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26 sex, not simply generic social stimulation, may be an important evolutionary driver for
27 cognitive ability in fruit flies.

28 **Keywords**

29 Cognition, sperm competition, learning, memory

30

31 **Introduction**

32 Cognition is defined as the neural processes needed to acquire, process, retain and use
33 information [1], including processes such as learning, memory and decision making [2].
34 However, whilst the processes by which brains have evolved, and how the environment
35 affects the cognition of animals has been widely researched, a consensus about the critical
36 drivers of cognitive evolution has not yet emerged [3].

37 Social contact has long been hypothesised to affect the evolution of cognition [4].
38 The Social Brain Hypothesis postulates that the evolution of improved cognition is driven by
39 increasing social group complexity [5]. However, types of social interactions are varied and
40 which are important for cognition to evolve is controversial [5, 6]. The role for sexual
41 competition, in particular, has proven to be problematic. In mammals an increase in sexual
42 competition was shown to have no, or a negative effect on brain size, the notional proxy for
43 cognitive ability [7]. However, in frogs [8] and pipefish [9], differing levels of sexual
44 competition has been associated with morphological differences in brain structure. Similarly,
45 in the rose bitterling, plastic male mating strategies are associated with cognitive
46 performance [10], and in bowerbirds increased complexity of displays correlates with an
47 enlarged cerebellum [11]. In addition, in species where females are under sexual selection
48 instead of males they display heavier brains [9]. In the fruit fly (*Drosophila melanogaster*)
49 and seed beetle (*Callosobruchus maculatus*), enforced monogamy over multiple generations
50 reduces cognitive performance compared to males kept in polygamous conditions [12, 13].
51 As the social environment is dynamic, males need to assess and predict competition after

52 mating (sperm competition), which is likely cognitively challenging. Indeed, it is predicted to
53 be an evolutionary driver of the ability to assess quantity i.e. the magnitude of competition
54 within the environment (quantity estimation [14]).

55 In *D. melanogaster*, males alter their mating duration and ejaculate [15, 16] in
56 response to exposure to other males, a cue of future sperm competition threat. Males can
57 track changes in their social environment and are sensitive to the amount of time spent with
58 other males [17]. Males also alter ejaculate depending on the number of males in the
59 environment [18]. This sophisticated, multifaceted response requires males to assess the
60 magnitude of competition akin to quantity estimation [14]. The response requires multiple
61 sensory inputs [19] and utilises processes known to be important in learning and memory
62 [17]. We therefore hypothesised that competitive sexual interactions may be a vitally
63 important social driver of cognitive ability in male *D. melanogaster*.

64 Any form of environmental enrichment could be cognitively stimulating [20], so to
65 determine whether social reproductive competition cues are particularly important, we
66 compared responses to conspecifics and heterospecifics. *Drosophila* live in multiple species
67 groups [21], and this requires the ability to interpret different forms of social contact. *D. virilis*
68 and *D. melanogaster* are generalists and are expected to compete for food and/or space
69 [22], however they are very distantly related and are likely to be fully reproductively isolated
70 as this is seen between more closely related species [23]. Indeed, *D. melanogaster* males
71 do not make a sperm competition response to *D. virilis* males [24] indicating they do not
72 perceive them as a reproductive threat. Therefore, any differences seen in *D. melanogaster*
73 males after contact with conspecific males not seen due to contact with heterospecific
74 contact are suggestive of the importance of sexual competition.

75 Female and male social mechanisms are thought to differ [25], therefore we also
76 studied the effects of conspecific and heterospecific same-sex social contact in females. As
77 females do not undergo the same sperm competition pressures it was expected that any

78 difference shown by females in response to conspecific contact would be mirrored when
79 exposed to heterospecific social contact.

80 We used multiple assays of olfactory and visual learning and decision-making ability
81 of males and female flies held in isolation or exposed to a conspecific or heterospecific
82 social partner. To assess potential underlying molecular mechanisms, we measured changes
83 in the expression of genes associated with synaptic plasticity at the neuromuscular junction
84 (*Neurexin-1* [26]), growth (*Futsch* [27]) or maintenance (*Bruchpilot* [28]) and that we had
85 previously identified to be socially responsive in male-male contact [29]. We also assessed
86 how activity patterns changed depending on the social environment, as this is associated
87 with sleep and cognitive processes.

