# Sexual rejection modulates social interaction and reproductive physiology

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**Abstract**

In highly polyandrous species, in which females mate with multiple males within a single fertility period, there is typically a high level of sperm competition. To cope with this challenge, males apply various behavioral and physiological strategies to maximize their reproductive success. Previous studies in *Drosophila melanogaster* established a link between the composition of the social environment and the reproductive success of individual male flies. While most studies until now focused on the adaptive responses of male flies to the presence of rival males, little is known about whether the outcomes of sexual interactions with female partners alter male-male social interactions. Here we show that repeated failures to mate promote coordinated physiological and behavioral responses that can serve to increase reproductive success over mating rivals in the future competition. We exposed male flies to sexual rejection, successful mating or no sexual experience, and analyzed the behavioral repertoires of individuals within groups and the structure of their emerging social networks. We discovered that failures to mate promote the formation of distinct emergent group interactions and structures, where rejected male flies form low density social networks and actively minimize their encounters with other group members, while increasing their aggressive behavior. In addition, sexually rejected male flies elevate the production of seminal fluid proteins and extend mating duration with receptive females, altogether leading to reduced re-mating rates. Our results demonstrate the existence of a flexible mating response as possible coping strategy for living in a highly dynamic and competitive environment as the social domain.

## 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[1–3]. This is particularly important in a dynamic environment such as the social domain composed of many behaving animals, the interaction with ultimately determines the reproductive success of individuals[2,4–6]. 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[5]. 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[7–9]. 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[9,10].

As a species with sociable lifestyle, *Drosophila melanogaster* exhibit communal living around freshly decaying fruits[11] and engage in diverse forms of social interactions[12]. This includes courtship and mating[13–16], fighting over resources[17], group interactions[18–20], coordinated responses to threats[18,21,22], cultural transmission of complex behaviors[23], learning from conspecifics[24,25], and synchronization of activity by social cues[26]. 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

62 and physiology in response to changes in their social environment. This includes the ability of male flies
63 to change their aggressive behavior in response to prior fighting experience[27–29], regulate sperm
64 composition and the duration of copulation events in response to perceived competition[30–32], and suppress
65 courtship efforts towards non-receptive female flies[33–35].

66 Recent studies in *Drosophila* demonstrate that fruit flies generate complex and rich group
67 structures that are sensitive to the density of the group, its composition, as well as to the prior experience
68 of its members[19,20,36,37]. We previously showed that sexual experience in male flies can modulate their
69 motivational state and, subsequently, their reward seeking behaviors[38,39]. However less is known about
70 the way by which prior sexual interactions that are experienced as success or failure to mate shape social
71 interaction of male flies in a group context. Furthermore, it is not clear whether sexually rejected male
72 flies exhibit loser-like responses, as in the case of social defeat[29], or rather actively increase their
73 competitive behavior to cope with mating rivals. Here we explored the effects of success or failure to
74 mate on the dynamics of social interaction in groups of male flies. We discovered that rejection promotes
75 the formation of discrete group interactions and structures, wherein rejected male flies actively minimize
76 their interactions with group members and at the same time enhance competitive behaviors and alter
77 reproductive physiology, presumably to increase their reproductive success.

## Results

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

We previously demonstrated that sexual experiences associated with different levels of mating success, such as repeated events of successful mating, or failures to mate in the form of repeated rejection events by non-receptive female flies, alter internal state and consequently motivational responses[38,39]. The negative valence of rejection, reflected by its capacity to induce courtship suppression and increase the consumption of ethanol, prompted us to explore the effects of sexual rejection on male-male social interaction and test whether failure to mate leads to loser-like state or rather facilitates competitive responses. To discriminate between these alternatives we analyzed the behavioral signature of male flies that experienced repeated failures to mate upon interaction with other male flies in a group context, compared to controls that experienced successful mating or lack of any sexual experience.

Wild type male flies (Canton S) were collected upon eclosion and kept in isolation for four days before being exposed to female flies. Rejected cohort (rejected-isolated) was generated by exposing 4 days old single male flies to 1h long interaction with a non-receptive female fly, 3 times a day for 4 days (Fig. 1A)[40]. As control, we used male flies that experienced successful mating, 1h long interactions with a receptive virgin female (1:1 ratio) , 3 times a day for 4 days (mated-isolated) and age matched male flies that lack any sexual experience (naïve isolated). At the end of this experience, their social group interactions was examined by introducing 10 flies from each cohort into the FlyBowl arenas, where they could move and interact in two dimensions. Their behavior was recorded for 30 min and analyzed using the FlyBowl suite of tracking and behavior analysis softwares[20,41,42] (Fig. 1A).

The tracking data obtained was used to calculate various kinetic features including velocities, distances and angles between flies and their relative differences across time (Fig. 1B). This set of per frame features served to train 8 types of behavior classifiers using JAABA (Fig. 1B)[20], and to analyze

101 their emergent social networks, by measuring the overall number or duration of interactions between pair
102 of flies. Interaction events were scored using the following parameters: >8mm between 2 flies and the
103 visual field of view of the focal fly is occupied by the other fly (angle subtended > 0), indicating that the
104 focal fly can see the other fly, and that these two requirements are maintained for at least 2 seconds. We
105 used these interaction criteria to calculate various properties of the emergent social networks of flies from
106 the rejected, mated, and naive cohorts.

