1 **Sexual deprivation modulates social interaction and reproductive physiology**

2

3 Liora Omesi[1], Mali Levi[1], Assa Bentzur[1], Yong-Kyu Kim[2a], Shir Ben-Shaanan[1], Reza Azanchi[2b], Ulrike

4 Heberlein[2] and Galit Shohat-Ophir[1,3]

5

6

7 [1]The Mina & Everard Goodman Faculty of Life Sciences, The Leslie and Susan Gonda Multidisciplinary

8 Brain Research Center and The Nanotechnology Institute, Bar-Ilan University, Ramat Gan 5290002

9 Israel

10

11 [2]Janelia Research Campus, Howard Hughes Medical Institute, Ashburn, VA 20147, USA

12

13 [a]Current Address: Department of Neurobiology, Northwestern University, Evanston, IL 60208, USA

14

15 [b]Current Address: Department of Neuroscience, Brown University, Providence, USA

16

17 [3]Lead contact

18

19 Correspondence should be addressed to G.S.O. (galit.ophir@biu.ac.il)

20    **Abstract**
21

22        In highly polyandrous species, where females mate with multiple males within a single fertility

23    period, there is typically a high level of sperm competition. To cope with this challenge, males apply

24    various behavioral and physiological strategies to maximize their paternity rates. Previous studies in

25    *Drosophila melanogaster* established a link between the composition of the social environment and the

26    reproductive success of individual male flies. While most studies until now focused on the adaptive

27    responses of male flies to the presence of rival males, little is known about whether the outcomes of

28    sexual interactions with female partners affect male-male social interactions in a competitive

29    environment such as the social group. Here we show that repeated failures to mate promote a coordinated

30    physiological and behavioral responses that can serve to increase paternity chances over mating rivals.

31    We exposed male flies to sexual deprivation or successful mating and analyzed the behavioral repertoires

32    of individuals within groups and the structure of their emerging social networks. We discovered that

33    failures to mate and successful mating generate distinct emergent group interactions and structures,

34    where sexually deprived males form low density social networks and actively minimize their encounters

35    with other group members, while increasing their aggressive behavior.  In addition, sexually deprived

36    male flies elevate the production of seminal fluid proteins (known to facilitate post-mating responses in

37    females) and extend mating duration upon mating with receptive females, altogether leading to reduced

38    re-mating rates. Our results demonstrate the existence of a flexible mating strategy that may provide a

39    short-term fitness advantage over competing rivals and pave the path for using simple model organisms

40    to dissect the neurobiology of social plasticity as coping strategy to living in a highly dynamic

41    environment as the social domain.

42


43

## Introduction

The ability to adapt to environmental changes is an essential feature of biological systems, achieved in multicellular organisms by a coordinated crosstalk between neuronal and hormonal programs that generate plastic physiological and behavioral responses to environmental challenges[1,2]. This is particularly important in a dynamic, ever-changing and unpredictable environment, such as the social domain composed of many behaving animals, the interaction with ultimately determines the reproductive success of individuals[2–4]. The intricate nature of social interaction requires the ability to recognize other members of the group in the right context, season, sex, age and reproductive state, and integrate this information with prior experience to produce the appropriate and optimal behavioral response[4]. Plastic social responses are seen in diverse animals, and include modulation of competitive sexual behaviors such as mating preferences and aggressive displays, and also the regulation of social foraging and parental care[5–7]. A remarkable example of social plasticity is evident in the African cichlid fish *Astatotilapia burtoni*, which live in a highly complex social environment consisting of many rival males that compete over limited food, territorial resources and female partners. Such a complex biotic and social environment produces a small number of dominant male fish and a large number of submissive males that closely monitor the social landscape in a constant search for opportunities to improve their social status, taking over mating territories and females[7].

As a species with sociable lifestyle, *Drosophila melanogaster* exhibit communal living around freshly decaying fruits[8] and engage in diverse forms of social interactions[9]. This includes courtship and mating[10,11], fighting over resources[12], group interactions[13], coordinated responses to threats[14–16], cultural transmission of complex behaviors[17], learning from conspecifics[18,19], and synchronization of activity by social cues[20]. Although some of these behaviors are considered innate responses, there are striking examples of the ability of fruit flies to exhibit social plasticity as they modulate their behavior and

67    physiology in response to changes in their social environment. This includes the ability of male flies to

68    change their aggressive behavior in response to prior fighting experience[21–23], regulate sperm

69    composition and the duration of copulation events in response to perceived competition[24–26], and suppress

70    courtship efforts towards non-receptive female flies[27–29].

71        Recent studies in *Drosophila* demonstrate that fruit flies generate complex and rich group

72    structures that are sensitive to the density of the group, its composition, as well as to the prior experience

73    of its members[30–33]. We previously showed that sexual experience in male flies can modulate their

74    motivational state and, subsequently, their reward seeking behaviors[34,35]. However less is known about

75    the way by which prior sexual interactions that are experienced as success or failure to mate shape social

76    interaction of male flies in a group context. Furthermore, it is not clear whether sexually deprived male

77    flies exhibit loser-like responses, as in the case of social defeat[23], or rather actively increase their

78    competitive behavior to cope with mating rivals. Here we explored the effects of success or failure to

79    mate on the dynamics of social interaction in groups of male flies. We discovered that sexual deprivation

80    and successful mating generate opposite emergent group interactions and structures, wherein sexually

81    deprived male flies actively minimize their interactions with group members. Moreover, sexual

82    deprivation enhances competitive behaviors and leads to changes in reproductive physiology, possibly

83    to increase paternity chances over mating rivals.

4

84    **Results**

85    **Failure to mate modifies action selection upon encounters with rival male flies**

86    We previously demonstrated that sexual experiences associated with different levels of mating

87    success, such as repeated events of successful mating, or sexual deprivation in the form of repeated

88    rejection events by non-receptive female flies, alter internal state and consequently motivational

89    responses[34,35]. The negative valence of rejection, reflected by its capacity to induce courtship suppression

90    and increase the consumption of ethanol, prompted us to ask whether sexually deprived male flies exhibit

91    loser-like responses[23] or rather actively increase their competitive behavior to cope with mating rivals.

92    To this end, we generated two cohorts of male flies that were exposed to repeated encounters with either

93    receptive virgin female flies (mated-isolated) or non-receptive female flies (rejected-isolated), consisting

94    of 1h sessions 3 times a day for 4 days (Fig. 1A). At the end of this experience, their interactions in group

95    context were tested by introducing 10 flies from each cohort into a shallow arena in which they could

96    move and interact in two dimensions. Their behavior was recorded for 30 min and analyzed using the

97    FlyBowl suite of tracking and behavior analysis softwares[32,36,37] (Fig. 1A). The tracking data obtained

98    was used to generate a comprehensive behavioral representation for each cohort composed of 60 distinct

99    features, including kinetic features, eight distinct complex behaviors, and six social network features

100   (Table 1)[32]. The overall differences between the two cohorts across all features are depicted in a scatter

101   plot of normalized differences and are divided into 4 main categories: activity-related features,

102   interaction-related features, coordination between individuals, and features associated with social

103   clustering (Fig. 1B). The two cohorts of male flies exhibited distinct repertoires of behavioral responses

104   upon first encounters with other male flies. Sexually deprived male flies exhibited increased activity

105   manifested as longer overall time spent walking, increased average velocity, and higher number of body-

106   turns (Fig. 1B, highlighted in pink, Supp Fig. 1A-C). When analyzing social-related behaviors, rejected

107   male flies exhibited lower rates of close touch encounters (Fig. 1B, highlighted in blue, Supp Figure 1D),

5

108    and while they displayed similar levels of active approaches towards other members of the group, the

109    duration of these encounters was significantly shorter (Fig. 1B, highlighted in blue, Supp. Fig. 1E,F). In

110    contrast, mated males exhibited long periods of quiescence (Fig. 2B, highlighted in blue, Supp. Fig. 1B),

111    and formed close-distance social (Fig. 1B, highlighted in blue, Supp Fig. 1G), reflected also by an

112    increase in the number of flies found in close proximity to one another (Fig. 1C).

113    **Failure to mate promotes social avoidance**

114        We next analyzed the properties of emerging social networks in both groups using weighted

115    networks as described by Bentzur el al.,[32] (Fig. 2A).  We calculated network weights according to the

116    overall duration of interactions (emphasizing long-lasting interactions) or the overall number of

117    interactions (emphasizing short interactions) between each pair of flies. Analysis by duration revealed

118    that social networks of rejected males are characterized by lower density (Fig. 2B), reduced modularity

119    (Fig. 2C), and reduced variation in individual strength levels across the group (SD strength, Fig. 2D).

120    These findings suggest that rejection promotes the formation of sparser groups containing fewer

121    subgroups and that individuals in those groups are more homogenous in the strength of their interactions.