88

89 **Materials and Methods**

90 **Fly stocks and rearing**

91 *Drosophila melanogaster* fruit flies were raised in a 25°C humidified room, with a 12:12 light
92 dark cycle. Flies were maintained in plastic vials containing 7ml sugar-yeast-agar medium
93 [30]. Wild-type flies are from a large laboratory stock population of the Dahomey strain [31].
94 Larvae were raised 100 per vial and supplemented with live yeast. As *Drosophila virilis* have
95 a slightly lower thermal preference than *D. melanogaster* [32], *D. virilis* were grown in vials at
96 20°C before collection. Upon eclosion sexes were separated using ice anaesthesia. Non-
97 focal flies were collected in single-sex groups of ten and the females supplemented with live
98 yeast. All focal flies were aged individually for 1 day before exposure to a social partner.
99 Non-focal flies used as social partners were aged-matched and identified with a small wing
100 clip.

101 Flies were maintained in their social treatments, singly, with a conspecific or a
102 heterospecific of the same sex, for 10 days before being used in one of the assays detailed

103 below (Figure S1). We assessed learning in a range of assays, both sexual and non-sexual
104 context. Where possible, the observer was blind to the social identity of the focal fly.

105

106 *Virgin Finding – decision making in a complex environment*

107 We tested a male's decision making in a complex social environment, whereby males target
108 courtship towards a virgin female in a group of mated females [12]. Focal males were
109 aspirated into a group of six females, one virgin and five mated 24 hours prior. Male courting
110 behaviour was recorded every minute for 20 min, or until mating occurred. Female type was
111 identified by a wing-clip administered 2 days before a trial. Virgin females were clipped in
112 50% of the trials. Trials were conducted on 10 individuals at a time, 5 of each social
113 treatment, to control for time of day effects. This assay was carried out at 25°C under
114 standard white light. Each block contained 30 males from each social treatment and was
115 repeated 3 times, with separate experiments for conspecific and heterospecific contact.

116

117 *Aversive olfactory associative learning*

118 We tested the ability of flies to learn to associate an odour with a mechanical shock [33].
119 Experiments were performed at 22-25°C under red light to remove visual cues. Odorants, 3-
120 octanol (2.7µL/mL) and 4-methylcyclohexanol (1µL/mL) diluted in light mineral oil, were
121 drawn through a T-maze with a vacuum pump. The side the odorant originated from was
122 switched every second trial.

123 Firstly, innate preference for either odour was derived from the time spent in either
124 arm (not the central section of the T maze) over 2 min. A fly was then conditioned to avoid its
125 preferred odour by exposing it to the preferred odour accompanied by mechanical shock for
126 1 sec every 5 sec for 1 min. This was followed by a 30 sec exposure to air and then 1 min
127 exposed to the non-preferred odour without shock. This training protocol was repeated once.
128 To test learning, flies were immediately moved to the central section choice point and given

129 both odours simultaneously. The time an individual spent showing preference for either
130 odour was recorded over 2 min. A learning index (LI) was calculated as:

131 Equation 1

132
$$\frac{\text{post-training time in unconditioned odour} - \text{pre-training time in unconditioned odour}}{\text{post-training total time making a choice} - \text{pre-training total time making a choice}}$$

133
134
135

136 Each testing period assessed 2-8 flies, half held singly, repeated 6-8 times per social
137 treatment. To control for day and time effects the LI of individuals kept with social partners
138 was standardised by the average LI of singles in the same block. Separate experiments
139 were conducted for conspecific and heterospecific exposure.

140

141 *Visual learning and reversal learning*

142 This assay tests the ability of flies to associate a visual cue, a green dot, with a “safe”
143 temperature zone on the floor of an arena. Learning was assessed as the time a fly took to
144 spend 20 sec consecutively in the “safe” zone. The total distance travelled by the fly was
145 measured to control for directional learning. An increase in distance travelled would suggest
146 that flies were not learning visual cues, but were instead learning the presence of a safe spot
147 within the arena. Flies underwent 3 trials of 10 min. A reversal “probe” trial was then
148 performed by moving the visual cue to an “unsafe” area of the arena, and measuring the
149 time taken to spend time in the “safe” zone and the mean distance from the visual cue.

150

151 *Gene expression*

152 Flies were snap frozen in liquid nitrogen, head and thorax dissected away from the abdomen
153 on dry ice and frozen at -80°C before extraction. RNA was extracted from a pools of 10 to 25
154 flies using Direct-zol™ RNA miniprep columns. cDNA was synthesised using the Life

155 Technologies First strand cDNA kit. Housekeeper genes *EF1* and *Rap21* were chosen as
156 likely to be unaffected by social environment [34].

157 Quantification of transcript levels relative to housekeeping genes were performed
158 using the Pfaffl method [35]. Ct values for samples were quantified against the lowest value
159 across 5 to 7 biological replicates, taking into account the efficiency of the primer used.
160 Relative expression was calculated by dividing the gene of interest by the geometric mean of
161 the housekeepers for each sample, averaged across biological replicates [35].

