

1 **Female fruitflies use gustatory cues to exhibit reproductive plasticity in response to the**  
2 **social environment**

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15

16 **Abstract**

17 Animals can exhibit remarkable reproductive plasticity in response to their social  
18 surroundings, with profound fitness consequences. The study of such plasticity in females,  
19 particularly in same-sex interactions, has been severely neglected. Here we measured the  
20 impact of variation in the pre-mating social environment on reproductive success in females  
21 and tested the underlying mechanisms involved. We used the *Drosophila melanogaster*  
22 model system to test the effect of varying female group size prior to mating and deployed  
23 physical and genetic methods to manipulate the perception of different social cues and  
24 sensory pathways. We found that socially isolated females were significantly more likely to  
25 retain unfertilised eggs before mating, but to show the opposite pattern and lay significantly  
26 more fertilised eggs in the 24h after mating, in comparison to grouped females. More than  
27 48h of exposure to other females was necessary for this socially-induced plasticity to be  
28 expressed. Neither olfactory nor visual cues were involved in mediating these responses.  
29 Instead, we found that females detected other females through direct contact with the deposits  
30 they leave behind, even in the absence of eggs. The results demonstrate that females show  
31 striking reproductive plasticity in response to their social surroundings and that the nature of  
32 their plastic reproductive responses, and the cues they use, differ markedly from those of  
33 males. The results emphasise the stark contrasts in how each sex realises reproductive  
34 success.

## 35 **Introduction**

36 Phenotypic plasticity (the expression of different phenotypes from the same genotype) is a  
37 widespread and important component of fitness, allowing individuals to adaptively alter their  
38 behaviour or physiology in response to environmental variation (Pigliucci, 2001; West-  
39 Eberhard, 2003). An organism's social surroundings (e.g. the local density and ratio of male  
40 and female conspecifics and heterospecifics) can vary considerably (Kasumovic & Brooks,  
41 2011). Sex differences in birth and death rates or sexual maturity can cause temporal shifts in  
42 sex ratio, either on an immediate, short-term basis or over seasons or successive years. Other  
43 factors such as immigration, dispersal and the level of predation also contribute to a dynamic  
44 social environment (Kasumovic & Brooks, 2011). The density and identity of individuals in  
45 the social milieu can signal resource quality or the expected likelihood of competition (Davis  
46 *et al.*, 2011). For example, the sex ratio of conspecifics could indicate the level of  
47 competition for mating opportunities, or for sex-specific resources such as oviposition sites.  
48 Detection of information from heterospecifics may also be beneficial if habitat requirements  
49 overlap between species. If this is the case, the overall density of individuals, independent of  
50 species, could signal expected levels of nutrient availability or quality, predation risk (Huang  
51 *et al.*, 2011) or oviposition sites. Given that variation in the social environment has  
52 significant consequences for the level of reproductive competition or resource availability,  
53 individuals with the ability to detect cues from their social environment and adjust their  
54 phenotype accordingly can increase their fitness (Bretman *et al.*, 2013).

55         The effect of the social environment on phenotypic plasticity in males has been well  
56 studied in the context of sperm competition (Bretman *et al.*, 2011; Dore *et al.*, 2018; Parker &  
57 Pizzari, 2010; Wedell *et al.*, 2002). *Drosophila melanogaster* fruitflies in particular have  
58 proved to be a valuable model in this context. Males can precisely and flexibly adjust their  
59 ejaculate composition and extend copulation duration in response to the presence of

60 conspecific rival males (Bretman *et al.*, 2011; Bretman *et al.*, 2013; Garbaczewska *et al.*,  
61 2013; Wigby *et al.*, 2009). These plastic adjustments enable males to secure a greater share of  
62 the paternity when sperm competition is perceived to be high, while conserving costly  
63 resources when sperm competition is unlikely (Bretman *et al.*, 2009).

64         Despite extensive studies into male social plasticity, we know very little about the  
65 corresponding context in females – i.e. whether and how they might adjust their reproductive  
66 output in response to the intrasexual environment. Naïve females can exhibit social learning  
67 and adjust their oviposition site preferences to match those of experienced mated females  
68 (Sarin & Dukas, 2009) and oviposition preference can be influenced both by pheromonal  
69 cues from conspecifics (Dumenil *et al.*, 2016; Malek & Long, 2020; Wertheim *et al.*, 2002)  
70 and the presence of predators (Kacsoh *et al.*, 2015). Female social plasticity has also been  
71 considered in the context of mate choice and differential responses to male characteristics  
72 (Bailey & Zuk, 2008; Billeter *et al.*, 2012; Filice & Long, 2017; Fox *et al.*, 2019). However,  
73 whether females can plastically optimise their reproductive output according to the general  
74 expectation of reproductive or resource competition (e.g. as signalled by the presence of other  
75 females) is not yet known and remains an important and unanswered question.

76         For fitness benefits of phenotypic plasticity to be accrued by either sex, and plasticity  
77 itself to evolve, mechanisms for the accurate perception of cues that reliably indicate the  
78 social or sexual environment are required. In male *D. melanogaster* cues of competition are  
79 detected via multiple, interchangeable olfactory, auditory and tactile sensory pathways  
80 (Bretman *et al.*, 2011). This multimodal strategy is predicted to decrease the risk of costly  
81 mismatches between environment and phenotype in highly variable environments (Dore *et*  
82 *al.*, 2018) enabling males to accurately perceive information on the species, sex and  
83 prevalence of other individuals, and respond appropriately to the level of sperm competition

84 (Bretman *et al.*, 2017). Whether females deploy any such multimodality via complex cues is  
85 also not yet known.

86 Here, we address these omissions by testing the hypothesis that *D. melanogaster*  
87 females plastically adjust their reproductive investment according to the con and hetero-  
88 specific intrasexual social environment. Focal females were either housed in isolation or with  
89 three other females before being given the opportunity to mate with a single male. We  
90 recorded mating times and the number of eggs (fecundity) laid in the 3 days before and in the  
91 24h after mating. During the social exposure phase, all females were virgins. This allowed us  
92 to test the response of females to the same sex environment without the confounding effects  
93 of previous mates or male pheromones. We thus investigated the effect of the proximate  
94 social environment on both virgin egg laying, and subsequent post-mating fecundity. We also  
95 probed the underpinning mechanisms involved by varying social exposure time and by  
96 restricting the perception of social cues by using genetic and physical manipulations.

97

## 98 **Results**

99 *Female fecundity responses to variation in the social environment and effect of exposure to*  
100 *con- vs hetero-specific females*

101 We measured the impact of pre-mating social isolation versus exposure to other females on  
102 the reproductive output of focal *D. melanogaster* females following a single mating. Virgin  
103 focal females were exposed to different social environments for 72h prior to mating, and  
104 fecundity was measured as the number of eggs laid in the 24h period following mating.  
105 During the post-mating period, focal females previously held in groups of four conspecifics  
106 laid significantly fewer eggs than previously socially isolated females (Figure 1a,  $F_{(1, 84)} =$   
107  $4.48, p = 0.037$ ). Similarly, *D. melanogaster* females held with three heterospecific females  
108 (either *D. simulans* or *D. yakuba*) prior to mating were also significantly less fecund

109 following mating than were socially isolated females (*simulans*:  $F_{(1, 76)} = 4.64$ ,  $p = 0.035$ ;  
110 *yakuba*:  $F_{(1, 90)} = 18.00$ ,  $p = 5.36 \times 10^{-5}$ ) (Figure 1b).