107 Examining features that reflect activity levels such as time spent walking, turning, time spent
108 stopping and average speed, revealed similar activity levels in rejected and naive males, that are
109 significantly higher than those of mated males (Fig. 1 C-F). Analysis of specific social behaviors
110 indicated overall reduced degree of social interaction in rejected males than in naive males (Fig. 1 G-J).
111 This included low levels of approach towards other individuals (Fig. 1G), low levels of male-male chase
112 behavior (Fig. 1H), reduced formation of chains composed of multiple chasing males (Fig. 1I), and
113 reduced rates of close touch behavior (Fig. 1 G-J).

114 **Failure to mate promotes social avoidance**

115 Next, we investigated the network structures of groups composed of rejected, mated and naive
116 male flies using features that describe the properties of the network and the individuals within it (Fig.
117 2A)[20]. We calculated network weights according to the overall duration of interactions (emphasizing
118 long-lasting interactions) or the overall number of interactions (emphasizing short interactions) between
119 each pair of flies (Fig. 2B-I). Analysis by duration indicated that the social networks of rejected males
120 are characterized by lower density (Fig. 2B), and that the average strength of individual flies in these
121 networks is lower compared to mated and naive male flies (Fig. 2C). Likewise analysis of network
122 features by number of interactions identified reduced density and low strength of the individuals than

123 naive males (Fig. 2 F,G). These findings suggest that rejection promotes the formation of sparser groups
124 containing individuals with reduced social interaction.

125 The reduced network density of rejected male flies prompted us to compare the average distance
126 between individuals of each group as indicated by the average field of view occluded by another fly
127 (anglesub), a feature that increases when distance between individuals decreases (Fig. 2J). Analyzing the
128 average degree of anglesub along the experiment discovered that rejected male flies maintain
129 significantly low values of anglesub, suggestive of maintaining long distances between flies across the
130 experiment (Fig. 2J). This finding is also supported by reduced number of flies found near a focal fly
131 compared to mated and naive male flies (Fig. 2K). Given that rejected male flies exhibit similar activity
132 levels as naive male flies, and presumably higher probability to encounter other flies, their reduced
133 network density and high inter-individual distance suggest that rejected individuals actively avoid social
134 interactions with other flies, resulting in low density groups.

135 The behavioral signatures of the three cohorts across all 60 parameters are depicted in a scatter
136 plot of normalized differences and are divided into 4 main categories: activity, interaction, coordination
137 between individuals and social clustering where male aggregate to form clusters (Fig. 3). While the
138 cohort composed of mated male flies is characterized by overall reduced activity and the formation of
139 stable social clusters, the cohort composed of naïve male flies displayed increased activity, high levels
140 of social interaction and low levels of social clustering (Fig. 3). In contrast, the rejected cohort depicted
141 similar activity levels to that of the naïve cohort, reduced formation of social clusters and overall reduced
142 behaviors that are associated with social interaction (Fig. 3). The existing similarities and differences
143 between the three cohorts indicate that sexual rejection induce a discrete behavioral state that differs from
144 successful mating and the lack of mating and point to sexual rejection as the major contributor to reduced
145 social interaction.

146 **Sexual rejection modulates competitive behaviors**

147 The discrete group signature associated with sexual rejection led us to hypothesize that the
148 behaviors exhibited by rejected males reflect behavioral response to coping with high sexual competition
149 over mating partners, where repeated encounters with mated females are indicative of a high male to
150 female sex ratio. To examine this hypothesis, we next tested whether rejection modulate competitive
151 behaviors that can affect reproductive success such as aggressive responses toward other males and
152 mating with virgin female flies. We compared rejected males to mated males, since both cohorts
153 experienced interaction with females, unlike naïve flies that were socially isolated, a condition known by
154 itself to promote aggression. We discovered that pairs of rejected male flies exhibited significantly higher
155 aggressive displays in comparison to pairs of mated male flies (Fig. 4A), and that in mixed pairs, rejected
156 males exhibited greater numbers of lunges compared to their mated opponents (Fig. 4B,C). To test
157 whether rejection modulates investment in mating behavior, we compared the mating duration of rejected
158 males to that of naïve male flies since mating investment of the mated cohort is shaped by their previous
159 mating events. When allowed to mate with virgin female flies, rejected male flies extended the duration
160 of copulation events by 25% (3.5 minutes longer) compared to naïve males (Fig. 4D). Thus, rejected
161 male flies exhibited a significant increase in behaviors that may enhance their reproductive success in a
162 competitive environment.

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

164 To determine whether prior rejection affects reproductive physiology such that it can promote
165 reproductive successes, the expression levels of genes related to sperm production and reproduction were
166 assessed. First, the expression of DON-JUAN (DJ), a protein that is specifically expressed in mature
167 male sperm cells[32,33], was measured using a GFP-based reporter line in which a GFP sequence was
168 inserted within the coding locus, so that the expression of GFP reflects the expression of the endogenous

169 DJ protein. The reliability of the DJ-GFP reporter as a sensitive measure for changes in sperm production
170 was first confirmed by comparing DJ-GFP levels in isolated and grouped housed male flies, since these
171 conditions are known to affect the amount of mature sperm[31,43](Supp Fig.1). The relative levels of GFP
172 were then measured in rejected and naïve male flies (Fig. 4E,F). We documented a two-fold decrease in
173 the levels of GFP in the rejected cohort compared to naïve males, suggesting that male flies decrease
174 their investment in sperm allocation in response to sexual rejection (Fig. 4E,F).