122    Analysis by number of interactions revealed that, although rejected networks have lower modularity and

123    SD strength, there is no significant differences in the density of their networks, suggesting that they

124    maintain an overall similar number of interactions as mated male flies (Fig. 2E-G). Together, these

125    differences indicate that mated male flies form networks with higher-order structures compared to those

126    formed by rejected male flies. Notably, although rejected male flies participate in a similar number of

127    interactions, their networks are simpler and sparser. The apparent differences in the density of networks

128    measured by duration are consistent with significant differences between the two cohorts in the average

129    distance between the two closest flies in each frame (dcenter), which is considerably higher in rejected

130    males (Fig. 2H). More importantly, while in mated males the average distance between flies decreased

131    along the experiment as flies adapt to the arena, it remained constantly high in groups of rejected male

132   flies (Fig. 2H).  Considering that the elevated activity of rejected male flies (Fig. 1B) is expected to

133   increase the opportunity to encounter others, the maintenance of a larger distance throughout the

134   experiment and the reduced density suggest that rejected individuals actively avoid social interactions

135   with other flies. Together, these experiments point to sexual deprivation as the major contributor to the

136   reduced social interaction. To further test the strength of this conclusion, we divided a cohort of rejected-

137   isolated males into two subgroups, one of which was left undisturbed, and the other subgroup was

138   allowed to mate with virgin females for 2.5 hours immediately before testing. The rejected, then mated

139   sub-group exhibited intermediate levels of activity related features such as walk, stop, turn and average

140   velocity when compared to subgroups that had only experienced rejection or successful mating (Fig. 2I).

141   The rejected and then mated subgroup exhibited also intermediate degrees of social interaction related

142   features such as social clustering, number of flies found in close proximity to one another, and the levels

143   of grooming behavior that is tightly associated with social clustering (Fig. 2I). The capacity of mating to

144   partially reverse the effects of sexual deprivation is consistent with sexual deprivation being the major

145   contributor to social avoidance.

146   **Sexual deprivation modulates competitive behaviors**

147   Considering the major differences in group behavior displayed by rejected and mated male flies,

148   we hypothesized that the responses exhibited by rejected males reflect behavioral adaptation to coping

149   with high sexual competition over mating partners, where repeated encounters with mated females are

150   indicative of high male to female sex ratio. If so, rejected male flies are expected to increase behaviors

151   that provide them with an adaptive competitive value over rival male flies.  This prediction can be tested

152   by measuring their aggressive responses toward other males in the presence of limited food resources or

153   their mating behavior upon opportunities to mate with virgin female flies. Indeed, pairs of rejected male

154   flies exhibited significantly higher aggressive displays in comparison to pairs of mated male flies (Fig.

155   3A), and that in mixed pairs, rejected males exhibited greater numbers of lunges compared to their mated

7

156    counterparts (Fig. 3B,C). When allowed to mate with virgin female flies, rejected male flies extended

157    the duration of copulation events by 25% (3.5 minutes longer) compared to naïve males (Fig. 3D). Thus,

158    rejected male flies exhibited an overall increase in behaviors that can provide them with an adaptive

159    competitive value over rival male flies.

160    **Failure to mate induce changes in sperm and seminal fluid composition**

161    The act of mating alone does not guarantee fitness benefits including known strategies that reflect

162    male investment in sperm and non-sperm components, such as fecundity-enhancing seminal fluid

163    proteins[38,39,40]. To determine whether prior rejection affects reproductive physiology in a manner that

164    may improve mating competitiveness, expression levels of genes related to sperm production and

165    reproduction were assessed.  First, the expression of DON-JUAN (DJ), a protein that is specifically

166    expressed in mature male sperm cells[32,33], was measured using a GFP-based reporter line in which a GFP

167    sequence was inserted within the coding locus, so that the expression of GFP reflects the expression of

168    the endogenous DJ protein. The reliability of the DJ-GFP reporter as a sensitive measure for changes in

169    sperm production was first confirmed in male flies raised among a high number of rival males (5 flies

170    for 4 days), compared to the flies that were housed in pairs (Supp Fig.2), social conditions known to

171    affect the amount of mature sperm[25,41] (Supp Fig.2). The relative levels of GFP were then measured in

172    rejected and naïve male flies (Fig. 4A-B). Surprisingly, there was a twofold decrease in the levels of GFP

173    in the rejected cohort compared to naïve males (with no prior sexual experience), suggesting that male

174    flies decrease their investment in sperm allocation in response to sexual deprivation (Fig. 4A-B). Next,

175    the relative expression of the following reproductive related genes was directly assessed in fly abdomens

176    by qRT-PCR. We measured the expression of *Sex-Peptide (Acp70A), Acp63, Acp53, Ovulin (Acp26Aa),*

177    which are responsible for the females' long-term post-mating responses and fertility[38]. We also measured

178    the expression of genes encoding the *Ejaculatory bulb protein (Ebp),* which is responsible for the

8

179    posterior mating plug formation at the end of mating[42], *don-juan (dj)*[40], the *corazonin (Crz)* neuropeptide,

180    which promotes sperm and seminal fluid ejaculation in males and its receptor *Crz-receptor*[35,43], and

181    finally *Esterase 6 (est-6),* an enzyme that is transferred to females during copulation and presumably

182    functions to degrade the pheromone cVA[44] (Fig. 4C). There was a two-fold increase in the levels of *Acp-*

183    *70A (Sex-Peptide)* and *Acp-63* in rejected male flies when compared to naïve males, suggesting that

184    rejected male flies increase their investment in the production of seminal fluid proteins that are

185    transferred to females flies during copulation (Fig. 4C). Nevertheless, in agreement with the observed

186    reduction in DJ-GFP reporter levels, there was a drastic decrease in the transcript levels of *don-Juan* in

187    rejected males. The transcript levels of *Ebp, Est-6, Crz* and its receptor were similar in both cohorts (Fig.

188    4C). Overall, these results suggest that rejected male flies respond to sexual deprivation by elevating

189    seminal fluid protein transcript levels, presumably to maximize their fitness. In addition to proteins

190    associated with the male reproductive system, levels of several genes expressed in the brain and antenna

191    were also assessed. These included the neuropeptides *Crz, Neuropeptide F* (*npf*) and its receptor (*npfr*),

192    and two olfactory related genes associated with aggression (the *Odorant binding protein 69a*[45], and

193    *Cyp6a20*[46]). In agreement with previous studies, the levels of *npf* were significantly lower in sexually

194    deprived male flies[34]; we also observed a reduction in *npfr* (Fig. 4D). Interestingly, sexually deprived

195    male flies also exhibited reduced levels of *Cyp6a20* in comparison to naïve male flies (Fig. 4D),

196    consistent with their enhanced aggression (Fig. 3 A-C).

197    **Females that mate with rejected male flies exhibit reduced re-mating behavior**

198         The molecular changes associated with the rejected condition support our initial hypothesis that

199    rejected male flies adjust their behavior and physiology to cope with high sexual competition. If this is

200    correct, the changes in seminal fluid composition and the extended copulation are expected to provide

201    rejected male flies with an advantage over rival male flies. To test this prediction, several aspects

202    associated with female fecundity were measured. First, the fertility of female flies was assessed by

9

203    counting the number of eggs they laid after one mating event with either rejected or naïve male flies.

204    There was no significant difference in the number of eggs laid across five days between the two cohorts

205    (Fig. 5A). The lack of difference in the amount of progeny suggested that lower sperm investment in

206    rejected males (as reflected by reduced DJ levels) does not affect the total offspring number, meaning

207    that there is no link between sperm investment and the number of offspring.

208        Next, we tested whether the increase in sex-peptide could facilitate enhanced post-mating

209    behavior (such as reduced receptivity) in females that mated with rejected male flies. Since the strongest

210    post-mating response is observed 24h post mating (data not shown), the proportion of female flies that

211    re-mated with new male flies 24h after they mated with either rejected or naïve male flies was measured.

212    A significant reduction was documented in the re-mating rates of females that mated initially with

213    rejected *versus* virgin male flies (Fig. 5B), suggesting that extended copulation time and increase in

214    seminal fluid proteins can lead to a stronger reduction in female receptivity.

215        During copulation, male flies transfer to female flies seminal fluid proteins and also anti-

216    aphrodisiac pheromones such as cVA[28]. The extended copulation observed in rejected male flies may

217    facilitate the transfer of larger amounts of cVA as a means to delay further courtship and copulation

218    events by female flies. As an indirect measure for possible changes in the amount of transferred cVA,

219    we analyzed the courtship behavior of male flies towards females that previously mated with either

220    rejected or naive male flies 1h after the initial mating.  No significant difference was observed in the

221    latency to court, i.e. the time it takes male flies to exhibit their first courtship action (wing vibration)

222    following introduction of the pair into the courtship arena (Fig. 5C). However, there was a significant

223    reduction in the number of male flies that courted females previously mated with rejected males than

224    those previously mated with naïve male flies (Fig. 5D), suggesting that mating with rejected male flies

225    results in females that are less attractive courtship targets.

226    **Discussion**

227         In this study we used the FlyBowl[37] as an agnostic tool to explore responses modulated by sexual

228    interaction and discovered that rejected male flies cope with their failures to mate by changing their

229    behavior and physiology to enhance their reproductive success. This is presumably achieved by avoiding

230    interaction with potential rival male flies and competing over mating partners via increased aggression

231    and prolonged copulation; this is known as mate guarding. The latter is strengthened by the increased

232    production of certain seminal fluid proteins that facilitate stronger post-mating responses in female flies.

233         The behavior of sexually deprived male flies was examined in this study under behavioral

234    contexts that illuminate different aspects of their action selection. Using the FlyBowl system, we

235    analyzed their emergent group interactions and social networks, and discovered that although rejected

236    males are highly active, they exhibit sparse networks and maintain large distance with other members,

237    as if they were actively minimizing or avoiding interaction with rival male flies. When tested in a social

238    context that promotes fighting over limited resources, rejected male flies exhibited enhanced aggression.