162

163 *Behavioural analysis – activity, social interactions and aggression*

164 We observed behaviour of flies within their social treatments. Focal flies were held in their
165 social treatment for 5 days and behaviour recorded on days 6, 8 and 10, at 9am, 12 pm and
166 3pm. This was replicated in 4 blocks; each block contained 10 vials per sex and social
167 treatment (final n = 40). Focal flies were scored as active or not, and for those with a social
168 partner, whether they were within one body length of that partner (proximity) and whether the
169 flies interacted aggressively (wing flicking and fencing) (females [36], male [37]) once every
170 5 min for 10 sec. Movement and proximity were not mutually exclusive; individuals could be
171 both stationary and close to another flies. However, any aggressive interactions were
172 deemed as activity whether flies were stationary or not. For this assay it was impossible to
173 blind the observer to the social treatment of the fly.

174

175 **Data analysis**

176 Statistical analysis was performed using SPSSv14 and R 3.3.1 [38].

177

178 *Virgin Finding – decision making in a complex environment*

179 To account for day/time of day effects, amount of time courting and courtship directed to the
180 virgin for paired flies was standardised by subtracting from each, the mean of their matched
181 single counterparts. This was then analysed using a Generalised Linear Mixed Model
182 (GLMM) with a binomial distribution. Social treatment was a fixed factor and ID of the fly
183 nested in repeat as a random factor. A model with effect of social treatment included was
184 compared to that only containing random factors using Analysis of Deviance.

185

186 *Aversive olfactory associative learning*

187 To account for day/time of day effects, innate odour preference and LI (Equation 1) for flies
188 kept with a social partner were standardised by matched groups of single flies tested
189 concurrently. Standardised values were then compared to 0 (i.e. no difference between
190 single and social partners) with one sample Wilcoxon signed ranks tests.

191

192 *Visual learning and probe trial analysis*

193 A learning index was calculated for both time taken to find a “safe” spot and for total distance
194 moved by subtracting the last learning trial from the first learning trial. The time taken to find
195 a “safe” spot was compared between individuals kept with a conspecific or heterospecific
196 social partner with Mann-Whitney U tests. For total distance moved, the difference between
197 the first and last trial was compared to zero with a Wilcoxon signed rank test.

198 To test for learning in reversal trails (both time taken to find “safe” spot and mean
199 distance from “safe” spot) learning was calculated as the difference between an individual
200 kept with a social partner and the average of individuals kept singly. Both measures were
201 compared to 0 (no difference between single and social partner males) with a one sample t-
202 test.

203

204 *Gene expression*

205 Relative expression differences were examined using ANOVA with gene identity and social
206 group as fixed factors. Pairwise comparisons with Bonferroni correction were performed to
207 investigate the difference between males kept singly and with a social partner for each gene.

208

209 *Social interactions and activity*

210 Separate analysis was conducted for each sex. Movement, proximity and interaction data
211 were analysed with generalised linear mixed models with zero inflation correction (package
212 glmmADMB and glmmTMB). For movement, social treatment and time of day were used as
213 fixed effects and Day and ID were random effects. Number of interactions with social
214 treatment and time as fixed factors, day and ID as random effects. Maximal models were
215 reduced to minimum explanatory factors through Analysis of Deviance, with post-hoc
216 comparisons between groups performed using the package emmeans with the Tukey
217 adjustment for multiple testing.

218

219 **Results**

220 After exposure to a conspecific, males performed better in both the virgin finding assay
221 (AOD: $X^2_1 = 29.212$, $N = 165$, $p < 0.001$ Figure 1A) and olfactory learning ($z = 2.753$, $N = 58$
222 $p = 0.006$ Figure 1B) compared to males held singly. There was a similar (though non-
223 significant) trend for visual reversal learning (Time: $X = 1.851$, $N = 19$, $p = 0.064$. Distance:
224 $t_{18} = 0.994$, $p = 0.355$ Figure S3A and B). Males kept with heterospecifics responded to
225 social contact through an increase in courting effort (AOD: $X^2_1 = 4.871$, $N = 176$, $p = 0.027$,
226 Figure S2) though showed none of the learning improvements seen when males were kept
227 with conspecifics when compared to single males (Virgin finding $X^2_1 = 8.1616$, $N = 176$, $p =$
228 0.004 Figure 1A; Olfactory learning: $z = 0.957$, $N = 30$, $p = 0.338$ Figure 1B, Visual reversal
229 Learning Time: $z = -1.461$, $N = 18$, $p = 0.144$. Distance: $t_{17} = 0.115$, $p = 0.115$ Figure S3A and

230 D). Importantly, the ability of males to detect cues needed to complete the learning tasks did
 231 not differ significantly between social environments (Figure S4).