175 Next, the relative expression of the following reproductive related genes was directly assessed in
176 fly abdomens by qRT-PCR. We measured the expression of *Sex-Peptide (Acp70A), Acp63, Acp53,*
177 *Ovulin (Acp26Aa)*, which are responsible for the females' long-term post-mating responses and fertility[44].
178 We also measured the expression of genes encoding the *Ejaculatory bulb protein (Ebp),* which is
179 responsible for the posterior mating plug formation at the end of mating[45], *don-juan (dj)*[46], the *corazonin*
180 *(Crz)* neuropeptide, which promotes sperm and seminal fluid ejaculation in males and its receptor *Crz-*
181 *receptor*[39,47] (Fig. 4G). There was a two-fold increase in the levels of *Acp-70A (Sex-Peptide)* and *Acp-63*
182 in rejected male flies when compared to naïve males, suggesting that rejected male flies increase their
183 investment in the production of seminal fluid proteins that are transferred to females flies during
184 copulation (Fig. 4G). Nevertheless, in agreement with the observed reduction in DJ-GFP reporter levels,
185 there was a drastic decrease in the transcript levels of *don-Juan* in rejected males. The transcript levels
186 of *Ebp and Crz* and its receptor were similar in both cohorts (Fig. 4G). Overall, these results suggest that
187 rejected male flies respond to sexual rejection by elevating seminal fluid protein transcript levels,
188 presumably to maximize their fitness.

189 In addition to proteins associated with the male reproductive system, levels of several genes
190 expressed in the brain and antenna were also assessed. These included the neuropeptides *Crz*,
191 *Neuropeptide F* (*npf*) and its receptor (*npfr*), and two olfactory related genes associated with aggression
192 (the *Odorant binding protein 69a*[48], and *Cyp6a20*[49]. In agreement with previous studies, the levels of *npf*

193 were significantly lower in sexually rejected male flies[38]; we also observed a reduction in *npfr* (Fig. 4H).
194 Interestingly, sexually rejected male flies also exhibited reduced levels of *Cyp6a20* in comparison to
195 naïve male flies (Fig. 4H), consistent with their enhanced aggression (Fig. 3 A-C).

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

197 While the molecular changes associated with rejection support our initial hypothesis that rejected
198 male flies adjust their behavior and physiology to cope with high sexual competition, it is not clear
199 whether these and the extended copulation improve their reproductive success. To test this direction,
200 several aspects associated with female fecundity were measured. First, the fertility of female flies was
201 assessed after one mating event with either rejected or naïve male flies, by counting the number of eggs
202 laid (fecundity) and the corresponding number of adult progeny (productivity). There was no significant
203 difference in the number of eggs laid or emerging progeny between the two cohorts 2, 4 and 6 days after
204 initial mating (Fig. 5A). The lack of difference in the amount of progeny suggested that lower sperm
205 investment in rejected males (as reflected by reduced DJ levels) does not affect the total offspring
206 number, meaning that there is no link between sperm investment and the number of offspring.

207 Finally, we tested whether the increase in sex-peptide could facilitate enhanced post-mating
208 behavior (such as reduced receptivity) in females that mated with rejected male flies. Since the strongest
209 post-mating response is observed 24h after mating (data not shown), the proportion of female flies that
210 re-mated with fresh male flies 24h after they mated with either rejected or naïve male flies was measured.
211 A significant reduction was documented in the re-mating rates of females that mated initially with
212 rejected males (Fig. 5B), suggesting that extended copulation time and increase in seminal fluid proteins
213 can lead to a stronger reduction in female receptivity.

214 During copulation, male flies transfer to female flies seminal fluid proteins and also anti-
215 aphrodisiac pheromones such as cVA[34]. The extended copulation observed in rejected male flies may

216 facilitate the transfer of larger amounts of cVA as a means to delay further courtship and copulation with
217 subsequent male flies. As an indirect measure for possible changes in the amount of transferred cVA, we
218 analyzed the courtship behavior of naïve male flies towards females that previously mated with either
219 rejected or naive male flies 1h after the initial mating. No significant difference was observed in the
220 latency to court, i.e. the time it takes male flies to exhibit their first courtship action (wing vibration)
221 following introduction of the pair into the courtship arena (Fig. 5C). However, there was a significant
222 reduction in the number of naïve male flies that courted females previously mated with rejected males
223 (Fig. 5D), suggesting that mating experience with rejected male flies results in mated females that are
224 less attractive courtship targets than females previously mated with naïve males.

225 **Discussion**

226 In this study we used the FlyBowl as an agnostic tool to explore responses altered by sexual
227 interaction, and discovered that rejected male flies cope with their failures to mate by changing their
228 behavior and physiology, possibly to enhance their reproductive success. This is presumably achieved
229 by avoiding interaction with potential rival male flies and competing over mating partners via increased
230 aggression and prolonged copulation (known as mate guarding). The latter is strengthened by the
231 increased production of certain seminal fluid proteins that facilitate stronger post-mating responses in
232 female flies.