239         The increased aggression displayed by the rejected cohort is associated with a significant decrease

240    in the levels of *Cyp6a20*. This is consistent with a previous study showing that *Cyp6a20* levels are

241    reduced in social conditions that promote aggression and that this reduction is responsible for the

242    observed increase in aggression[46]. Interestingly, exposure to female flies prior to male-male interactions

243    was previously shown to suppress aggression[47]. However, our findings suggest that not all types of

244    interactions with female flies are sufficient for suppressing aggression, but rather that the quality of the

245    interactions (i.e., the male's sexual success) determines the resulting aggression levels when

246    encountering another male fly.

247         There are two possible explanations for the behavioral responses exhibited by rejected male flies.

248    First, failure to mate could enhance aggression to improve the chances of successful mating and, upon

11

249    eventual mating, the increased duration of copulation could increase the relative paternity share. Second,

250    repeated rejection experiences could be perceived by male flies as an indication for high density of sexual

251    competition over mating partners, where encountering mated females is suggestive of high male to

252    female sex ratio. Consistent with the second hypothesis, several studies have described a link between

253    pre-exposure to rival male flies and an extension of copulation events[24,48]. One study also demonstrated

254    that male flies use multiple sensory cues such as auditory, olfactory and gustatory signals to estimate the

255    level of mating competition[48]. Although rejected males were not exposed directly to other male flies

256    during the training phase, the observed extension of their copulation events suggests that they can assess

257    the level of competition by evaluating the quality of their sexual interaction with female flies. Studies

258    performed in *Pieris rapae* butterflies, in which virgin males were shown to allocate their sperm

259    investment by assessing not only the mating status of the female, but also her previous mating history[49],

260    are consistent with this hypothesis.

261        The behavioral responses to sexual deprivation were accompanied by changes in the repertoire

262    of genes expressed in the brain and reproductive system in the form of increased expression of several

263    accessory gland protein genes (*Acps*). This, together with the increased copulation duration, supports the

264    idea that the observed extension in mating duration serves to transfer a higher amount of Acps to intensify

265    the females' post- mating responses[23,79]. Unlike previous studies that demonstrated a link between the

266    presence of rival male flies and an increase in both copulation duration and sperm allocation (measured

267    by increase number of sperm cells)[25], rejected male flies exhibited a significant reduction in the levels of

268    DJ, a protein expressed in mature sperm cells. Although this finding is limited to only one protein, this

269    is surprising in light of sperm competition theory, which predicts that males should strategically increase

270    their investment in sperm allocation when in competition[50]. Furthermore, our findings are different from

271    studies in crickets, sunfish, birds and rats, which showed that the perceived risk of sperm competition, in

272    the form of the presence of rival males or their odors before and during mating, led to an increase in

12

273    sperm investment[49,51,52]. The unexpected uncoupling between the investment in sperm and non-sperm

274    components and the regulation of investment in copulation time, demonstrates that sexually deprived

275    male flies regulate each of these processes independently.

276    Functionally, the observed decrease in sperm quantity with increasing seminal fluid protein *(Acp)*

277    expression in rejected males did not affect the amount of progeny produced in females. This observation

278    suggests that there is no link between the observed behavioral and physiological changes and the amount

279    of progeny. Nevertheless, females that mated with rejected males were less attractive to naïve male flies,

280    as reflected by the reduced number of male flies that courted these females. The combination of reduced

281    female attractiveness in subsequent mating encounters, and reduced motivation of the female to re-mate,

282    may reduce the odds for a second mating and thus increase the rejected male's paternity rate despite the

283    lack of an effect on progeny number.

284    In summary, our results demonstrate a plastic mating strategy by males that experienced repeated

285    events of rejection that gives them a short-term advantage, promoting reproductive fitness when

286    competing with rival male flies. We postulate that rejected males invest more energy in the production

287    of seminal fluid proteins over sperm; these Acps are known to have important roles in modulating

288    different aspects of female mating physiology and behavior. Furthermore, at low population density, the

289    chances to meet a receptive female are low, therefore an investment in sperm ejaculate may be more

290    costly[53]. Further research is needed to dissect the molecular and neuronal mechanisms that mediate these

291    adaptive responses, identify the sensory modalities that perceive failure to mate, which encode this

292    information within the nervous system leading to ejaculate plasticity.

293

294

13

## Materials and methods:

299

300 <u>Fly lines and culture</u>

301 Canton S flies were used as the wild-type strain. Flies were raised at 25°C in a 12-h light/12-h dark cycle

302 in 60% relative humidity and maintained on cornmeal, yeast, molasses, and agar medium, and were tested

303 as 3–4-day old adults, unless otherwise specified. The DJ-GFP and White Berlin (WB) lines were

304 obtained from the HHMI Janelia Farm Research Campus.

305 <u>Sexual experience paradigm</u>

306 Male and female flies were anesthetized under $CO_2$ and isolated immediately after eclosion. Flies were

307 reared as single-housed in vials (23 mm by 94 mm) containing 7 ml of medium and were aged separately

308 for 3–4 day. Rejected and mated cohorts were generated as previously described[34].  In the naïve cohort,

309 male flies were isolated for 4 days.

310

311 <u>Social group interaction using the FlyBowl system</u>

312 At the end of the sexual experience phase, rejected and mated male flies were inserted in groups of 10

313 into Fly Bowl arenas[36], and their behavior was recorded for 30 minutes and analyzed using CTRAX,

314 FixTrax[32] and JAABA[36]. For kinetic features, scripts were written in MATLAB to use the JAABA code

315 to generate the statistical features as specified in Kabra et al. [36]. Time series graphs (per frame) were

316 created using JAABA Plot[36]. Quantification of complex behavios was done using JAABA Classifiers[36]

317 to identify specific behaviors: Walk, Stop, Turn, Approach, Touch, Chase, Chain, Song, Social

318 Clustering and Grooming. Bar graphs were created using JAABA Plot[36]. Network analysis was

319 performed using an interaction matrix according to the interaction parameters described previously[32].

320 Two interaction matrices were created for each movie, one with the total number of frames each pair of

321 flies were interacting divided by the number of frames in the movie and another with the number of

15

322 separate interactions between each pair of flies divided by the maximum number of possible interactions,

323 calculated as:

324

325
$$max \text{ \# of interaction possible } \frac{\text{\# of frames} - min \text{ \# of frames for interaction}}{min \text{ \# of frames for interaction} + min \text{ \# of gap frames}} + 1$$

326

327 The parameters to define an interaction are: angle subtended by the other fly $> 0$, distance between the

328 nose of current fly to any point on the other fly $\leq 8$ mm, number of frames for interaction $\geq 60$ and

329 number of gap frames $\geq 120$. Interaction end is defined when distance or angle conditions are not

330 maintained for 4 seconds. Networks and their features were generated from the interaction matrix in R

331 using the igraph package. The function that was used to the generate networks is

332 "graph_from_adjacency_matrix" with parameters "mode = undirected" and "weighted = TRUE".

333 Density was calculated on all movies with the formula:

334

335
$$density = \frac{sum \text{ of weights}}{[number \text{ of vertices} * (number \text{ of vertices} - 1)] * 0.5}$$

336

337 Modularity was calculated using the "modularity" function on output from the "cluster_walktrap"

338 function[54]. Strength was calculated using "strength" function and SD Strength was calculated on all

339 movies using "sd" function on the strength value. Betweenness Centrality was calculated on all flies

340 using the "betweenness" function and SD Betweenness Centrality was calculated on all movies using

341 "sd" function on the Betweenness Centrality value. Box plots were created using R.

342

343 Each feature of the FlyBwol experiment was standardized according to all values calculated in our

344 experiments for that feature to generate a z-score. Scatter plots were created using R.

345

346     Aggression

347     Pairs of rejected or mated male flies were introduced into aggression arenas (circular chambers, about

348     0.08 cm3 in volume), which contained a mixture of agarose and apple juice (1% agarose, 50% apple

349     juice) that was placed in arenas to enhance aggressive behavior. Flies were filmed for 30 min with Point-

350     Grey Flea3 ($1080 \times 720$ pixels) at 60 fps. Aggressive behavior was later quantified by counting the number

351     of lunges for each pair using CADABRA software (ref). The $\log_2$ ratio between the number of lunges in

352     rejected and mated flies was calculated for each pair, and then a one-sample t-test was performed to test

353     whether the mean ratio is significantly different from 0.

354

355     Copulation duration

356     Rejected and naïve male flies were put into courtship arenas (circular chambers, about 0.04 cm3 in

357     volume) with virgin females and were allowed to mate for 1 hour. They were recorded for the whole

358     experiment using a Point-Grey firefly camera. Courtship arenas consist of 25 flat arenas each arena

359     containing only one pair of male-female flies. The copulation duration was measured from the moment

360     the mating began until it ended. We calculated the time in seconds for each fly and the average for each

361     group.

362

363     Egg laying assay

364     Egg production was determined for females that had been allowed to copulate with rejected or naive

365     males for 1 hour at the end of the conditioning (as described above). Every female was put in a glass vial

366     containing fresh food every day for 5 days in total and was kept in the incubator. Days 3 and 4 have

367     received approximated values since day 3 was Saturday and we couldn't replace the vail that day;

368     therefore, we tried to divide the number of eggs equally. Eggs can be spotted easily as circular white dots

369     on the surface of the medium. The sum of the number of eggs in the vials of each female was used for

370     analysis.