111

### 112 *Effect of length of social exposure period on post-mating fecundity*

113 The response of *D. melanogaster* female fecundity to the pre-mating social environment was  
114 affected by the length of exposure to conspecific females. When focal females were exposed  
115 to the different social environment treatments for 2, 4, 8, 24, 48 or 72h prior to mating, only  
116 those exposed for 72h showed a significant reduction in fecundity compared to isolated  
117 females ( $F_{(1, 120)} = 20.85$ ,  $p = 1.21 \times 10^{-5}$ ). The effect of social treatment on eggs was  
118 marginally non-significant for the 48h exposure period ( $F_{(1, 115)} = 3.68$ ,  $p = 0.058$ ), and not  
119 significant for all other shorter periods (2h:  $F_{(1, 87)} = 0.80$ ,  $p = 0.37$ ; 4h:  $F_{(1, 86)} = 0.03$ ,  $p =$   
120  $0.87$ ; 8h:  $F_{(1, 75)} = 1.28$ ,  $p = 0.26$ ; 24h:  $F_{(1, 115)} = 0.30$ ,  $p = 0.59$ ) (Figure 2).

121

### 122 *Investigation of whether exposure to eggs or to female deposits in the absence of eggs are* 123 *required for social exposure effects on post-mating fecundity*

124 To identify the cues that *D. melanogaster* females use to respond to the presence of others,  
125 we analysed whether a female's post-mating fecundity responded to the physical presence of  
126 other females, to their eggs or to the deposits they leave behind even in the absence of egg  
127 laying. We compared the post-mating fecundity of females subjected to the following  
128 treatments: 'isolation', 'group', 'group - eggless females', 'isolation - female deposits',  
129 'isolation - egg-spiked'. Consistent with the previous experiments, 'group' females laid  
130 significantly fewer eggs than females from the 'isolation' treatment (*OvoD1* control:  $F_{(1, 81)} =$   
131  $26.40$ ,  $p = 1.88 \times 10^{-6}$  (Figure 3A); egg-spiked control:  $F_{(1, 76)} = 20.45$ ,  $p = 2.22 \times 10^{-5}$  (Figure  
132 3B)). Furthermore, females from the 'group - eggless females', 'isolation - female deposits',  
133 and 'isolation - egg-spiked' treatments also laid significantly fewer eggs in comparison to

134 females from the ‘isolation’ treatment (deposits:  $F_{(1, 88)} = 8.20, p = 0.0052$ ; eggless:  $F_{(1, 77)} =$   
135 4.29,  $p = 0.042$  (Figure 3A); egg-spiked:  $F_{(1, 69)} = 7.11, p = 0.0010$  (Figure 3B)).

136

137 *Investigation of the sensory pathways required to detect cues of social exposure effects on*  
138 *post-mating fecundity*

139 To identify the sensory pathways used by focal females to detect the cues contained within  
140 female deposits identified as important above, we restricted olfactory, tactile/gustatory and  
141 visual inputs. Each sensory input test included socially isolated and group control treatments.  
142 In the olfactory restriction experiments, antennaless females laid significantly fewer eggs in  
143 the group versus isolation treatment ( $F_{(1, 62)} = 6.43, p = 0.014$ ), consistent with the  
144 unmanipulated controls (though in this control the group versus isolation comparison was  
145 marginally non-significant ( $F_{(1, 83)} = 3.58, p = 0.062$ ; Figure 4a). Antennal removal only  
146 partially restricts olfactory sensory pathways, since a secondary olfactory system is located in  
147 the maxillary palps which thus remained intact (Laissue & Vosshall, 2008). Therefore, to  
148 restrict olfactory senses more precisely, we complemented the antennal removal experiment  
149 by testing the responses of focal females with a knockout mutation in the broadly expressed  
150 olfactory receptor, *Orco*, which is associated with volatile pheromone sensing (Larsson *et al.*,  
151 2004). As with antennaless females, *Orco* knockout females maintained significant fecundity  
152 responses to their social environment comparable with those of wild type controls (*Orco*:  $F_{(1,$   
153  $66)} = 5.13, p = 0.027$ , control:  $F_{(1, 88)} = 4.22, p = 0.043$ ; Figure 4b).

154 In tests of tactile and gustatory cues, focal females were separated from non-focals in  
155 the same vial using a perforated acetate divide. When direct contact with other females was  
156 restricted in this way, there was no significant difference in fecundity between grouped and  
157 isolated females ( $F_{(1, 84)} = 0.05, p = 0.82$ ), in contrast to the control ( $F_{(1, 81)} = 9.31, p =$   
158 0.0031; Figure 4c).

159 To manipulate visual input cues, we used either wild-type focal females held in  
160 darkness throughout the social exposure period, or vision-defective *white* focal females held  
161 under normal conditions (Ferreiro *et al.*, 2018). Females held in darkness showed the same  
162 significant fecundity responses to social environment as did the control (darkness:  $F_{(1, 86)} =$   
163  $11.56, p = 0.001$ ; control:  $F_{(1, 82)} = 15.97, p = 1.40 \times 10^{-4}$ ; Figure 4d). In contrast, *white* focal  
164 female fecundity was unaffected by social environment (*white*:  $F_{(1, 87)} = 0.21, p = 0.65$ ;  
165 Figure 4d).

166

### 167 *Effect of social environment on virgin egg retention*

168 To test for any potential associations of pre- and post-mating fecundity plasticity we also  
169 examined the number of eggs laid by isolated and grouped females prior to mating. Eggs laid  
170 by the focal female in the group treatment were distinguished from those of the non-focal by  
171 dyeing non-focal females with Sudan Red. Thus focal eggs were white and non-focal eggs  
172 were pink. We analysed the egg count data in two steps. First, we split the data into two  
173 groups – ‘layers’ ( $\geq 1$  egg laid by focal) or ‘retainers’ (zero eggs laid by focal) and compared  
174 the likelihood of focal females from the two social treatments to lay at least one egg. Second,  
175 we excluded all zero-counts from the data and compared the numbers of eggs laid by ‘layers’  
176 between the social treatments. For days 1 and 3 of social exposure, isolated females were  
177 significantly more likely to retain virgin eggs (i.e. lay zero eggs) than were grouped females  
178 (day 1:  $X^2_1 = 17.8, p = 2.43e-05$ ; day 3:  $X^2_1 = 11.5, p = 0.0007$ ; Table S2). There was no  
179 significant difference on day 2 ( $X^2_1 = 1.3, p = 0.26$ ). Combining data across the 72h period,  
180 isolated females were more likely to retain their eggs than were grouped females ( $X^2_1 = 12.2,$   
181  $p = 0.00048$ ; Figure 5a). Of the ‘layers’, isolated females laid significantly more eggs on day  
182 1 than did grouped females ( $F_{(1, 53)} = 6.31, p = 0.015$ ). However, egg counts did not vary  
183 significantly with social treatment on days 2 or 3 or when all days were combined (day 2:  $F$