233 The behavior of sexually rejected male flies was examined in this study under behavioral contexts
234 that illuminate different aspects of their action selection. Using the FlyBowl system, we analyzed their
235 emergent group interactions and social networks, and discovered that although rejected males are highly
236 active, they exhibit sparse networks and maintain large distance with other members, as if they were
237 actively minimizing or avoiding interaction with rival male flies. When tested in a social context that
238 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[49]. Interestingly, exposure to female flies prior to male-male interactions
243 was previously shown to suppress aggression[50]. 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 mating success) determines aggression levels when encountering another
246 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
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 a 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[30,51]. 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[51]. 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[52],
260 are consistent with this hypothesis.

261 The behavioral responses to sexual rejection were accompanied by changes in the repertoire of
262 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 increased number of sperm cells)[31], rejected male flies exhibited a significant reduction in the levels
268 of DJ expressed in mature sperm cells. Although this finding is limited to only one protein, this is
269 surprising in light of sperm competition theory, which predicts that males should strategically increase
270 their investment in sperm allocation when in competition[53]. This finding is also different from previous

271 studies that mating with non-preferred males produces smaller number of offspring and that non-
272 preferred males deliver larger amounts of sperm, presumably as a compensation mechanism[54–56].
273 Furthermore, our findings are different from studies in crickets, sunfish, birds rats and also flies, which
274 showed that the perceived risk of sperm competition, in the form of the presence of rival males or their
275 odors before and during mating, led to an increase in sperm investment[52,57,59,61,63]. The uncoupling
276 between the investment in sperm and non-sperm components and the regulation of investment in
277 copulation time demonstrates that sexually rejected male flies regulate each of these processes
278 independently.

279 Functionally, the observed decrease in sperm quantity with increasing seminal fluid protein *(Acp)*
280 expression in rejected males did not affect the amount of progeny produced in females. This observation
281 suggests that there is no link between the observed behavioral and physiological changes and the number
282 of progeny. Nevertheless, females that mated with rejected males were less attractive to naïve male flies,
283 as reflected by the reduced number of male flies that courted these females. The combination of reduced
284 female attractiveness in subsequent mating encounters, and reduced motivation of the female to re-mate,
285 may reduce the odds for a second mating and thus increase the rejected male's paternity rate despite the
286 lack of an effect on progeny number.

287 In summary, our results demonstrate a plastic mating strategy by males that experienced repeated
288 events of rejection which improves their reproductive success when competing with rival male flies. We
289 postulate that rejected males invest more energy in the production of seminal fluid proteins over sperm;
290 these Acps are known to have important roles in modulating different aspects of female mating
291 physiology and behavior. Furthermore, at low population density, the chances to meet a receptive female
292 are low, therefore an investment in sperm ejaculate may be more costly[58]. In this respect, Cameron et al.,
293 developed models that can explain the tradeoff between sperm and seminal fluid protein production and
294 their regulation under various conditions[60]. Their model predicts that competing males should evolve the

295 ability to adjust ejaculate composition based on competition, and whether they are the first or second
296 male to mate with a particular female[60]. Further research is needed to test ejaculate plasticity in the
297 context of sexual rejection, including whether mRNA levels of ACPs correlate with protein levels as
298 there are studies suggesting a weak correlation between the two[62], the proteome composition of ejaculate
299 that is transferred to females during copulation, and the ability of females to actively control this
300 process[64].

**Acknowledgments:** We thank all members of the Shohat-Ophir lab for fruitful discussions and technical support. We specially thank Kristin Branson and Alice Robie (HHMI Janelia research campus) for their valuable advice and guidance with the experimental and computational design. This work was supported by the Israel Science Foundation grant 384/14 and Israel Science Foundation grant 174/19.

305 **Materials and methods:**

306 Fly lines and culture

307 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
308 in 60% relative humidity and maintained on cornmeal, yeast, molasses, and agar medium, and were tested
309 as 3–4-day old adults, unless otherwise specified. The DJ-GFP and White Berlin (WB) lines were
310 obtained from the HHMI Janelia Farm Research Campus.

311 Sexual experience paradigm

312 Male and female flies were anesthetized under $CO_2$ and isolated immediately after eclosion. Male flies
313 were reared as single-housed in vials (23 mm by 94 mm) containing 7 ml of medium. Female flies were
314 kept in groups of 10 flies/vial. Both males and females were aged for 3–4 day before the beginning of
315 the experiment and kept in the incubator at 25°C, ~70% humidity, and light/dark of 12:12 hours. Mated
316 females to be used as trainers, were generated by pair groups of 10 virgin female flies with 15 male flies
317 16 - 18 h before the training phase and were separated from male flies each morning, before the beginning
318 of the training phase.

319 Experimental groups consisted of naive male, rejected and mated male flies: Rejected and mated cohorts
320 were generated by pairing single male flies with either virgin female (mated cohort) or mated female
321 (rejected cohort), for three one-hour sessions spaced by one hour rest, for consecutive days. During these
322 one-hour sessions, pairs were monitored closely to make sure that the males interacting with virgin
323 females mate successfully within the first hour, and that the males interacting with the previously-mated
324 females do not mate. Males from the rejected cohort that managed to mate and males from the mated
325 cohort that did not end up mating during all sessions were discarded. At the end of each session, the
326 female fly was removed, and the males that experienced rejection were kept in the original vial for one
327 hour rest. Naive male cohort was kept in the behavior chamber during the training phase, and the vials

containing single virgin males were handled as similar as possible to the rejected and mated cohorts, without inserting female trainers. Detailed protocol is previously described[40].