17

371    <u>Receptivity assay</u>

372    3-4-day old White Berlin (WB) females were allowed to mate once with rejected or naïve males at the

373    end of the conditioning for 1 h. After mating, the males were disposed and the mated females were kept

374    in the incubator for 24h. Afterward, the mated females were exposed to 5-day-old WT naïve males for 1

375    h to measure their receptivity to mate. Approximately 40 pairs of each group (rejected or naive) were set

376    up in every biological repeat.

377

378    <u>Latency to court assay</u>

379    1 hour after allowing WB females to mate with rejected or naïve males, we transferred the females into

380    courtship arenas and paired them with new WT naïve males. The pairs were recorded for 15 min to

381    measure courtship latency. Latency was defined as the time elapsing between the introduction of the pair

382    into the chamber and the first appearance of wing vibration made by the courting male fly. We also

383    quantified the number of males who did and did not try to court in this assay.

384

385    <u>Courtship Index</u>

386    Courtship index for a given male is the fraction of time a male fly spent in courtship activity in the 10

387    min observation period (600 sec). It is calculated by dividing the number of seconds the male courted

388    over the total observation time and is been exhibit in percentage (CI = courtship behavior [sec] · 100 /

389    total observation [sec]).

390

391    <u>Molecular methods</u>

392    Western blot analysis: Sperm allocation in male flies carrying the DJ-GFP reporter was determined by

393    Western blotting. DJ protein size is ~29 kDa, and GFP size is ~25 kDa. We also determined the levels of

394    Sex-peptide (SP), a protein of size ~7 kDa, and the levels of Tubulin for normalization. The primary

395    antibodies used were mouse anti-GFP, rabbit anti-SP and rabbit anti-Tubulin, and the secondary

18

396    antibodies that were used are rabbit α-mouse HRP and mouse α-rabbit HRP, respectively. Virgin females

397    were used as negative controls.

398

399    Quantitative Real-Time PCR analysis

400    Frozen flies were placed on ice and decapitated using a scalpel. Total RNA was extracted from ~15

401    frozen heads and bodies (separately), using TRIZOL reagent according to the manufacturer's protocol.

402    mRNA was reverse transcribed using BIORAD cDNA synthesis kit. cDNA was analyzed by quantitative

403    real-time PCR (BIORAD CFX96) using specific primers for the head and for the body. Relative

404    expression was quantified by ΔΔCT method using RPL32[55] as a loading control. We run each sample in

405    triplicates. Each experiment was repeated four times using independent sets of experimental flies.

406

407    Statistical analysis

408    For each experiment, Shapiro–Wilk test was done on each experiment to test for normal distribution.

409    Statistical significance was determined by t-test for experiments that were distributed normally, and by

410    Wilcoxon test for experiments that were not distributed normally. For experiments with three or four

411    conditions: statistical significance determined by one-way ANOVA followed by Tukey's range test for

412    experiments that were distributed normally, and by Kruskal–Wallis test followed by Wilcoxon signed-

413    rank test for experiments that were not distributed normally.

414

**References**

1. Robinson, G.E., Fernald, R.D., and Clayton, D.F. (2008). Genes and Social Behavior. Science *322*, 896–900.

2. Oliveira, R.F. (2009). Social behavior in context: Hormonal modulation of behavioral plasticity and social competence. Integr. Comp. Biol. *49*, 423–440.

3. Cardoso, S.D., Teles, M.C., and Oliveira, R.F. (2015). Neurogenomic mechanisms of social plasticity. J. Exp. Biol. *218*, 140–149.

4. Taborsky, B., and Oliveira, R.F. (2012). Social competence: an evolutionary approach. Trends Ecol. Evol. *27*, 679–688.

5. Jennions, M.D., and Petrie, M. (1997). Variation in Mate Choice and Mating Preferences: A Review of Causes and Consequences. Biol. Rev. *72*, 283–327.

6. O'Connell, L.A., and Hofmann, H.A. (2012). Evolution of a Vertebrate Social Decision-Making Network. Science *336*, 1154–1157.

7. Fernald, R.D. (2017). Cognitive skills and the evolution of social systems. J. Exp. Biol. *220*, 103–113.

8. Soto-Yéber, L., Soto-Ortiz, J., Godoy, P., and Godoy-Herrera, R. (2018). The behavior of adult Drosophila in the wild. PloS One *13*, e0209917.

9. Sokolowski, M.B. (2010). Social interactions in "simple" model systems. Neuron *65*, 780–794.

10. Dickson, B.J. (2008). Wired for Sex: The Neurobiology of Drosophila Mating Decisions. Science *322*, 904–909.

11. Anderson, D.J. (2016). Circuit modules linking internal states and social behaviour in flies and mice. Nat. Rev. Neurosci. *17*, 692–704.

12. Wang, L., Han, X., Mehren, J., Hiroi, M., Billeter, J.-C., Miyamoto, T., Amrein, H., Levine, J.D., and Anderson, D.J. (2011). Hierarchical chemosensory regulation of male-male social interactions in Drosophila. Nat. Neurosci. *14*, 757–762.

13. Ramdya, P., Schneider, J., and Levine, J.D. (2017). The neurogenetics of group behavior in Drosophila melanogaster. J. Exp. Biol. *220*, 35–41.

14. Elliott, K.H., Betini, G.S., and Norris, D.R. (2017). Fear creates an Allee effect: experimental evidence from seasonal populations. Proc. Biol. Sci. *284*.

15. Ramdya, P., Lichocki, P., Cruchet, S., Frisch, L., Tse, W., Floreano, D., and Benton, R. (2015). Mechanosensory interactions drive collective behaviour in Drosophila. Nature *519*, 233–236.

16. Ferreira, C.H., and Moita, M.A. (2020). Behavioral and neuronal underpinnings of safety in numbers in fruit flies. Nat. Commun. *11*, 4182.

20

17. Danchin, E., Nöbel, S., Pocheville, A., Dagaeff, A.-C., Demay, L., Alphand, M., Ranty-Roby, S., van Renssen, L., Monier, M., Gazagne, E., et al. (2018). Cultural flies: Conformist social learning in fruitflies predicts long-lasting mate-choice traditions. Science *362*, 1025–1030.

18. Kacsoh, B.Z., Bozler, J., Ramaswami, M., and Bosco, G. (2015). Social communication of predator-induced changes in Drosophila behavior and germ line physiology. eLife *4*.

19. Mery, F., Varela, S.A.M., Danchin, E., Blanchet, S., Parejo, D., Coolen, I., and Wagner, R.H. (2009). Public versus personal information for mate copying in an invertebrate. Curr. Biol. CB *19*, 730–734.

20. Levine, J.D., Funes, P., Dowse, H.B., and Hall, J.C. (2002). Resetting the Circadian Clock by Social Experience in Drosophila melanogaster. Science *298*, 2010–2012.

21. Yurkovic, A., Wang, O., Basu, A.C., and Kravitz, E.A. (2006). Learning and memory associated with aggression in Drosophila melanogaster. Proc. Natl. Acad. Sci. U. S. A. *103*, 17519–17524.

22. Liu, W., Liang, X., Gong, J., Yang, Z., Zhang, Y.-H., Zhang, J.-X., and Rao, Y. (2011). Social regulation of aggression by pheromonal activation of Or65a olfactory neurons in Drosophila. Nat. Neurosci. *14*, 896–902.

23. Kim, Y.-K., Saver, M., Simon, J., Kent, C.F., Shao, L., Eddison, M., Agrawal, P., Texada, M., Truman, J.W., and Heberlein, U. (2018). Repetitive aggressive encounters generate a long-lasting internal state in Drosophila melanogaster males. Proc. Natl. Acad. Sci. U. S. A. *115*, 1099–1104.

24. Kim, W.J., Jan, L.Y., and Jan, Y.N. (2013). A PDF/NPF neuropeptide signaling circuitry of male Drosophila melanogaster controls rival-induced prolonged mating. Neuron *80*, 1190–1205.

25. Garbaczewska, M., Billeter, J.-C., and Levine, J.D. (2013). Drosophila melanogaster males increase the number of sperm in their ejaculate when perceiving rival males. J. Insect Physiol. *59*, 306–310.

26. Hopkins, B.R., Sepil, I., Thézénas, M.-L., Craig, J.F., Miller, T., Charles, P.D., Fischer, R., Kessler, B.M., Bretman, A., Pizzari, T., et al. (2019). Divergent allocation of sperm and the seminal proteome along a competition gradient in Drosophila melanogaster. Proc. Natl. Acad. Sci. U. S. A. *116*, 17925–17933.

27. Siegel, R.W., and Hall, J.C. (1979). Conditioned responses in courtship behavior of normal and mutant Drosophila. Proc. Natl. Acad. Sci. U. S. A. *76*, 3430–3434.

28. Keleman, K., Vrontou, E., Krüttner, S., Yu, J.Y., Kurtovic-Kozaric, A., and Dickson, B.J. (2012). Dopamine neurons modulate pheromone responses in Drosophila courtship learning. Nature *489*, 145–149.

29. Ejima, A., Smith, B.P.C., Lucas, C., van der Goes van Naters, W., Miller, C.J., Carlson, J.R., Levine, J.D., and Griffith, L.C. (2007). Generalization of courtship learning in Drosophila is mediated by cis-vaccenyl acetate. Curr. Biol. CB *17*, 599–605.