184  $(1, 35) = 1.98, p = 0.17$ ; day 3:  $F_{(1, 40)} = 0.74, p = 0.39$ ; combined:  $F_{(1, 67)} = 0.13, p = 0.72$ ;  
185 Figure 5b). Analysis of the fecundity of these same females after mating showed that,  
186 consistent with previous experiments, grouped females laid significantly fewer eggs post-  
187 mating than did isolated females ( $F_{(1, 86)} = 13.35, p = 4.43 \times 10^{-4}$ ; Figure S1). In both social  
188 treatments, there was a negative relationship between the number of pre- and post-mating  
189 eggs laid (isolation:  $F_{(1, 45)} = 18.16, p = 1.03 \times 10^{-4}$ ; group:  $F_{(1, 39)} = 4.34, p = 0.044$ ; Figure  
190 6). This was true for isolated females when both layers and retainers were included in the  
191 analysis, and when only layers were considered (Figure S2).

192

### 193 *Effect of social environment on mating latency and duration*

194 Mating latency varied significantly with social environment in the control groups in five of  
195 the nine experiments (Figure S3, Table S3). In those five cases, previously grouped females  
196 were slower to mate than isolated females. Mating duration did not vary with social treatment  
197 in eight of the nine control experiments (Table S4). The exception was the 72h timepoint  
198 from the “length of social exposure” experiment in which previously grouped females had a  
199 significantly shorter mating duration than isolated females (Figure S4). Overall, there  
200 appeared to be no consistent effect of social exposure treatment on mating latency or mating  
201 duration.

202

## 203 **Discussion**

204 The results show that female fecundity is strikingly plastic and varies according to the  
205 intrasexual social environment. Females exposed to groups of con- or heterospecific females  
206 in the pre-mating social environment showed significantly reduced post-mating fecundity  
207 compared to isolated females. Between 48-72h of exposure was required for fecundity to vary  
208 plastically. Direct contact with deposits left behind by previous females was sufficient to

209 stimulate this plastic response, suggesting that the relevant cues are detected using tactile or  
210 gustatory pathways. Virgin egg retention was significantly higher among isolated in  
211 comparison to grouped females, leading to a negative relationship between virgin and post-  
212 mating fecundity, regardless of social treatment.

213

214 *Female fecundity varies plastically according to the con- and heterospecific social*  
215 *environment*

216 The results reveal that the pre-mating social environment of female *D. melanogaster*  
217 significantly affects post-mating fecundity (see also Churchill *et al.*, 2021). Such plasticity is  
218 expected to have profound fitness consequences for both the female experiencing the social  
219 environment and her mate. Females responding to others in their environment may gain  
220 benefits by optimising oviposition sites and food availability for offspring or through access  
221 to antimicrobials or anti-cannibalistic molecules deposited by other females or on the surface  
222 of eggs (Marchini *et al.*, 1997; Narasimha *et al.*, 2019). The presence of other adults and  
223 larvae at oviposition sites is known to have a significant impact on larval survival. Higher  
224 adult densities at oviposition sites lead to increased larval survival (Ashburner, 1989;  
225 Wertheim *et al.*, 2002), likely through the suppression of fungal growth, but very high larval  
226 densities create competition and also lead to a lower larval survival rate (Wertheim *et al.*,  
227 2002). Therefore, a potential benefit of plasticity is that females adjust their oviposition rate  
228 in grouped situations to balance benefits of the suppression of microbial infection versus  
229 competition experienced by their larvae. The pattern we observed is consistent with potential  
230 benefits for grouped females in avoiding competition at oviposition sites by laying fewer  
231 eggs, and for isolated females to achieve density-dependent benefits by laying more. It is also  
232 possible that females alter their fecundity in order to benefit explicitly from the production of  
233 public goods. For example, in grouped situations, females might calibrate their fecundity to

234 the level where they optimise benefits from the amount of tunnelling in the food medium and  
235 production of diffusible antimicrobials or anticannibalistic molecules (Marchini *et al.*, 1997;  
236 Narasimha *et al.*, 2019). Another explanation for grouped females laying fewer eggs after  
237 mating could be that they trade off offspring quantity for quality in environments where they  
238 expect their offspring to be in competition. It would be interesting to test for any such  
239 maternal effects by measuring offspring fitness traits.

240         Interestingly, the fecundity effect was not restricted to the conspecific social  
241 environment, as exposure of *D. melanogaster* females to either *D. simulans* or *D. yakuba*  
242 females also resulted in significantly reduced post-mating fecundity. Both *D. simulans* and *D.*  
243 *yakuba* are members of the *melanogaster* species subgroup, there is geographical overlap in  
244 the ranges of their populations, and all three species are generalists requiring rotting fruit for  
245 oviposition (Markow & O'Grady, 2005). The cues required for eliciting social responses may  
246 be conserved across this subgroup, with fecundity plasticity being triggered by the presence  
247 of any other females displaying these cues. Other types of sensory cues, such as chemical or  
248 pheromonal are known to be shared across closely related species. For example, aggregation  
249 pheromones across *D. melanogaster*, *yakuba* and *simulans* appear identical (Symonds &  
250 Wertheim, 2005) and attract heterospecifics as well as conspecifics in the field (Jaenike *et al.*,  
251 1992; Wertheim, 2001). There could be benefits to individuals from responding to cues  
252 emanating from heterospecifics if resources are shared and thus if the heterospecific cues  
253 signal resource quality or expected levels of competition for those limited resources. For  
254 example, larval resources may be exploited by several different species and so oviposition  
255 decisions based on the presence of heterospecifics could minimise over exploitation and have  
256 important fitness effects (Wertheim, 2005; Wertheim *et al.*, 2002; Wertheim *et al.*, 2002). We  
257 suggest that plasticity allows females to optimise their egg laying when oviposition and larval  
258 resources are likely to be utilised by closely-related species in sympatry. Interestingly, male

259 *D. melanogaster* respond plastically to the presence of con- and some heterospecific males  
260 (*D. simulans* and *D. pseudoobscura*) but not others (*D. yakuba* or *D. virilis*) by increasing  
261 mating duration. However, the heterospecific responses when present do not occur to the  
262 same extent as following conspecific exposure (Bretman *et al.*, 2017), likely because male  
263 responses to heterospecifics would carry costs but apparently little benefit (since  
264 heterospecifics pose minimal sperm competition). For females however, the consequences of  
265 basing oviposition decisions on the presence of heterospecifics or conspecifics may not differ  
266 markedly.