Social group interaction using the FlyBowl system

At the end of the sexual experience phase, rejected and mated male flies were inserted in groups of 10 into Fly Bowl arenas[41], and their behavior was recorded for 30 minutes and analyzed using CTRAX, FixTrax[20] and JAABA[41]. For kinetic features, scripts were written in MATLAB to use the JAABA code to generate the statistical features as specified in Kabra et al. [41]. Time series graphs (per frame) were created using JAABA Plot[41]. Quantification of complex behavios was done using JAABA Classifiers[41] to identify specific behaviors: Walk, Stop, Turn, Approach, Touch, Chase, Chain, Song, Social Clustering and Grooming. Bar graphs were created using JAABA Plot[41]. Network analysis was performed using an interaction matrix according to the interaction parameters described previously[20]. Two interaction matrices were created for each movie, one with the total number of frames each pair of flies were interacting divided by the number of frames in the movie and another with the number of separate interactions between each pair of flies divided by the maximum number of possible interactions, calculated as:

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

The parameters to define an interaction are: angle subtended by the other fly > 0, distance between the nose of current fly to any point on the other fly ≤ 8 mm, number of frames for interaction ≥ 60 and number of gap frames ≥ 120. Interaction end is defined when distance or angle conditions are not maintained for 4 seconds. Networks and their features were generated from the interaction matrix in R using the igraph package. The function that was used to the generate networks is

351 “graph_from_adjacency_matrix” with parameters “mode = undirected” and “weighted = TRUE”.
352 Density was calculated on all movies with the formula:
353

354 $$density = \frac{sum\ of\ weights}{[number\ of\ vertices * (number\ of\ vertices - 1)] * 0.5}$$

355

356 Modularity was calculated using the “modularity” function on output from the “cluster_walktrap”
357 function[65]. Strength was calculated using “strength” function and SD Strength was calculated on all
358 movies using “sd” function on the strength value. Betweenness Centrality was calculated on all flies
359 using the “betweenness” function and SD Betweenness Centrality was calculated on all movies using
360 “sd” function on the Betweenness Centrality value. Box plots were created using R.
361

362 Each feature of the FlyBwol experiment was standardized according to all values calculated in our
363 experiments for that feature to generate a z-score. Scatter plots were created using R.
364 Aggression
365 Pairs of rejected or mated male flies were introduced into aggression arenas (circular chambers, about
366 0.08 cm3 in volume), which contained a mixture of agarose and apple juice (1% agarose, 50% apple
367 juice) that was placed in arenas to enhance aggressive behavior. Flies were filmed for 30 min with Point-
368 Grey Flea3 (1080×720 pixels) at 60 fps. Aggressive behavior was later quantified by counting the number
369 of lunges for each pair using CADABRA software (ref). The $\log_2$ ratio between the number of lunges in
370 rejected and mated flies was calculated for each pair, and then a one-sample t-test was performed to test
371 whether the mean ratio is significantly different from 0.
372 Copulation duration
373 Rejected and naïve male flies were put into courtship arenas (circular chambers, about 0.04 cm3 in
374 volume) with virgin females and were allowed to mate for 1 hour. They were recorded for the whole

375 experiment using a Point-Grey firefly camera. Courtship arenas consist of 25 flat arenas each arena
376 containing only one pair of male-female flies. The copulation duration was measured from the moment
377 the mating began until it ended. We calculated the time in seconds for each fly and the average for each
378 group.

379 Egg laying assay

380 Egg production was determined for females that had been allowed to copulate with rejected or naive
381 males for 1 hour at the end of the conditioning (as described above). Every female was put in a glass vial
382 containing fresh food every two days for 6 days in total and was kept in the incubator. The sum of the
383 number of eggs in the vials of each female was used for analysis.

384 Receptivity assay

385 3-4-day old White Berlin (WB) females were allowed to mate once with rejected or naïve males at the
386 end of the conditioning for 1 h. After mating, the males were disposed and the mated females were kept
387 in the incubator for 24h. Afterward, the mated females were exposed to 5-day-old WT naïve males for 1
388 h to measure their receptivity to mate. Approximately 40 pairs of each group (rejected or naive) were set
389 up in every biological repeat.

390 Latency to court assay

391 1 hour after allowing WB females to mate with rejected or naïve males, we transferred the females into
392 courtship arenas and paired them with new WT naïve males. The pairs were recorded for 15 min to
393 measure courtship latency. Latency was defined as the time elapsing between the introduction of the pair
394 into the chamber and the first appearance of wing vibration made by the courting male fly. We also
395 quantified the number of males who did and did not try to court in this assay.