30. Rooke, R., Rasool, A., Schneider, J., and Levine, J.D. (2020). Drosophila melanogaster behaviour changes in different social environments based on group size and density. Commun. Biol. *3*, 304.

21

483 31. Schneider, J., Dickinson, M.H., and Levine, J.D. (2012). Social structures depend on innate
484     determinants and chemosensory processing in Drosophila. Proc. Natl. Acad. Sci. U. S. A. *109 Suppl*
485     *2*, 17174–17179.

486 32. Bentzur, A., Ben-Shaanan, S., Benichou, J.I.C., Costi, E., Levi, M., Ilany, A., and Shohat-Ophir, G.
487     (2020). Early Life Experience Shapes Male Behavior and Social Networks in Drosophila. Curr. Biol.
488     CB.

489 33. Battesti, M., Pasquaretta, C., Moreno, C., Teseo, S., Joly, D., Klensch, E., Petit, O., Sueur, C., and
490     Mery, F. (2015). Ecology of information: social transmission dynamics within groups of non-social
491     insects. Proc. Biol. Sci. *282*, 20142480.

492 34. Shohat-Ophir, G., Kaun, K.R., Azanchi, R., Mohammed, H., and Heberlein, U. (2012). Sexual
493     deprivation increases ethanol intake in Drosophila. Science *335*, 1351–1355.

494 35. Zer-Krispil, S., Zak, H., Shao, L., Ben-Shaanan, S., Tordjman, L., Bentzur, A., Shmueli, A., and
495     Shohat-Ophir, G. (2018). Ejaculation Induced by the Activation of Crz Neurons Is Rewarding to
496     Drosophila Males. Curr. Biol. CB *28*, 1445-1452.e3.

497 36. Kabra, M., Robie, A.A., Rivera-Alba, M., Branson, S., and Branson, K. (2012). JAABA: interactive
498     machine learning for automatic annotation of animal behavior. Nat. Methods *10*, 64–67.

499 37. Branson, K., Robie, A.A., Bender, J., Perona, P., and Dickinson, M.H. (2009). High-throughput
500     ethomics in large groups of Drosophila. Nat. Methods *6*, 451–457.

501 38. Sirot, L.K., LaFlamme, B.A., Sitnik, J.L., Rubinstein, C.D., Avila, F.W., Chow, C.Y., and Wolfner,
502     M.F. (2009). Molecular social interactions: Drosophila melanogaster seminal fluid proteins as a case
503     study. Adv. Genet. *68*, 23–56.

504 39. Wigby, S., Brown, N.C., Allen, S.E., Misra, S., Sitnik, J.L., Sepil, I., Clark, A.G., and Wolfner, M.F.
505     (2020). The *Drosophila* seminal proteome and its role in postcopulatory sexual selection. Philos.
506     Trans. R. Soc. B Biol. Sci. *375*, 20200072.

507 40. Santel, A., Winhauer, T., Blümer, N., and Renkawitz-Pohl, R. (1997). The Drosophila don juan (dj)
508     gene encodes a novel sperm specific protein component characterized by an unusual domain of a
509     repetitive amino acid motif. Mech. Dev. *64*, 19–30.

510 41. Moatt, J.P., Dytham, C., and Thom, M.D.F. (2014). Sperm production responds to perceived sperm
511     competition risk in male Drosophila melanogaster. Physiol. Behav. *131*, 111–114.

512 42. Avila, F.W., Cohen, A.B., Ameerudeen, F.S., Duneau, D., Suresh, S., Mattei, A.L., and Wolfner,
513     M.F. (2015). Retention of Ejaculate by Drosophila melanogaster Females Requires the Male-Derived
514     Mating Plug Protein PEBme. Genetics *200*, 1171–1179.

515 43. Tayler, T.D., Pacheco, D.A., Hergarden, A.C., Murthy, M., and Anderson, D.J. (2012). A
516     neuropeptide circuit that coordinates sperm transfer and copulation duration in Drosophila. Proc.
517     Natl. Acad. Sci. U. S. A. *109*, 20697–20702.

518  44. Chertemps, T., François, A., Durand, N., Rosell, G., Dekker, T., Lucas, P., and Maïbèche-Coisne,
519      M. (2012). A carboxylesterase, Esterase-6, modulates sensory physiological and behavioral response
520      dynamics to pheromone in Drosophila. BMC Biol. *10*, 56.

521  45. Bentzur, A., Shmueli, A., Omesi, L., Ryvkin, J., Knapp, J.-M., Parnas, M., Davis, F.P., and Shohat-
522      Ophir, G. (2018). Odorant binding protein 69a connects social interaction to modulation of social
523      responsiveness in Drosophila. PLoS Genet. *14*, e1007328.

524  46. Wang, L., Dankert, H., Perona, P., and Anderson, D.J. (2008). A common genetic target for
525      environmental and heritable influences on aggressiveness in Drosophila. Proc. Natl. Acad. Sci. U. S.
526      A. *105*, 5657–5663.

527  47. Yuan, Q., Song, Y., Yang, C.-H., Jan, L.Y., and Jan, Y.N. (2014). Female contact modulates male
528      aggression via a sexually dimorphic GABAergic circuit in Drosophila. Nat. Neurosci. *17*, 81–88.

529  48. Bretman, A., Westmancoat, J.D., Gage, M.J.G., and Chapman, T. (2011). Males use multiple,
530      redundant cues to detect mating rivals. Curr. Biol. CB *21*, 617–622.

531  49. Wedell, N., and Cook, P.A. (1999). Butterflies tailor their ejaculate in response to sperm competition
532      risk and intensity. Proc. R. Soc. Lond. B Biol. Sci. *266*, 1033–1039.

533  50. Parker, G.A., Ball, M.A., Stockley, P., and Gage, M.J. (1997). Sperm competition games: a
534      prospective analysis of risk assessment. Proc. R. Soc. B Biol. Sci. *264*, 1793–1802.

535  51. Gage, M.J.G. (1991). Risk of sperm competition directly affects ejaculate size in the Mediterranean
536      fruit fly. Anim. Behav. *42*, 1036–1037.

537  52. Nicholls, E.H., Burke, T., and Birkhead, T.R. (2001). Ejaculate Allocation by Male Sand Martins,
538      Riparia riparia. Proc. Biol. Sci. *268*, 1265–1270.

539  53. Parker, G.A., and Pizzari, T. (2010). Sperm competition and ejaculate economics. Biol. Rev. *85*,
540      897–934.

541  54. Pons, P., and Latapy, M. (2005). Computing communities in large networks using random walks. In
542      Lecture Notes in Computer Science (including subseries Lecture Notes in Artificial Intelligence and
543      Lecture Notes in Bioinformatics) (Springer, Berlin, Heidelberg), pp. 284–293.

544  55. Zheng, Y.T., Li, H.B., Lu, M.X., and Du, Y.Z. (2014). Evaluation and validation of reference genes
545      for qRT-PCR normalization in *Frankliniella occidentalis* (Thysanoptera: Thripidae). PLoS ONE *9*.
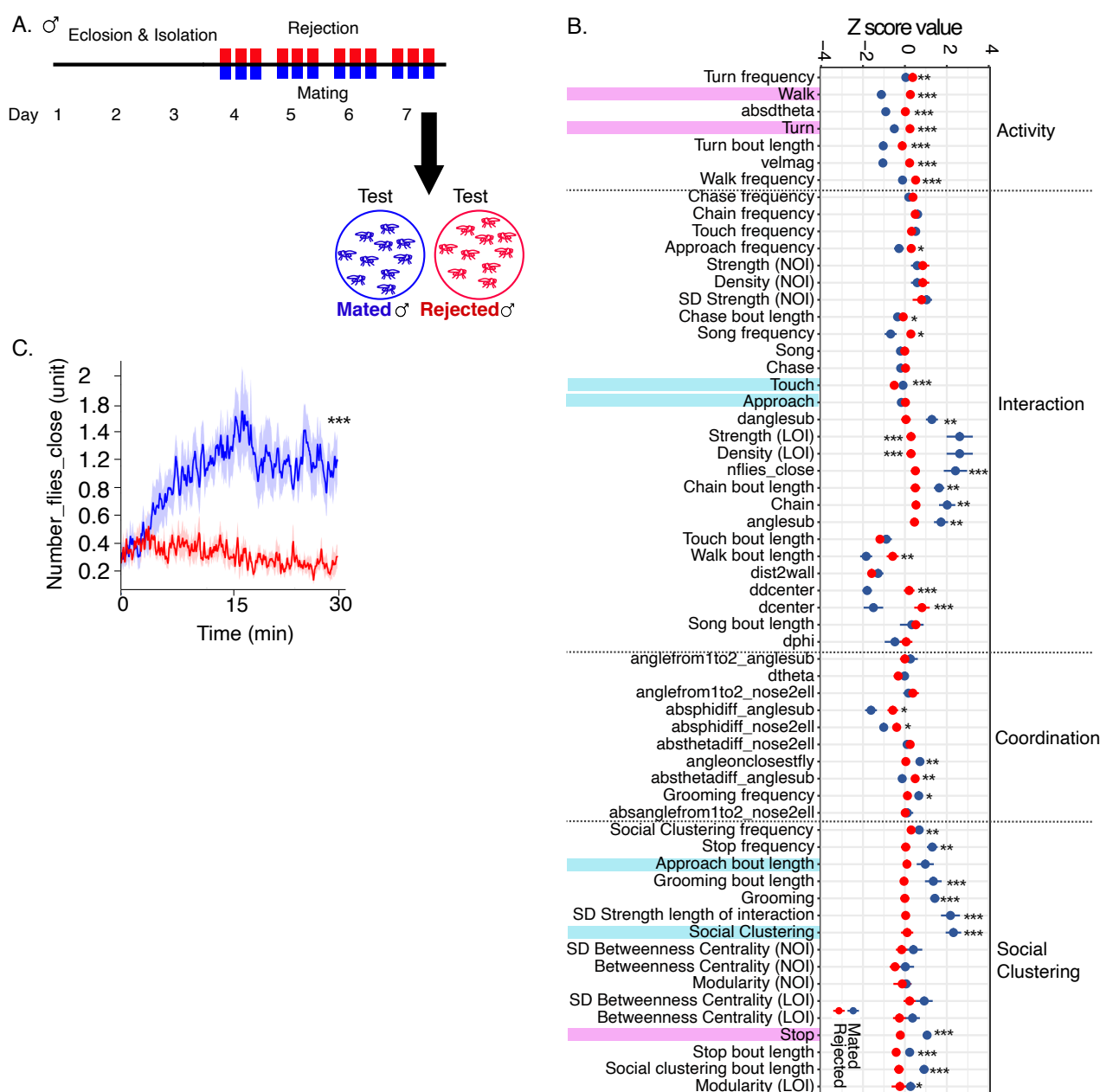
546
547