232 Females did not statistically differ in olfactory learning ability when kept with
 233 conspecifics ($t_{30} = -0.308$, $p = 0.760$ Figure 1D) but significantly improved when exposed to
 234 heterospecifics ($t_{32} = 2.675$, $p = 0.012$ Figure 1D). However, this may be partly due to a
 235 change in female olfaction preference for training odours used in the assay. Females
 236 significantly changed their preference for 3-Octanol compared to single females when kept
 237 with both conspecifics ($z = -2.079$, $N = 31$, $p = 0.038$, Figure S5A) and heterospecifics ($z =$
 238 2.010 , $N = 33$, $p = 0.044$, Figure S5A). There was no statistical difference in visual reversal
 239 learning dependent on social treatment (conspecific time: $z = -1.492$, $N = 19$, $p = 0.136$.
 240 Distance $X^2_1 = 0.604$, $N = 19$, $p = 0.546$, heterospecific time: $z = 1.099$, $N = 14$, $p = 0.272$.
 241 Distance $X^2_1 = 2.291$, $N = 14$, $p = 0.022$ Figure S3C and D).

242 We found a general pattern of increased expression across all genes in males kept
 243 with conspecifics compared to single males ($F_{1,29} = 11.349$, $p = 0.002$ Figure 2A). *Post-hoc*
 244 analysis showed both *futsch* ($t_9 = -3.299$, $p = 0.012$) and *Neurexin-1* ($t_{10} = -3.424$, $p = 0.006$)
 245 were significantly upregulated in males kept with conspecifics. Expression did not
 246 significantly differ between males held singly or with heterospecific males ($F_{1,22} = 2.589$, $p =$
 247 0.122 Figure 2B). Female gene expression was not affected by conspecific contact ($F_{1,24} =$
 248 3.351 , $p = 0.080$ Figure 2C). However, females kept with heterospecifics significantly
 249 increased expression overall ($F_{1,24} = 8.209$, $p = 0.009$ Figure 2D), though not for individual
 250 genes after *post-hoc* testing.

251 Male movement was dependent on an interaction between social environment and
 252 time of day ($X^2_4 = 11.297$, $p = 0.004$, Figure S6A). Males with conspecifics moved less than
 253 males kept singly and with heterospecifics. Conspecific partners were significantly closer in
 254 proximity than heterospecific partners ($X^2_1 = 11.575$, $p < 0.001$, Figure S6B), and also
 255 interacted significantly more ($X^2_1 = 31.94$, $p < 0.001$, Figure S6C).

256 Females held with any social partner moved less than single females ($X^2_4 = 10.761$,
257 $p < 0.004$; Figure S7A). Females kept with conspecifics moved less than single females at
258 9am only ($t_{334} = 2.666$, $p = 0.0219$), whilst with heterospecifics they were less active at 9am
259 ($t_{334} = 2.504$, $p = 0.034$) and 12pm ($t_{334} = 3.248$, $p = 0.003$). Type of social contact did not
260 affect partner proximity ($X^2_1 = 0.4253$, $p = 0.514$, Figure S7B). Interactions were seen too
261 infrequently to analyse.

262

263 Discussion

264 Our data supports the idea that competitive interactions, specifically mating competition, is
265 important for male cognitive ability. Males exposed to conspecifics, but not heterospecifics
266 that acted as a general social contact, showed general cognitive improvement, including in a
267 visual learning task, a sensory modality not required for a response to sperm competition
268 [19]. Interestingly, the opposite pattern was true for females who increased olfactory learning
269 ability in response to exposure to heterospecifics but not conspecifics.

270 When exposed to a conspecific, males increased their ability to identify a virgin
271 female. Though males exposed to heterospecifics failed to improve within this assay, they did
272 increase courtship effort. *D. melanogaster* males increase the volume of their courtship song
273 after social contact [39] and experience of heterospecifics strengthens conspecific mating
274 preferences [40]. In line with this, our results reflect that social experience increases male's
275 preference for conspecifics. Indeed, males involved in virgin finding, although requiring
276 learning, would also be expected to draw on other cognitive processes that could lead to a
277 change in social preference.