267

#### 268 *Females require between 48-72h of social exposure to express fecundity plasticity*

269 Responses by females to their social environments were not instantaneous, and appear to be  
270 longer than for the behavioural plasticity reported in males (Bretman *et al.*, 2010). The  
271 precise social environment adult flies experience in the wild is likely to be subject to rapid  
272 changes, as flies eclose, move between patchy food resources or die. Such rapid variation  
273 may not provide a reliable indication of resource levels for females, thus setting up the  
274 requirement for a longer threshold of exposure to cues before decisions about potentially  
275 costly reproductive investment are triggered. Therefore, it is likely that the types of social  
276 responses seen in this study only benefit females if the social environment is sustained and  
277 thus accurately signals resource levels. We suggest that transient changes in social  
278 environment are unlikely to represent accurate indicators of resource quality to an even  
279 greater extent for females than males (Rouse & Bretman, 2016).

280

#### 281 *Non-egg deposits from previous vial occupants stimulate the fecundity response*

282 Interestingly, non-egg derived deposits left behind by other females were sufficient to  
283 stimulate post-mating fecundity responses. Of relevance is the observation that residual cues

284 from either sex can also influence egg placement decisions in *D. melanogaster* (Malek &  
285 Long, 2020). Cues could include pheromones or microbes deposited from the cuticle or in the  
286 insect excreta (frass). Reproductively mature, virgin females harbour 50 types of cuticular  
287 hydrocarbon (CHC) and fatty acid molecules (Billeter & Wolfner, 2018). Female frass also  
288 contains CHCs such as methyl laurate, methyl myristate and methyl palmitate, and responses  
289 to deposited frass are reported to lead to increased feeding and aggregation (Keesey *et al.*,  
290 2016). Chemical cues are likely to be sensed by olfactory or gustatory sensory pathways, and  
291 indeed olfactory receptors were found to be partly responsible for behavioural changes in  
292 response to frass (Keesey *et al.*, 2016). Frass deposits could provide a persistent and accurate  
293 indicator of the local population density and composition, and thus a more accurate indicator  
294 of potential resource levels as opposed to detection of the numbers of flies present at any  
295 given time, which could fluctuate rapidly.

296

297 *Direct contact with social cues is required, suggesting the use of gustatory sensory pathways*

298 Females that were physically separated from other flies and eggs did not differ in fecundity  
299 from isolated females. Combined with our finding that non-egg derived female deposits are  
300 sufficient to stimulate plastic fecundity responses, these results suggest the gustatory (rather  
301 than tactile) pathways are used by females to respond to their social environment. Previous  
302 studies have found that female flies use sensory receptors located in their legs, ovipositor and  
303 proboscis to sample egg laying sites (Yang *et al.*, 2008) and integrate olfactory and gustatory  
304 cues to make egg-laying decisions. Visual cues appeared not to be necessary; however,  
305 visually compromised *white* females did not exhibit fecundity plasticity. Possible  
306 explanations include pleiotropic effects of the *white* eye mutation such as impaired memory  
307 (Sitaraman *et al.*, 2008), or compromised gravitaxis (Armstrong *et al.*, 2006). That gustatory  
308 cues alone appear to be sufficient for females to assess and respond to social cues is in

309 contrast to the multimodal strategy seen in males (Bretman *et al.*, 2011). This may reflect the  
310 complexity of information required to make the appropriate response in each sex or the type  
311 of plastic phenotype involved.

312

313 *The social environment alters virgin egg retention*

314 Isolated virgin females were more likely to retain eggs than those held in a group. This may  
315 be an adaptive strategy to conserve resources during long non-reproductive periods  
316 (Bouletreau-Merle & Fouillet, 2002) or when high quality oviposition sites are unavailable.  
317 Our finding that female *D. melanogaster* are more likely to retain virgin eggs in social  
318 isolation is consistent with observations for the tephritid *Rhagoletis pomonella* (Prokopy &  
319 Bush, 1973) and may indicate that a social stimulus is required for females to initiate  
320 ovulation. A benefit of high virgin egg retention was increased fecundity following mating,  
321 consistent with previous findings (Edward *et al.*, 2014).

322

323 *Mating behaviour was not consistently affected by social environment in females*

324 The effects of social exposure on mating latency were inconsistent, as is also found in males  
325 (Bretman *et al.*, 2009; Bretman *et al.*, 2013; Bretman *et al.*, 2013; Dore *et al.*, 2020).  
326 Individuals may be differentially susceptible to environmental differences between  
327 experiments or changing population dynamics in the stock cages from which they were  
328 collected. In almost all cases mating duration was unaffected by female social environment.  
329 This contrasts with the corresponding plasticity seen in males (Bretman *et al.*, 2009) and  
330 reflects the finding that mating duration is largely under male control (Bretman *et al.*, 2013).  
331 Additionally, it suggests that males do not respond to the social environment of their mate  
332 despite potential fitness costs if the female has lowered fecundity.

333

334 **Conclusions**

335 These results represent a significant advance in knowledge of how the intrasexual social  
336 environment affects female reproduction. We investigated responses to both con- and  
337 heterospecifics, the length of exposure required to express plasticity, and the cues and  
338 mechanisms underlying the fecundity response. We found that the social environment does  
339 indeed have the potential to affect female fitness. A key, important outcome is that the  
340 responses, timing and nature of cues used are markedly different in females vs males, and this  
341 likely reflects the contrasting benefits of reproductive plastic behaviour between the sexes.

342

343

344 **Methods**

345 *Fly stocks and handling*

346 Wild type *D. melanogaster* flies were from a large laboratory population originally collected  
347 in the 1970s in Dahomey (Benin) and maintained in stock cages with overlapping  
348 generations. Wild type *D. simulans* and *D. yakuba* were obtained from the San Diego  
349 *Drosophila* Stock Center and KYORIN-Fly *Drosophila* species stock centre (stock #k-s03),  
350 respectively. Flies were reared on standard sugar yeast (SY) medium (100 g brewer's yeast,  
351 50 g sugar, 15 g agar, 30 ml Nipagin (10% w/v solution), and 3 ml propionic acid, per litre of  
352 medium) in a controlled environment (25°C, 50% humidity, 12:12 hour light:dark cycle). For  
353 the Sudan Red food medium, 800 ppm Sudan Red 7B (*Sigma Aldrich*) dye was added to the  
354 SY diet before dispensing. Eggs were collected from population cages on grape juice agar  
355 plates (50 g agar, 600 ml red grape juice, 42 ml 10% w/v Nipagin solution per 1.1 l H<sub>2</sub>O)  
356 supplemented with fresh yeast paste, and first instar larvae were transferred to SY medium at  
357 a standard density of 100 per vial (glass, 75x25mm, each containing 7ml medium). Male and  
358 female adults were separated within 6h of eclosion under ice anaesthesia and stored in single

359 sex groups of 10/vial. *White* females were from a stock carrying the  $w^{1118}$  allele that had been  
360 backcrossed three times into the Dahomey wild type. *Orco* females were generated from  
361 backcrossing *Orco*<sup>1</sup> (Bloomington Drosophila Stock Centre, stock #23129) stock for three  
362 generations into a Dahomey stock carrying the *TM3 sb ry* balancer on chromosome 3.  
363 Eggless females were generated by crossing males from the *Ovo*<sup>D1</sup> stock (Bath *et al.*, 2017)  
364 with wild type Dahomey females.

