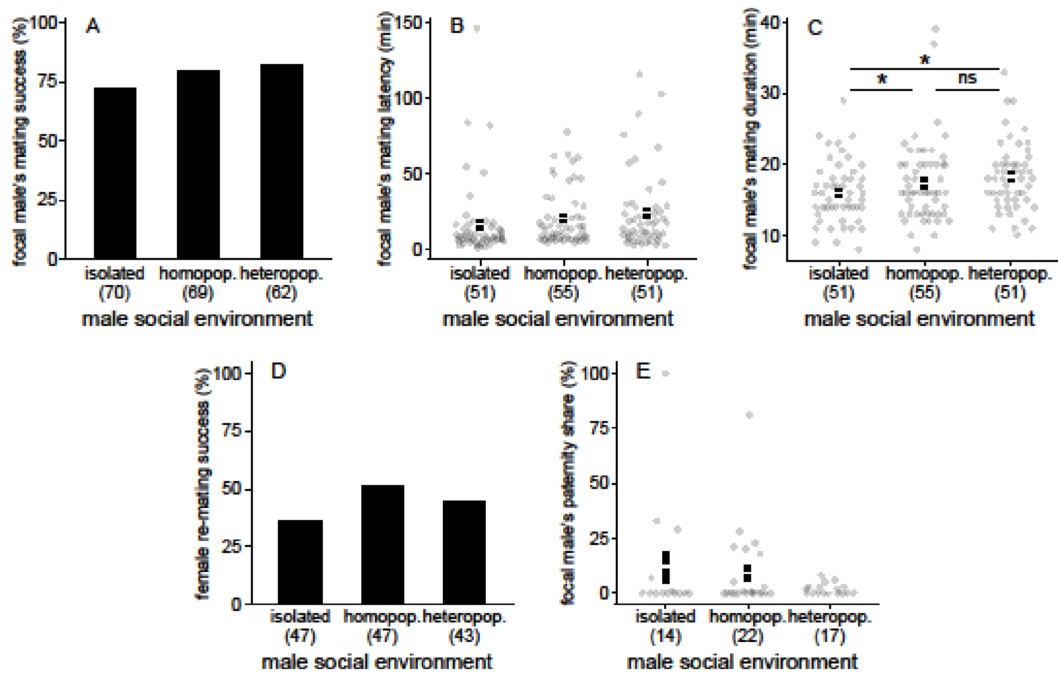
525 # FIGURES

526 **Figure 1.**



527

528 **Figure 2.**



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