396 Courtship Index

397 Courtship index for a given male is the fraction of time a male fly spent in courtship activity in the 10
398 min observation period (600 sec). It is calculated by dividing the number of seconds the male courted

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

401 Molecular methods

402 Western blot analysis: Sperm allocation in male flies carrying the DJ-GFP reporter was determined by
403 Western blotting. DJ protein size is ~29 kDa, and GFP size is ~25 kDa. We also determined the levels of
404 Sex-peptide (SP), a protein of size ~7 kDa, and the levels of Tubulin for normalization. The primary
405 antibodies used were mouse anti-GFP, rabbit anti-SP and rabbit anti-Tubulin, and the secondary
406 antibodies that were used are rabbit α-mouse HRP and mouse α-rabbit HRP, respectively. Virgin females
407 were used as negative controls.

408 Quantitative Real-Time PCR analysis

409 Frozen flies were placed on ice and decapitated using a scalpel. Total RNA was extracted from ~15
410 frozen heads and bodies (separately), using TRIZOL reagent according to the manufacturer's protocol.
411 mRNA was reverse transcribed using BIORAD cDNA synthesis kit. cDNA was analyzed by quantitative
412 real-time PCR (BIORAD CFX96) using specific primers for the head and for the body. Relative
413 expression was quantified by ΔΔCT method using RPL32[66] as a loading control. We run each sample in
414 triplicates. Each experiment was repeated four times using independent sets of experimental flies.

415 Statistical analysis

416 For each experiment, Shapiro–Wilk test was done on each experiment to test for normal distribution.
417 Statistical significance was determined by t-test for experiments that were distributed normally, and by
418 Wilcoxon test for experiments that were not distributed normally. For experiments with three conditions:
419 statistical significance determined by one-way ANOVA followed by Tukey's range test for experiments
420 that were distributed normally, and by Kruskal–Wallis test followed by Wilcoxon signed-rank test for
421 experiments that were not distributed normally. FDR correction for multiple testing was performed for
422 all analyses.

423 **References**


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

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

427 3. Baker, M.R., Hofmann, H.A., and Wong, R.Y. (2017). Neurogenomics of Behavioural Plasticity in
428 Socioecological Contexts. In eLS, John Wiley & Sons, Ltd, ed. (Wiley), pp. 1–11.

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

431 5. Taborsky, B., and Oliveira, R.F. (2012). Social competence: an evolutionary approach. Trends Ecol. Evol. *27*,
432 679–688.

433 6. Solomon-Lane, T.K., and Hofmann, H.A. (2019). Early-life social environment alters juvenile behavior and
434 neuroendocrine function in a highly social cichlid fish. Horm. Behav. *115*, 104552.

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

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

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

440 10. Wallace, K.J., Choudhary, K.D., Kutty, L.A., Le, D.H., Lee, M.T., Wu, K., and Hofmann, H.A. (2022). Social
441 ascent changes cognition, behaviour and physiology in a highly social cichlid fish. Philos. Trans. R. Soc. B
442 Biol. Sci. *377*, 20200448.

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

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

446 13. Dickson, B.J. (2008). Wired for Sex: The Neurobiology of Drosophila Mating Decisions. Science *322*, 904–
447 909.

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

450 15. Cheriyamkunnel, S.J., Rose, S., Jacob, P.F., Blackburn, L.A., Glasgow, S., Moorse, J., Winstanley, M.,
451 Moynihan, P.J., Waddell, S., and Rezaval, C. (2021). A neuronal mechanism controlling the choice between
452 feeding and sexual behaviors in Drosophila. Curr. Biol. *31*, 4231-4245.e4.

453 16. von Philipsborn, A.C. (2020). Neuroscience: The Female Art of Saying No. Curr. Biol. *30*, R1080–R1083.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

40. Zer, S., Ryvkin, J., Wilner, H.J., Zak, H., Shmueli, A., and Shohat-Ophir, G. (2016). A Simple Way to Measure Alterations in Reward-seeking Behavior Using Drosophila melanogaster. JoVE J. Vis. Exp., e54910.

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

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

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

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

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

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

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

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

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

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

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

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

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

540 54. Gowaty, P.A., Anderson, W.W., Bluhm, C.K., Drickamer, L.C., Kim, Y.-K., and Moore, A.J. (2007). The
541 hypothesis of reproductive compensation and its assumptions about mate preferences and offspring viability.
542 Proc. Natl. Acad. Sci. U. S. A. *104*, 15023–15027.

543 55. Gowaty, P.A., Kim, Y.-K., Rawlings, J., and Anderson, W.W. (2010). Polyandry increases offspring viability
544 and mother productivity but does not decrease mother survival in Drosophila pseudoobscura. Proc. Natl. Acad.
545 Sci. U. S. A. *107*, 13771–13776.

546 56. Anderson, W.W., Kim, Y.-K., and Gowaty, P.A. (2007). Experimental constraints on mate preferences in
547 Drosophila pseudoobscura decrease offspring viability and fitness of mated pairs. Proc. Natl. Acad. Sci. *104*,
548 4484–4488.

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

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

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

554 60. Cameron, E., Day, T., and Rowe, L. (2007). Sperm Competition and the Evolution of Ejaculate Composition.
555 Am. Nat. *169*, E158–E172.

556 61. Sirot, L.K. (2019). Modulation of seminal fluid molecules by males and females. Curr. Opin. Insect Sci. *35*,
557 109–116.

558 62. Smith, D.T., Sirot, L.K., Wolfner, M.F., Hosken, D.J., and Wedell, N. (2012). The consequences of genetic
559 variation in sex peptide expression levels for egg laying and retention in females. Heredity *109*, 222–225.