**Figure 1. Failure to mate modifies action selection upon encounters with rival male flies. A.** Schematic representation of the behavioral paradigm. **B.** Behavioral signatures of mated versus rejected WT male flies. Data is represented as normalized Z scores of 60 behavioral parameters, n = 18. Statistical significance was determined by t-test for normally distributed parameters or Wilcoxon test for non-normally distributed parameters. LOI: calculated according to the length of interactions. NOI: calculated according to the number of interactions. Features mentioned in the results section are highlighted in pink and blue. **C.** Average number of flies close to any fly (threshold ≤ 1.5 body length) along the experiment.
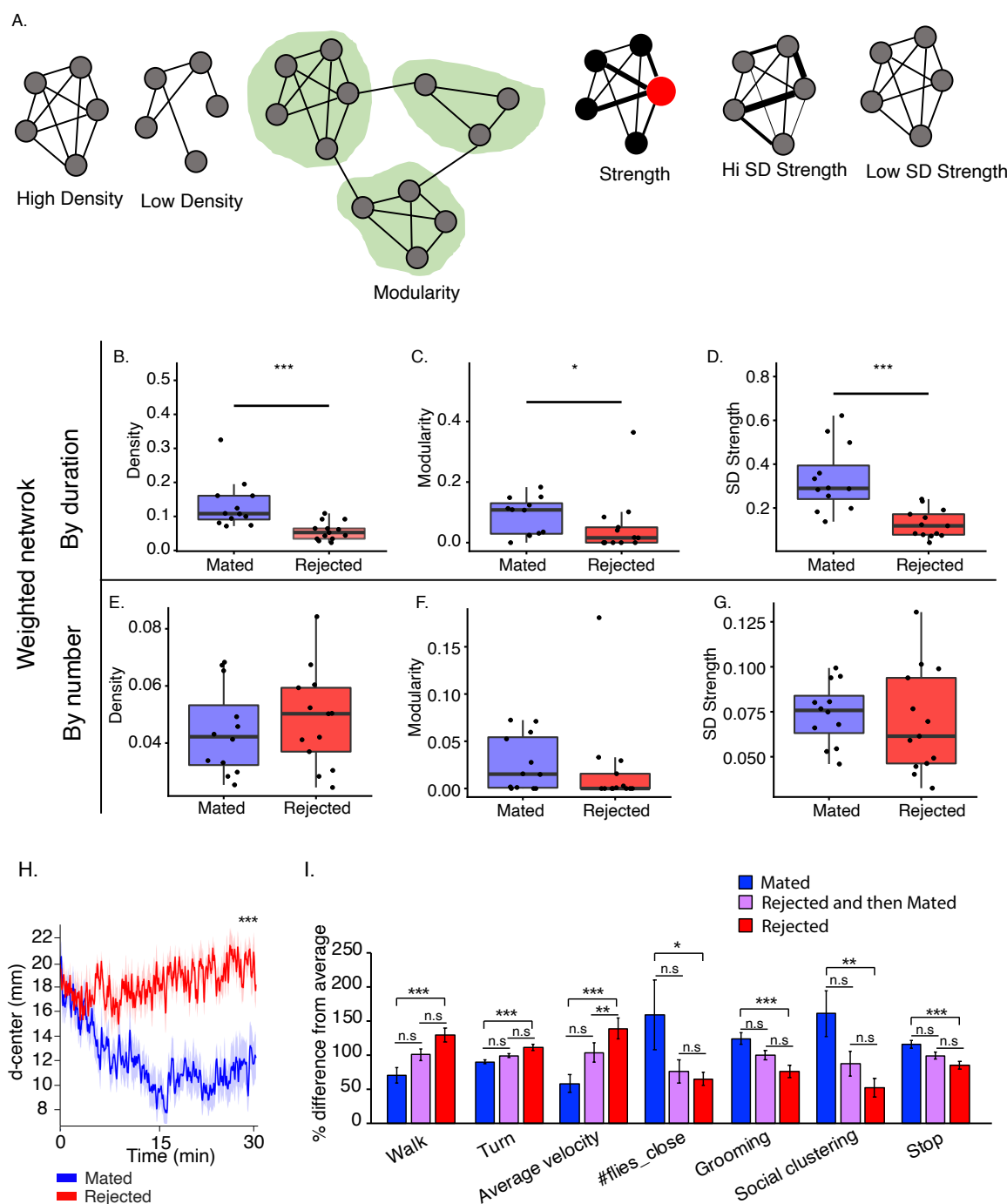
**Figure 2. Sexual deprivation promotes social avoidance. A.** Illustration of network parameters. Density of networks represents how saturated they are compared to the maximum possible. Modularity is a measure of the division of a network into sub-networks. Strength is proportional to vertex size (high in red individual). Standard deviation (SD) strength is a measure of the heterogeneity of the connections between individuals. **B-G.** Social network analysis of groups composed of rejected (red) and mated (blue) male flies. Network density, modularity, and SD strength calculated by network weights according to duration (A-C) or number of interactions (D-F), n = 18. Statistical significance was determined by Wilcoxon test and FDR correction for multiple tests, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Error bars signify SEM. **H.** Rejected male flies maintain large distances between flies along time, n=18 Statistical significance was determined by Wilcoxon test. Data is presented as mean±SEM. **I .** Rejected and then mated male flies depict intermediate levels of activity and social interaction features when compared to rejected or mated cohorts. n = 8. Statistical significance was determined by Wilcoxon test and FDR correction for multiple tests, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Error bars signify SEM.
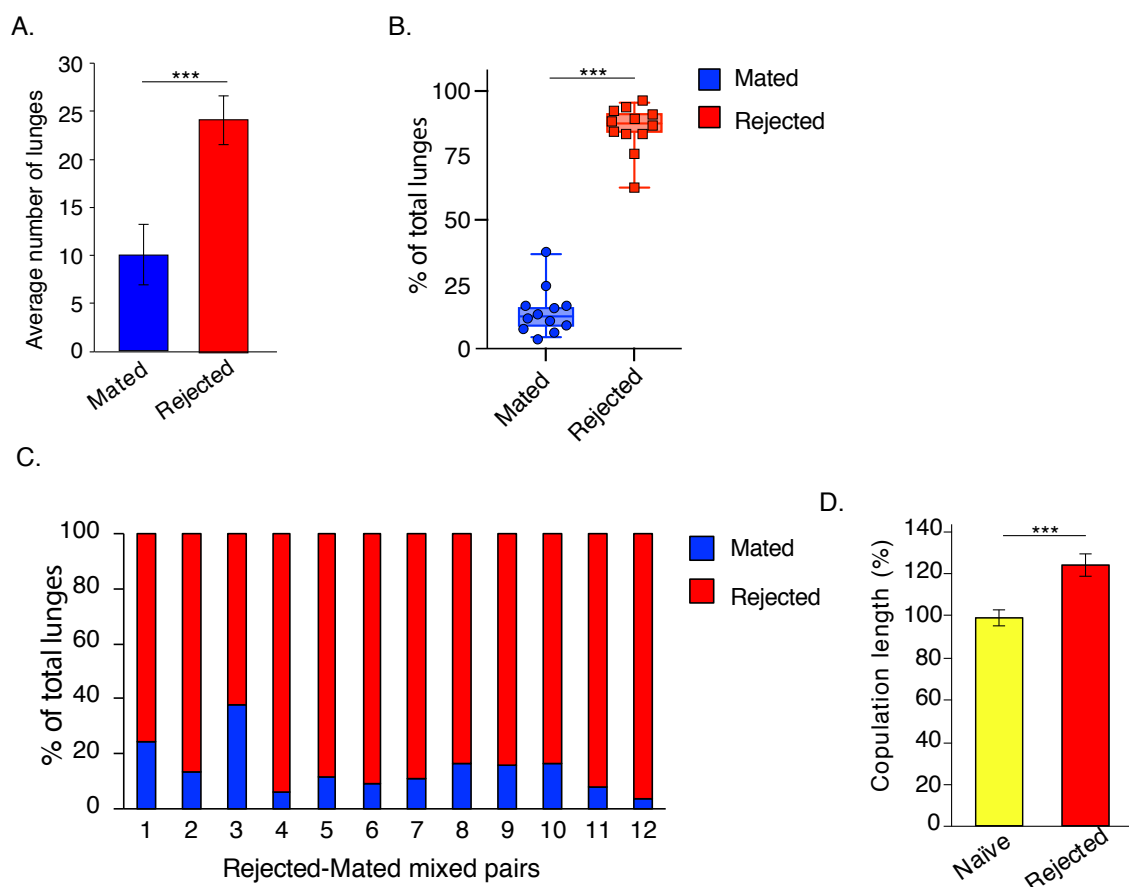