365

366 *Effect on female mating behaviour and fecundity of variation in pre-mating social*  
367 *environment*

368 In all experiments, virgin focal *D. melanogaster* females were CO<sub>2</sub> anaesthetised at 3-4 days  
369 old and assigned to isolation (1 female per vial) or group (1 focal and 3 virgin non-focal  
370 females per vial) social treatments. Females were exposed to these social environments for a  
371 period of 72h (unless stated otherwise) prior to mating. Wildtype males were aspirated  
372 individually into fresh SY vials the day prior to the mating trial. Mating trials were conducted  
373 at 25°C at 50% RH, always starting at 9 am in the morning unless otherwise stated. On the  
374 day of mating, focal females were aspirated into vials containing a single male. Pairs were  
375 observed and the introduction time, start and end of mating were recorded. Any flies that did  
376 not start mating within 90 min were discarded. Males were removed immediately following  
377 the end of copulation and females left to oviposit for 24h before being discarded. Eggs laid  
378 on the surface of the SY medium in this 24h period were counted under a Leica MZ7.5  
379 stereomicroscope. Sample sizes for all experiments are shown in Table S1.

380

381 *Female fecundity responses to variation in the social environment and effect of exposure to*  
382 *con- vs hetero-specific females*



383 Following the protocol as described above, focal wildtype *D. melanogaster* females were  
384 kept in isolation or housed with 3 non-focal females of the same or two different *Drosophila*  
385 species. We chose as heterospecific treatments two species of the *melanogaster* subgroup - *D.*  
386 *simulans* and *D. yakuba*, which shared their last common ancestor with *D. melanogaster* ~5  
387 MYA and ~13 MYA, respectively (Tamura *et al.*, 2004). Non-focal females were wing-  
388 clipped under CO<sub>2</sub> anaesthesia prior to setting up the social exposure treatments, in order to  
389 distinguish them from the focal *D. melanogaster* individuals.

390

#### 391 *Effect of length of social exposure period on post-mating fecundity*

392 The experiment was set up following the standard protocol above, with wildtype Dahomey  
393 focal and non-focal females, but with varying lengths of social exposure before mating. To  
394 test the effect on post-mating female fecundity from shorter term exposure, all females were  
395 placed into the social environments in parallel (between 9 and 10am on the day of the mating  
396 trials), then subsets of focal females were mated after 2, 4 or 8h. Therefore, these matings  
397 were conducted at different times of the day (2h at 12pm, 4h at 2pm, and 8h at 6pm). Longer-  
398 term exposure was tested in a separate experiment. Again, all social environments were set up  
399 in parallel, then mating trials on subsets of focal females were conducted after 24, 48 and  
400 72h, all at 9am each day.

401

#### 402 *Investigation of whether exposure to eggs or to female deposits in the absence of eggs are* 403 *required for social exposure effects on post-mating fecundity*

404 This experiment was carried out in two sets. In the first, we tested whether exposure to eggs  
405 of other females, or deposits of other females in the absence of eggs, were required for  
406 females to show plastic fecundity responses after mating. To do this we used non-focal  
407 females from the *Ovo<sup>Dl</sup>* (eggless) genotype. Wildtype focal females were kept alone

408 (isolation), exposed to 3 wildtype non focal conspecifics (group), 3 eggless *Ovo<sup>D1</sup>* non-focal  
409 females (group - eggless females), or to an SY vial that had previously housed 3 eggless  
410 *Ovo<sup>D1</sup>* females for the preceding 24h (isolation - female deposits). In the second set, wildtype  
411 focal females were again kept alone (isolation), exposed to 3 wildtype non focal conspecifics  
412 (group) or exposed to eggs laid in the previous 24h by three wildtype non-focals (isolation -  
413 egg-spiked). In both experiment sets, all focal females were moved to “fresh” (deposits, egg-  
414 spiked or clean food) vials every 24h of the exposure period to maintain the strength of the  
415 specific cues involved.

416

417 *Investigation of the sensory pathways required to detect cues of social exposure effects on*  
418 *post-mating fecundity*

419 To identify the sensory pathways used by females to detect the proxies of female presence  
420 described above, we conducted three sets of experiments, each with standard isolation and  
421 group control treatments. To test the effect on post mating fecundity of manipulating visual  
422 inputs, we used either wildtype females held in darkness, or visually-defective *white* focal  
423 females held under normal light conditions (Ferreiro *et al.*, 2018). Non-focal females were all  
424 wildtype. To test the effect of manipulating olfactory cues we used focal females with a  
425 knockout mutation in the *Orco* gene (encoding a broadly expressed odorant receptor,  
426 essential for olfaction of a wide range of stimulants (Larsson *et al.*, 2004)), or we surgically  
427 removed the third antennal segment of wildtype focal females under CO<sub>2</sub> anaesthesia one day  
428 prior to setting up the social treatments. The antennal segment contains sensillae bearing  
429 odorant receptors, but also aristaes that detect sound (Göpfert & Robert, 2001; van der Goes  
430 van Naters & Carlson, 2007). Non-focal females for both olfactory experiments were  
431 wildtype females with intact antennae, which were wing-clipped under CO<sub>2</sub> anaesthesia one  
432 day prior to social exposure. Finally, to test the effect of manipulating tactile cues, we

433 physically separated wildtype focal females from non-focals using a perforated acetate  
434 divider to create two chambers within a standard vial. Perforations allowed the transmission  
435 of sound and odours, and the dividers were translucent which allowed for the perception of  
436 visual cues.

437

#### 438 *Effect of social environment on virgin egg retention*

439 In the final experiment we used a novel egg marking procedure to test the effect of isolation  
440 and group treatments on pre-mating (virgin) egg production and retention. Wild type focal  
441 females were reared according to the standard protocol. Non-focal females were reared from  
442 the 1<sup>st</sup> instar larval stage on SY food containing 800 ppm oil-based Sudan Red dye, which  
443 stains lipids, resulting in the production and laying of visibly pink eggs as adults. Dyed  
444 females were collected upon eclosion and maintained on Sudan Red food for 3-4 days prior  
445 to setting up the social treatments. Social treatments were set up according to the standard  
446 protocol, above. For the group treatment, one focal female was housed in a vial with three  
447 dyed non-focals. Females were then moved every 24h to fresh food until mating. The number  
448 of white and dyed (pink) eggs laid by the focal and non-focal females, respectively, was  
449 recorded for each 24h period of social exposure. Mating trials and post-mating egg counts  
450 were conducted as above.