560 63. De Nardo, A.N., Roy, J., Sbilordo, S.H., and Lüpold, S. (2021). Condition-dependent interaction between
561 mating success and competitive fertilization success in Drosophila melanogaster. Evol. Int. J. Org. Evol. *75*,
562 2014–2026.

563 64. Kerwin, P., Yuan, J., and von Philipsborn, A.C. (2020). Female copulation song is modulated by seminal fluid.
564 Nat. Commun. *11*, 1430.

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

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

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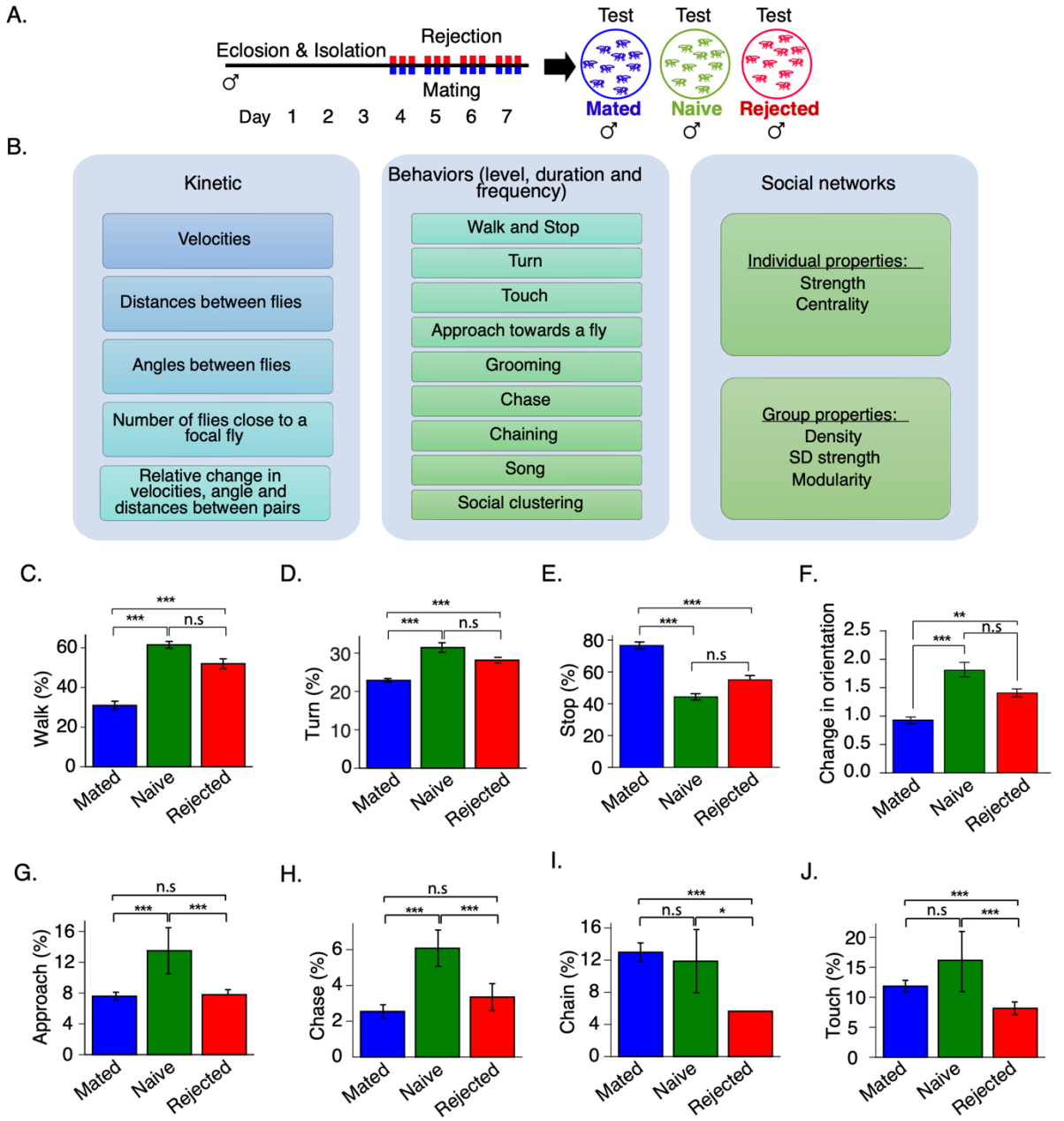



**Figure 1. Failure to mate modifies action selection upon encounters with rival male flies. A.** Schematic representation of sexual experience and behavioral analysis. Rejected males experienced 1h long interaction with non-receptive female flies, 3 times a day for 4 days. Mated males experienced 1h long interactions with a receptive virgin female, 3 times a day for 4 days. Male flies with no sexual experience are kept in social isolation (naïve). The social group interaction of the 3 cohorts is recorded for 30 min using the FlyBowl system. **B.** List of kinetic features, 8 behaviors and network parameters produced using the FlyBowl system. **C-J** Average percentage of time mated (blue) naive (green) and rejected (red) took to perform walk (C), turn (D), stop (E), change orientation (F), approach (G), Chase (H), chain (I) and touch (J) behaviors. n = 15, 10, 15 for mated, naïve and rejected respectively. one-way ANOVA followed by Tukey's and FDR correction for multiple tests *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Error bars signify SEM.