278 Social environments that produced increases in learning ability for both sexes were
279 associated with increased expression of synapse-related genes and a decline in
280 movement(used as a proxy for sleep). Decreases in synapse number is linked to decreased
281 cognition in multiple species [41], and chemically induced increases in synapse connectivity

282 in mice improves cognitive performance [42]. Sleep is thought to be vital in developing and
283 consolidating synaptic circuitry [43, 44]. Consistent with this, *Neurexin-1*, a gene involved in
284 synaptic plasticity is significantly increased in heads when males are kept with conspecifics.
285 *Neurexin-1* acute overexpression in adulthood is associated with synaptic growth and an
286 increase in sleep in *D. melanogaster* [45]. In this study we cannot separate out the exact
287 mechanisms leading to an increase in learning. However, we observe a coordinated change
288 in activity patterns and increased expression of genes involved in synaptic growth [45] due to
289 changes in the sexual environment, suggesting the socio-sexual environment has a
290 significant role to play in cognitive development.

291 Females also display less movement (likely more sleep) and an increase in synaptic
292 gene expression when kept with the type of contact leading to an increase in learning, in this
293 case heterospecific. We interpret the male responses caused by conspecific contact a
294 response to the sperm competition environment [15], however, what may be causing
295 females to increase memory after contact with heterospecifics is unknown. In addition to an
296 increase in learning, females were found to have changed their olfactory preference in
297 response to heterospecific exposure. During the assay this preference was taken into
298 account by training individuals against their preferred odour. However, the change would
299 also suggest heterospecific partners could impact future choice influenced by smell, for
300 example, of oviposition sites [46]. Female *D. melanogaster* use learning and memory
301 processes to choose oviposition sites avoiding parasitism [47] and for preferred substrates
302 [48]. Cues of heterospecific competition may similarly affect female *D. melanogaster* egg
303 laying decisions, though this is yet to be tested.

304 Examples of difference in cognitive abilities between the sexes are relatively common
305 [49, 50] and are often connected to sexually specific fitness benefits arising from different
306 selective pressures [10]. Here, cognitive differences are seen in how the sexes react to
307 different same-sex social pressures. This serves to again highlight that sexual competition is
308 especially important for males to develop cognitive abilities. It also suggests that cognitive

309 evolution is driven by sex specific pressures in *D. melanogaster*. Indeed, female *D.*
310 *melanogaster* base some mating decisions on public information [51], whereas males only
311 seem to use cues directly related to their own experience to modulate behaviour [52]. In
312 insects the evolution of cognition is intimately linked to increasing complexity of Mushroom
313 Bodies, a structure which is analogous to the mammalian central cortex [53], leading to
314 greater behavioural complexity [54, 55]. Previously, the primary driver of cognitive
315 development in insects was thought to be complex foraging behaviour [56]. Here we present
316 evidence that intra-sexual competition, and specifically sperm competition responses
317 previously linked to the MBs [17], are also likely important in driving cognitive development in
318 males. We also find that cues of another species may drive female cognitive development,
319 though we know much less about the underlying processes. Overall, our data, together with
320 other recent studies [10, 12] highlight competitive interactions as a key social driver of
321 cognitive evolution [4, 5], at least outside of mammals. We therefore suggest the
322 requirement for plasticity in male and female responses to fluctuating socio-sexual
323 environments may be more generally important to cognitive development than previously
324 thought.

325

326 Figure 1: Effect of social environment on learning. A) Change in percentage courting
327 directed towards a virgin female (“Correct” courting) by males kept with *D. melanogaster* or
328 *D. virilis* rivals. Change in olfactory learning ability of B) males or C) females kept with *D.*
329 *melanogaster* or *D. virilis* partners. All data are standardised by comparison to the group
330 average of single flies assayed at the same time * < 0.05, ** < 0.01, *** < 0.001.

331 Figure 2: Gene expression changes depending on social environment. Males (A and B) or
332 females (C and D) were held singly (white bars) or with social partners (grey bars) that were
333 conspecific (A and C) or heterospecific (B and D). Significant effects are indicated across the
334 whole model or in pairwise comparisons where * < 0.05, ** < 0.01, *** < 0.001.

335

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512

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519 Experimental design, JR and AB. Investigation, JR, LM. Analysis, JR, ZW and ED. Writing,
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521 JR designed experiment, carried out lab work, analysed data and drafted the manuscript; LM
522 carried out lab work, contributed to analysis and drafted manuscript; ZW contributed to
523 analysis; ED contributed to analysis, drafted the manuscript and provided supervision to JR;
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527 **Competing interests**

528 Authors declare no competing interests

529 **Data and materials availability**

530 The datasets generated and analysed during the current study will be available in the open
531 access Dryad repository doi:10.506/dryad.gqnk98sk4 upon acceptance.

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