451

#### 452 *Statistical analysis*

453 Statistical analyses were carried out in R v 3.6.3 (R Core Team, 2013). Post-mating egg  
454 counts were analysed using a generalised linear model (GLM) with a log link and quasi-  
455 Poisson errors to account for over-dispersion. The total number of virgin ‘egg layers’  
456 (females that laid  $\geq 1$  egg on a given day) versus ‘retainers’ (no eggs laid on a given day) in  
457 each social treatment was analysed using a Chi-square test. The number of virgin eggs laid by

458 ‘egg-layers’ (non-zero counts) across social treatments was analysed using a GLM with  
459 quasi-Poisson errors. Significance values for GLMs were derived from an anova F test of the  
460 model. Mating latency was analysed using Cox Proportional Hazards models, fitted using the  
461 “coxph” function from the “survival” package. Individuals that did not mate within 90  
462 minutes were treated as censors. For mating duration, times of < 6 min and > 30 min were  
463 excluded from the analysis. These data points represent extremely short copulations, in which  
464 genitalia were unlikely to have been fully engaged or sperm transferred (Gilchrist &  
465 Partridge, 2000). Very long copulations can result if genitalia become “stuck” and flies fail to  
466 disengage. In total, 11 such outliers were removed from across five of the mating duration  
467 experiments (supplementary table S2). Mating duration data were normally distributed for  
468 each experiment (Shapiro-Wilk tests,  $p > 0.05$ ) and were analysed using Welch two sample  $t$ -  
469 tests.

470

471

472 **Authors’ contributions.** EKF, AB and TC conceived the study, EKF, SL, WR and AT  
473 conducted the experiments and analyses, EKF analysed the data and EKF, SL and TC wrote  
474 the paper. All authors read and approved the final version of the manuscript.

475

476 **Competing interests.** We declare we have no competing interests.

477

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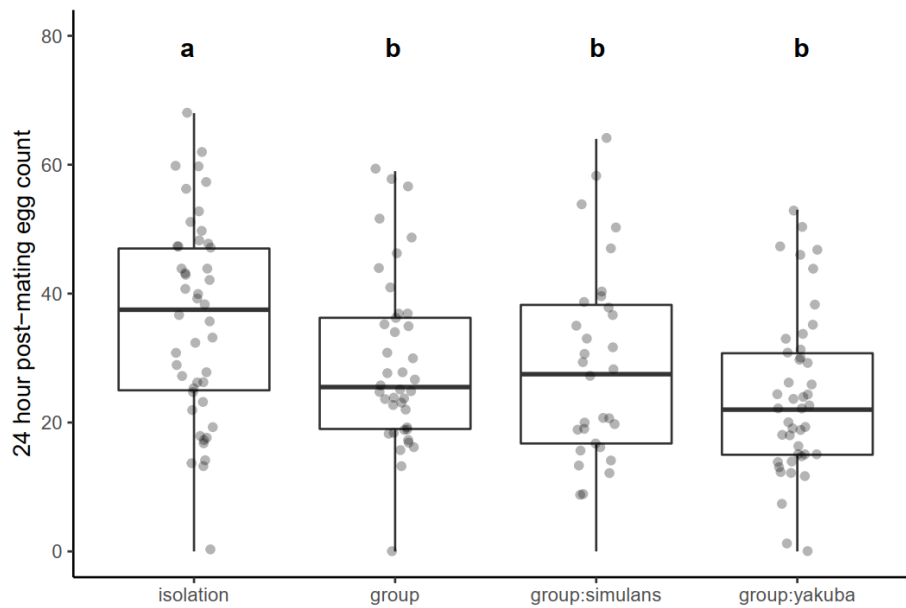
483 assistance and Ellie Bath for sending us the *OvoDI* strain.

484

485 **Statement on data sharing.** All raw data will be made available on the DRYAD data  
486 repository upon acceptance. We will also provide a private data sharing link to the raw data, if  
487 requested by the reviewers.

488

489



490

491 **Figure 1. *D. melanogaster* females exposed to con- or hetero-specific females prior to**

492 **mating show significantly decreased post-mating fecundity. *D. melanogaster* females**

493 were kept socially isolated ('isolation') or exposed to con- ('group') or hetero-specific

494 females ('group:simulans' or 'group:yakuba') for 72h prior to mating. Fecundity was

495 measured as the number of eggs laid by each female in the 24h period following mating.

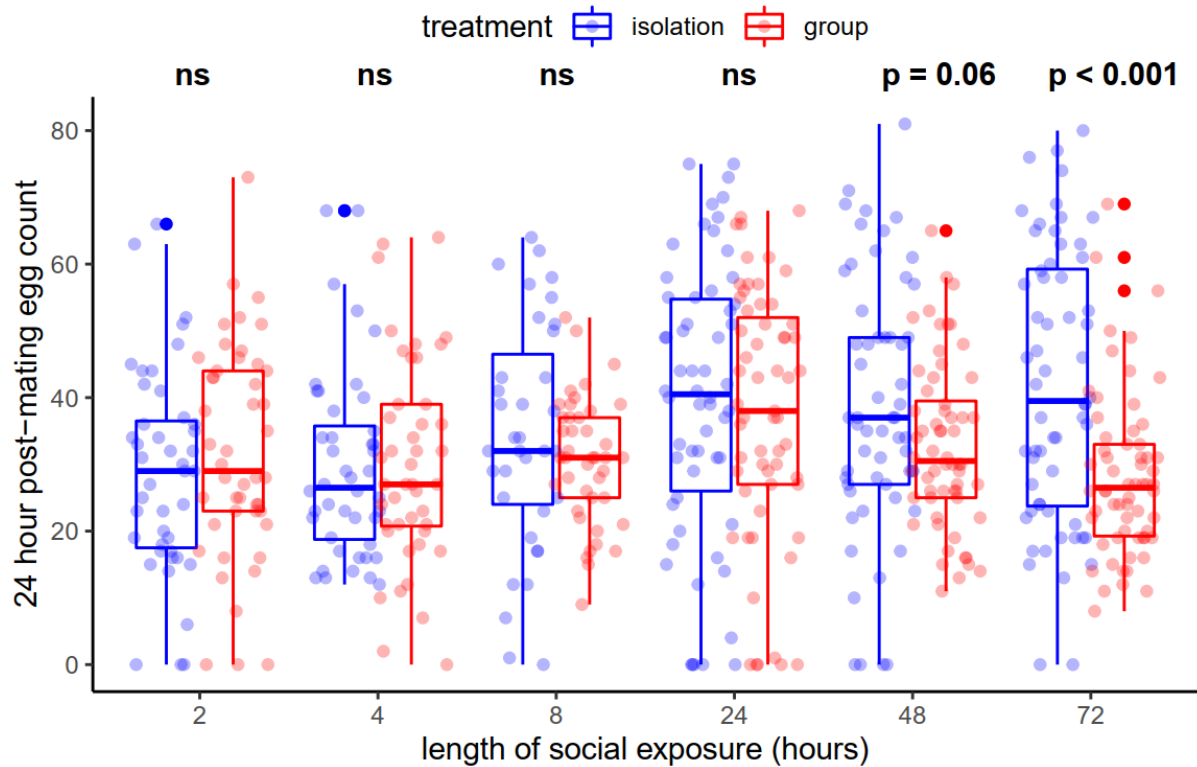
496 Boxplots show interquartile range (IQR) and median in the box, and whiskers represent the

497 largest and smallest values within 1.5 times the IQR above and below the 75<sup>th</sup> and 25<sup>th</sup>

498 percentiles, respectively. Raw data points are plotted with jitter. Treatments not sharing a

499 letter are significantly different from one another ( $p < 0.05$ ).