**Figure 3. Sexual deprivation modulates competitive behaviors. A.-C.** Aggression display (number of lunges) was compared between pairs of rejected and mated male flies (n=16, statistical significance determined by T-test, $p< 0.005$ **(A),** and mixed pairs (n=12) **(B-C).** The log2 ratio between the number of lunges in rejected and mated flies was calculated for each pair, and then a one-sample T-test was performed to test whether the mean ratio was significantly different than 0, $p<0.005$. Data is presented as the mean ± SEM. **D.** Duration of copulation in rejected vs. naïve male flies. Statistical significance was determined by T-test, $p < 0.001$. Data is presented as mean ± SEM, n=25.
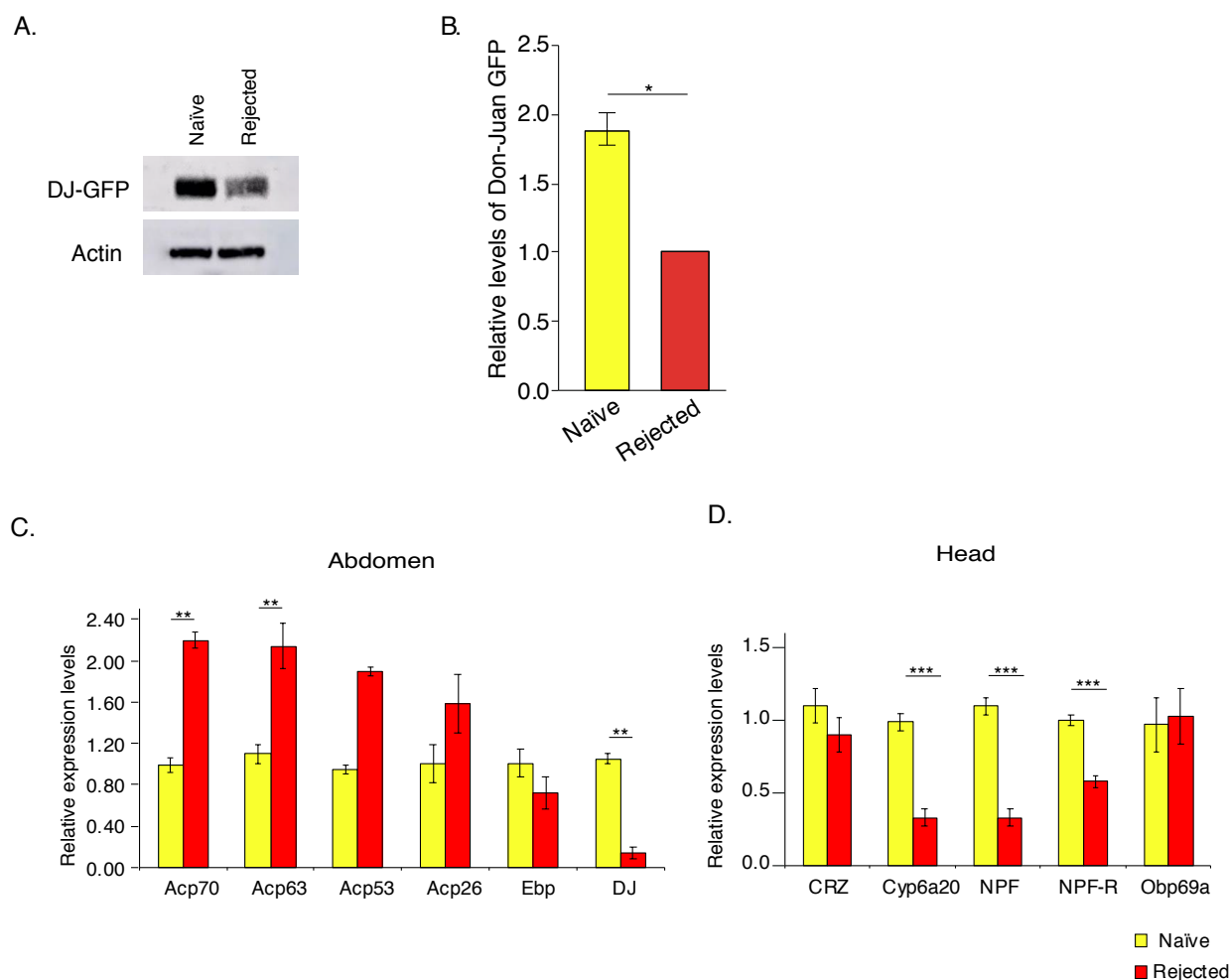
577
578 **Figure 4. Failure to mate modulate sperm and seminal fluid composition. A,B.** Protein lysates prepared from
579 abdomen of rejected and naïve male flies and were analyzed for the relative levels of Don-Juan-GFP using western
580 blot, actin was used as a loading control. Expression levels of Don-Juan-GFP protein were quantified and
581 normalized to actin levels (n=3), Statistical significance was determined by T-test, $p<0.05$ (F). **C.D.** Relative
582 transcript levels of candidate genes expressed in abdomen (G) and heads (H) of rejected and naïve male flies were
583 quantified by qRT-PCR, n = 6 independent experiments of 15–20 fly heads and abdomen. Statistical significance
584 was determined by Student's T-test with Bonferroni correction for multiple comparisons. **, $p <0.01$? ***, $p <$
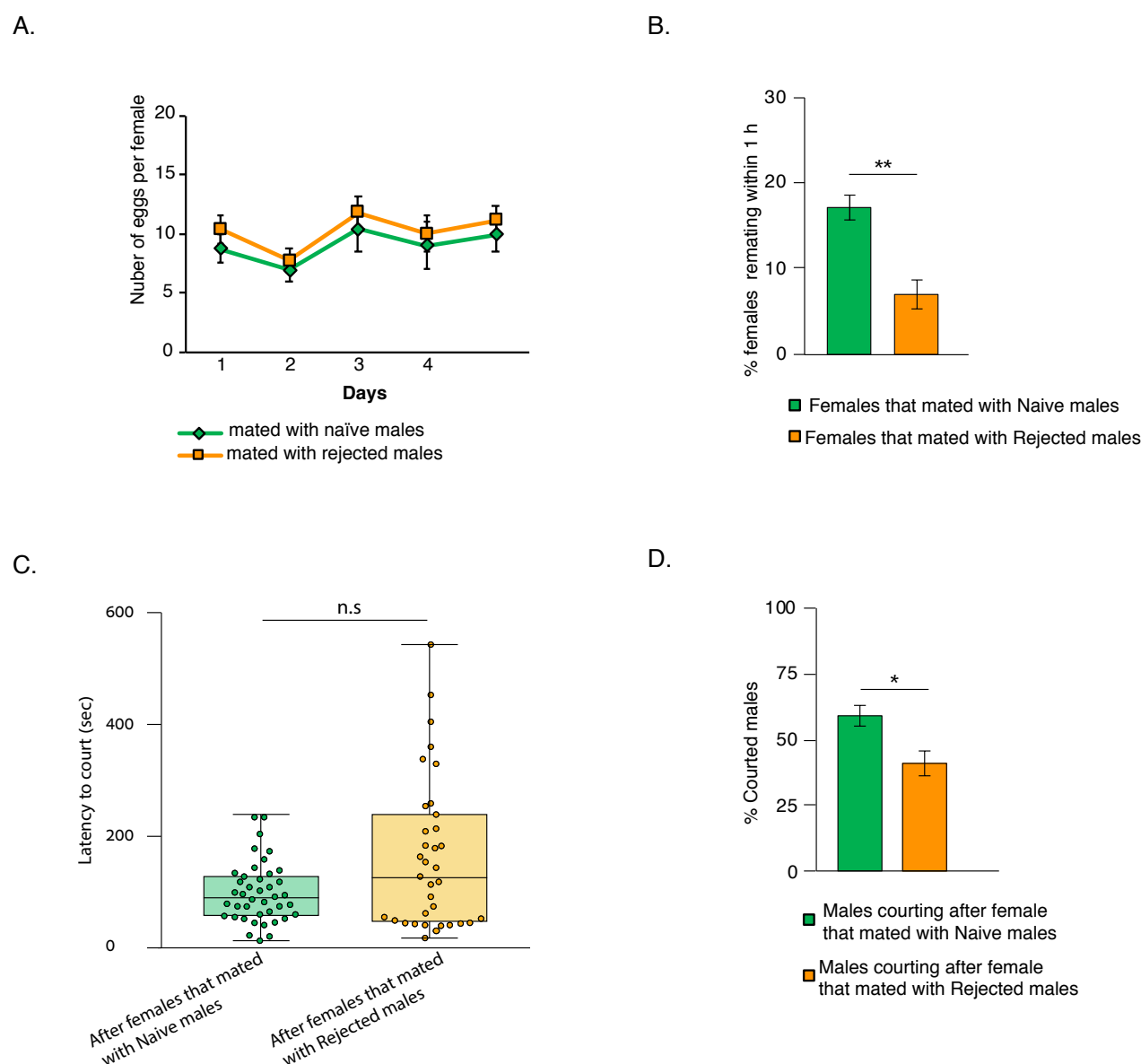585 0.005.