500



501

502 **Figure 2. *D. melanogaster* females require 72h of exposure to conspecifics to express**

503 **fecundity plasticity.** Females were housed in 'isolation' (blue) or in 'group' (red boxes)

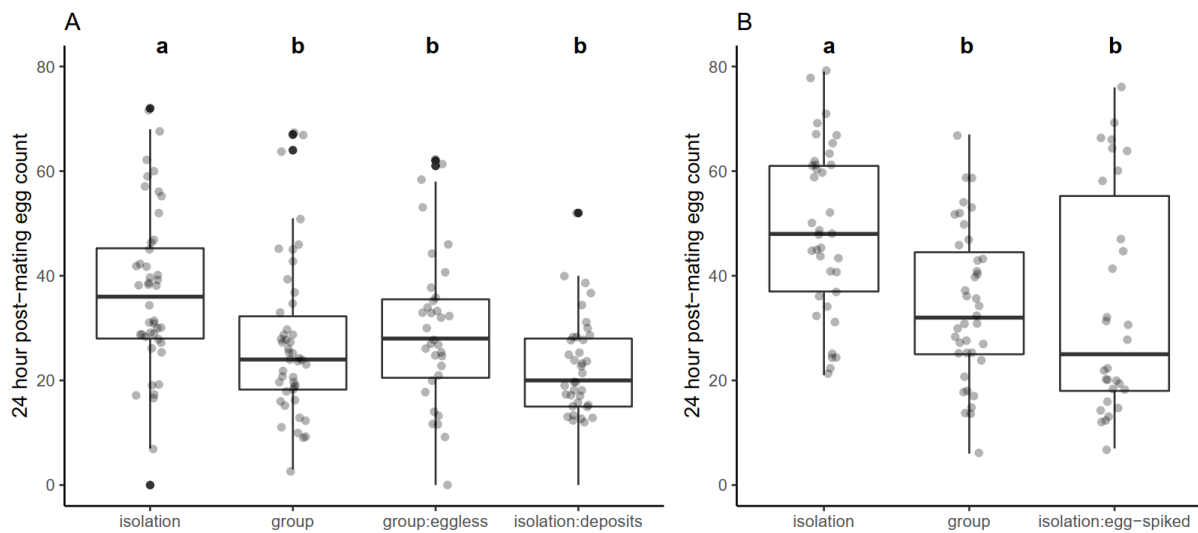
504 treatments, for between 2h and 72h prior to mating. Fecundity was measured as the number

505 of eggs laid in the 24h period following mating. Statistical significance indicated above box

506 pairs (ns:  $p < 0.1$ ). Boxplots as in Figure 1.

507

508



509

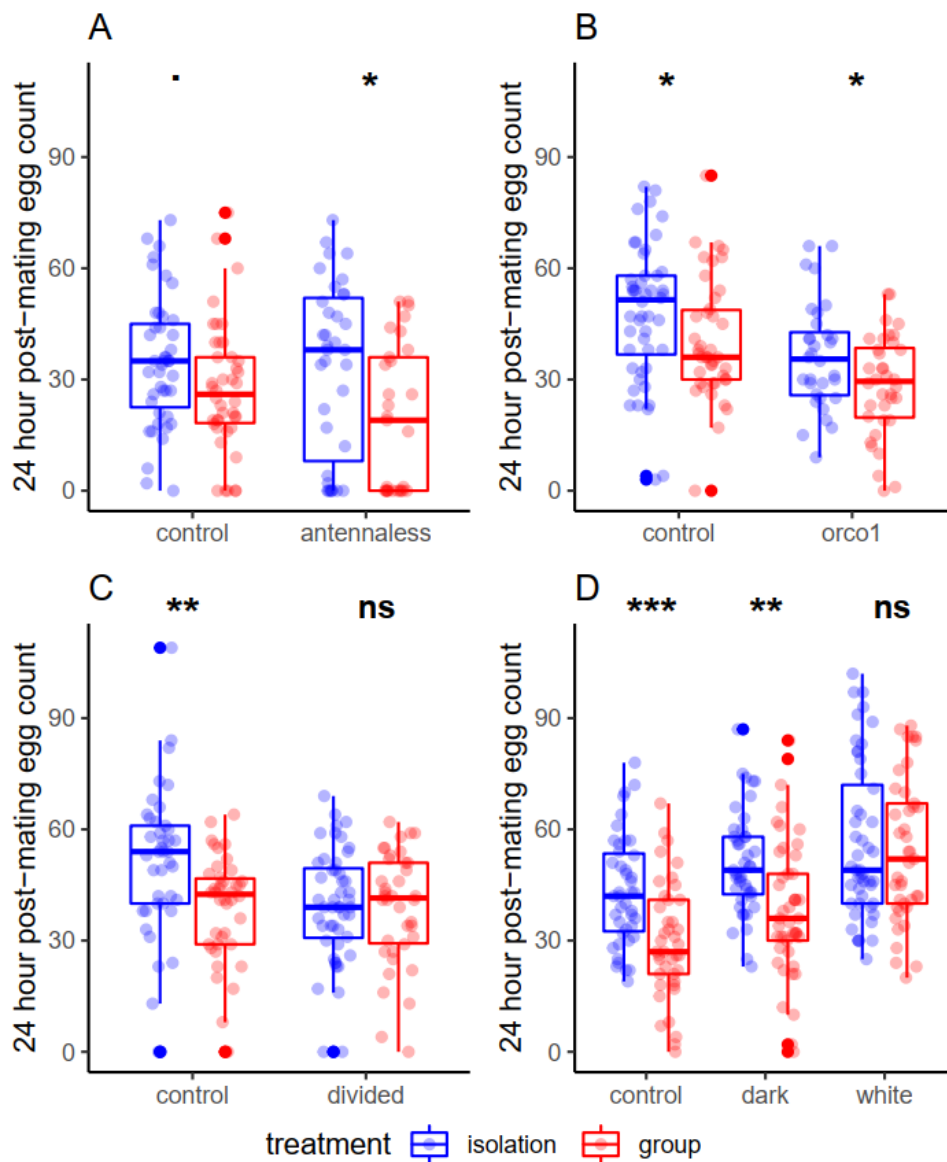
510 **Figure 3. *D. melanogaster* females respond to their social environment by detecting the**  
511 **deposits left by other females, even in the absence of eggs.** (A) Wildtype focal females  
512 were either isolated in clean vials ('isolation'), housed in groups of four in clean vials  
513 ('group'), housed with three *OvoD1* females ('group:eggless') or housed in vials previously  
514 occupied by three *OvoD1* females ('isolation:deposits'). (B) Wildtype focal females housed  
515 in isolation, in groups of four or in vials containing eggs laid by previous wildtype occupants  
516 ('isolation:egg-spiked'). Fecundity was measured as the number of eggs laid by the focal  
517 female in the 24h period following a single mating. Boxplots as in Figure 1. Within each plot,  
518 treatments not sharing a letter are significantly different from one another ( $p < 0.05$ ).

519

520



521



522

523 **Figure 4. *D. melanogaster* females respond to their social environment by using tactile /**

524 **gustatory sensory pathways. (A)** Olfactory restriction through antennal removal. Intact

525 focal females ('control') and olfactory-manipulated focal females with no third antennal

526 segment ('antennaless') were kept in isolation or in a group with three intact non-focal

527 females. (B) Olfactory restriction through *Orco* knockout. Wildtype Dahomey females

528 ('control') or females lacking the general olfactory receptor *Orco* (*orco*<sup>1</sup>) were kept in

529 isolation or in a group with three Dahomey non-focal females. (C) Tactile/gustatory

530 restriction. Focal females were housed in a standard vial ('control') or in a vial with a

531 transparent, perforated divide ('divided'). For the divided group treatment, focal females  
532 were physically separated from the three non-focals by the divide. (D) Visual restriction.  
533 Wildtype females held under standard light conditions ('control'), wildtype females held in  
534 darkness ('dark') and *white* females ('white') were kept in isolation or exposed to three  
535 wildtype non-focal females. Fecundity was measured as the number of eggs laid in the 24h  
536 period following mating. Boxplots as in Figure 1.

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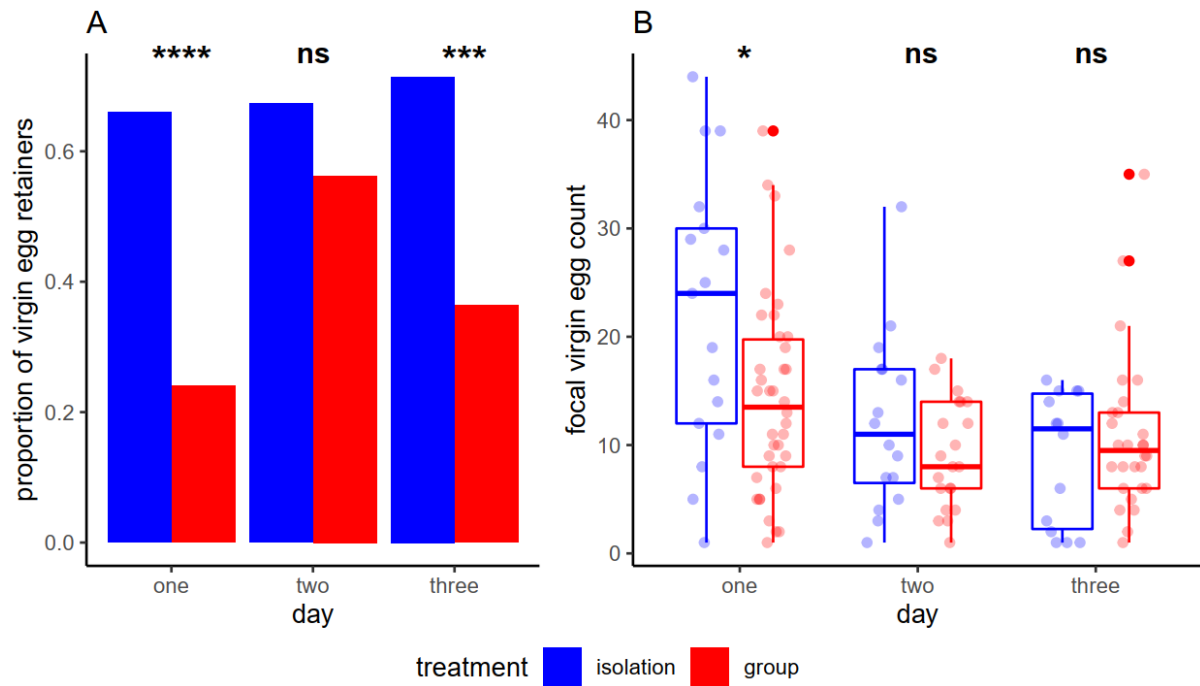
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548 **Figure 5. *D. melanogaster* females housed in isolation are more likely to retain virgin**

549 **eggs.** Virgin egg laying responses of *D. melanogaster* to the current social environment are

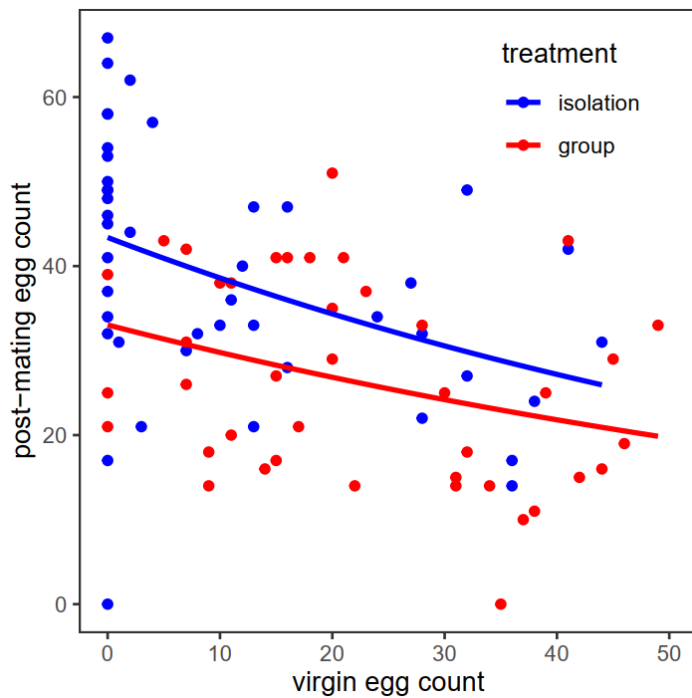
550 shown. Focal females were kept in ‘isolation’ (blue bars/boxes) or ‘group’ (housed with three

551 dyed non-focal females, red bars/boxes) treatments, for three days. (A) The proportion of

552 female egg retainers (laying no eggs) on days one, two or three of social exposure. (B) Virgin

553 egg counts of laying females (laying  $\geq 1$  egg on any given day) over three days of social

554 exposure. Boxplots as in Figure 1.



555

556 **Figure 6. Negative relationship between pre- and post-mating fecundity in socially**  
557 **isolated and grouped females.** Shown is the relationship between the total number of virgin  
558 eggs laid by a focal female in the three days prior to mating, and the number of post-mating  
559 eggs laid for 24h after mating. Focal females were held in either ‘isolation’ (blue) or in  
560 ‘group’ (with three Sudan red dyed non-focal females prior to mating, shown in red)  
561 treatments.

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