27

A.

B.

C.

D.



586

**Figure 5. Effect of male rejection on female's fertility and remating tendencies**. **A.** Number of eggs laid by females that copulated with rejected or naïve male flies over the course of 5 days. Statistical significance was determined using two-way ANOVA repeated measure, n=28 $p>0.05$. **B.** Female receptivity to re-mate with male flies 24h after the first mating with rejected or naïve male flies was scored bycounting the precent of female flies that mated during 1 hour of test. Data is presented as the mean ± SEM, n=4 repeats. Statistical significance was determined by Cochran-Mantel-Haenszel Chi-square test, $p<0.005$. **C.** Mean courtship latencies of rejected or naïve male flies towards mated female flies (24 hours post first mating), n=25. Statistical significance was determined by Mann-Whitney U-test, N.S., $p> 0.05$. **D.** Number of new males that courted females that were previously mated with rejected or naïve male flies, n=25. Statistical significance was determined by T-test, $p< 0.05$.

28

| Definition | Description | Definition | Description |
|---|---|---|---|
| dnose2ell | Minimum distance from any point of this animal nose to the ellipse of other flies. | Walk | Fly moves. |
| | | Stop | Fly is still. |
| absanglefrom1to2nose2ell | Absolute difference between direction to closest animal based on dnose2ell and current animal's orientation (rad). | Turn | Changes in fly's direction. |
| | | Touch | Fly actively touches another fly. |
| absdtheta | Angular speed (rad/s). | Approach | Fly approaches another fly and perform interaction (active or passive). |
| absphidiff anglesub | Absolute difference in velocity direction between current animal and closest animal based on anglesub (rad). | Aggregation | Fly sits in a group of 3 or more flies. |
| | | Grooming | Fly grooms. |
| | | Chase | Fly chases another fly. |
| | | Chain | Chase with 3 or more flies. |
| absphidiff nose2ell | Absolute difference in velocity direction between current animal and closest animal based on dnose2ell (rad). | Song | Fly moves one wing next to another fly. |
| | | Behavior bout length | Length of the longest sequence of frames in which the behavior occurred per fly. |
| absthetadiff anglesub | Absolute difference in orientation between current animal and closest animal based on anglesub (rad). | Behavior frequency | Length of the movie minus the length of the longest sequence of frames in which the behavior didn't occurred for each fly. |
| absthetadiff nose2ell | Absolute difference in orientation between this animal and closest animal based on dnose2ell (rad). | Density SD by length of interactions (LOI) | Accumulated interactions' length relative to the maximum interactions' length possible. |
| anglefrom1to2 anglesub | Angle to closest (based on angle subtended) animal's centroid in current animal's coordinate system (rad). | Modularity by length of interactions (LOI) | Representation of how much the network is divided into modules according to interactions' length. |
| anglefrom1to2 nose2ell | Angle to closest (based on distance from nose to ellipse) animal's centroid in current animal's coordinate system (rad). | Strength by length of interactions (LOI) | Length of interactions of a certain fly. |
| | | SD Strength according to length of interactions (LOI) | Standard deviation of the strengths according to interactions' length of flies from the same movie. |
| angleonclosestfly | Angle of the current animal's centroid in the closest (based on distance from nose to ellipse) animal's coordinate system (rad). | Betweenness Centrality by length of interactions (LOI) | A measure of centrality of a certain fly based on shortest paths according to interactions' length. |
| anglesub | Maximum total angle of animal's field of view (fov) occluded by another animal (rad). | SD Betweenness Centrality by length of interactions (LOI) | Standard deviation of the betweenness centralities according to interactions' length of flies from the same movie. |
| danglesub | Change in maximum total angle of animal's view occluded by another animal (rad/s). | Density by number of interactions (NOI) | Interactions' number relative to the maximum interactions' number possible. |
| dcenter | Minimum distance from this animal's center to other animal's center (mm). | Modularity Strength by number of interactions (NOI) | Representation of how much the network is divided into modules according to interactions' number. |
| ddcenter | Change in minimum distance between this animal's center and other flies' centers (mm/s). | Strength by number of interactions (NOI) | Number of interactions of a certain fly. |
| dist2wall | Distance to the arena wall from the animal's center (mm). | SD Strength by number of interactions (NOI) | Standard deviation of the strengths according to interactions' number of flies from the same movie. |
| dphi | Change in the velocity direction (rad/s). | Betweenness Centrality by number of interactions (NOI) | A measure of centrality of a certain fly based on shortest paths according to interactions' number. |
| dtheta | Angular velocity (rad/s). | | |
| nflies_close | Number of flies within 2 body lengths (4a). | | |
| velmag | Speed of the center of rotation (mm/s). | SD Betweenness centrality (by number of interactions (NOI) | Variance of the betweenness centralities according to interactions' number of flies from the same movie. |

597     Table 1: description of kinetic features, behaviors and social network related features.
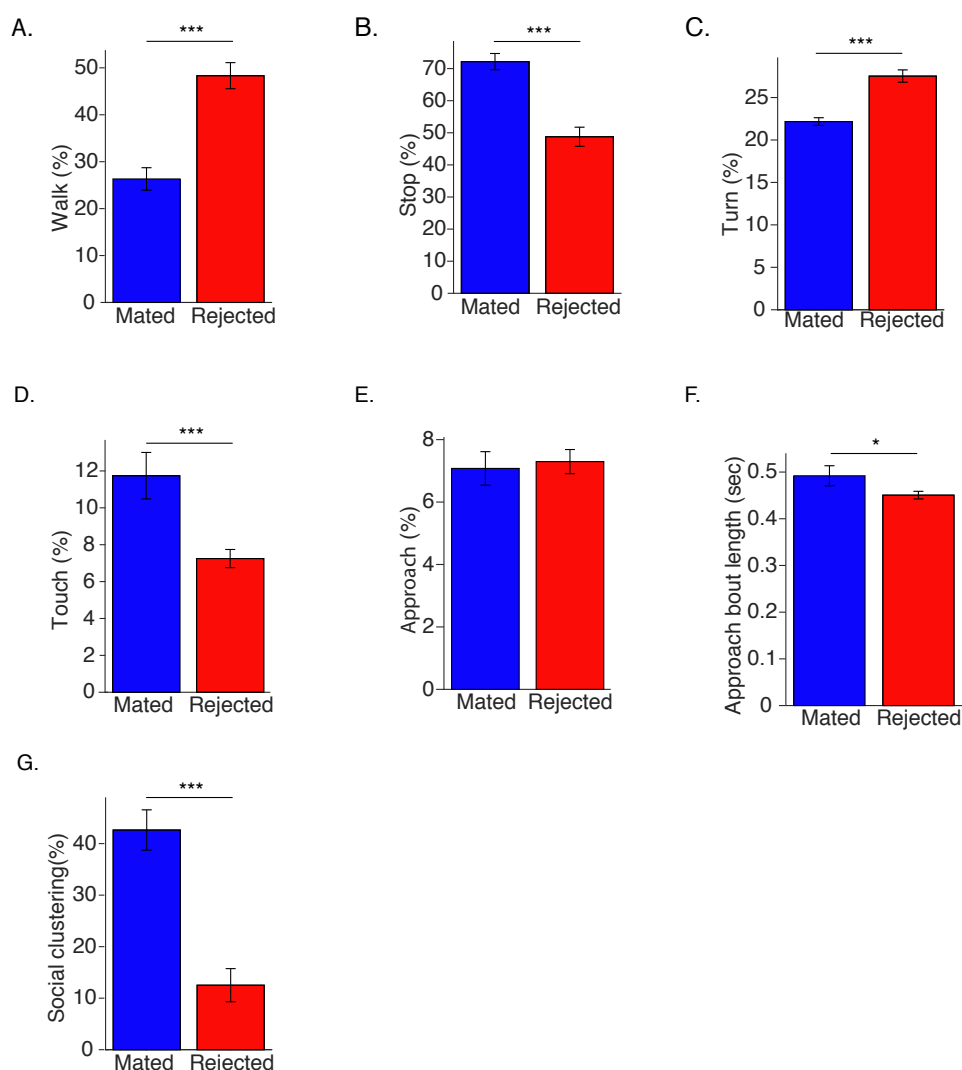
**Supplementary Figure 1.** Behavior classifier analysis depicts mean values of the behaviors averaged across the experiment: walking (A), Stop (B) body turns (C), close touch behavior (D), approach, bout duration of approach behavior (F) and social aggregation (G). n=18 t test for normally distributed parameters or Wilcoxon test for non-normally distributed parameters.

Number of housed male flies

Don-Juan GPF

Actin

602
**Supplementary Figure 2. The expression of Don-Juan protein in sensitive to the presence of rival male flies.**
Relative expression levels of Don-Juan-GFP in male flies in single or grouped housed male flies.