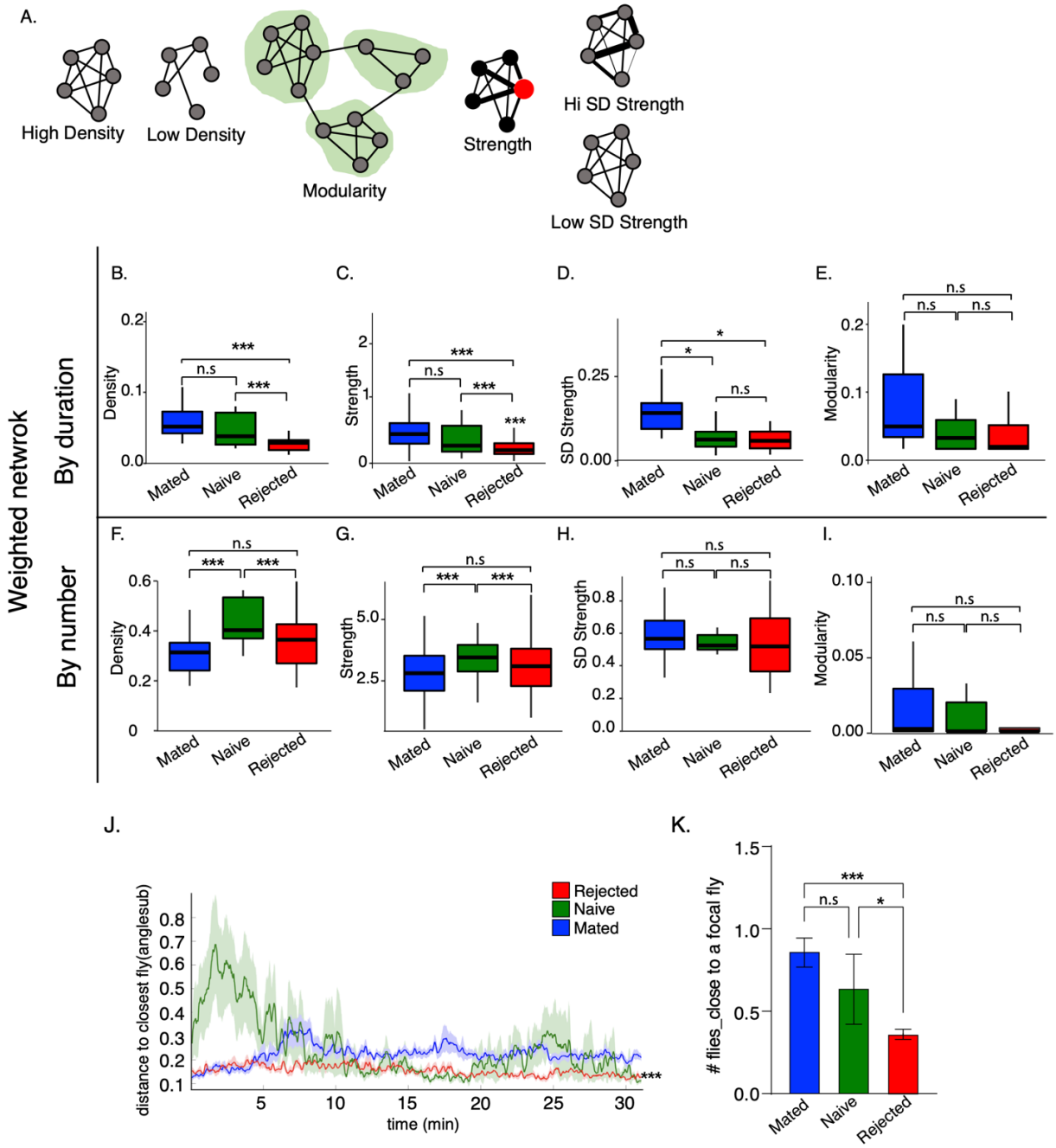



**Figure 2. Sexual rejection promotes social avoidance. A.** Illustration of network parameters. Density of networks represents how saturated they are compared to the maximum possible. Modularity is a measure of the division of a network into sub-networks. Strength is proportional to vertex size (high in red individual). Standard deviation (SD) strength is a measure of the heterogeneity of the connections between individuals. **B-I.** Social network analysis of groups composed of mated (blue) naive (green) and rejected (red). Network density, modularity, and SD strength calculated by network weights according to duration (B-E) or number of interactions (F-I). Kruskal–Wallis test followed by Wilcoxon signed-rank test and FDR correction for multiple tests *p < 0.05, **p < 0.01, ***p < 0.001, Box plot with median IQR. **J.** Rejected male flies maintain large distances between flies along (anglesub), statistical significance was tested on averages across time. one-way ANOVA followed by Tukey's and FDR correction for multiple tests *p < 0.05, **p < 0.01, ***p < 0.001. Error bars signify SEM. . **K.** Average number of flies close to a focal fly. n = 15, 10, 15 for mated, naïve and rejected respectively. one-way ANOVA followed by Tukey's and FDR correction for multiple tests *p < 0.05, **p < 0.01, ***p < 0.001. Error bars signify SEM.

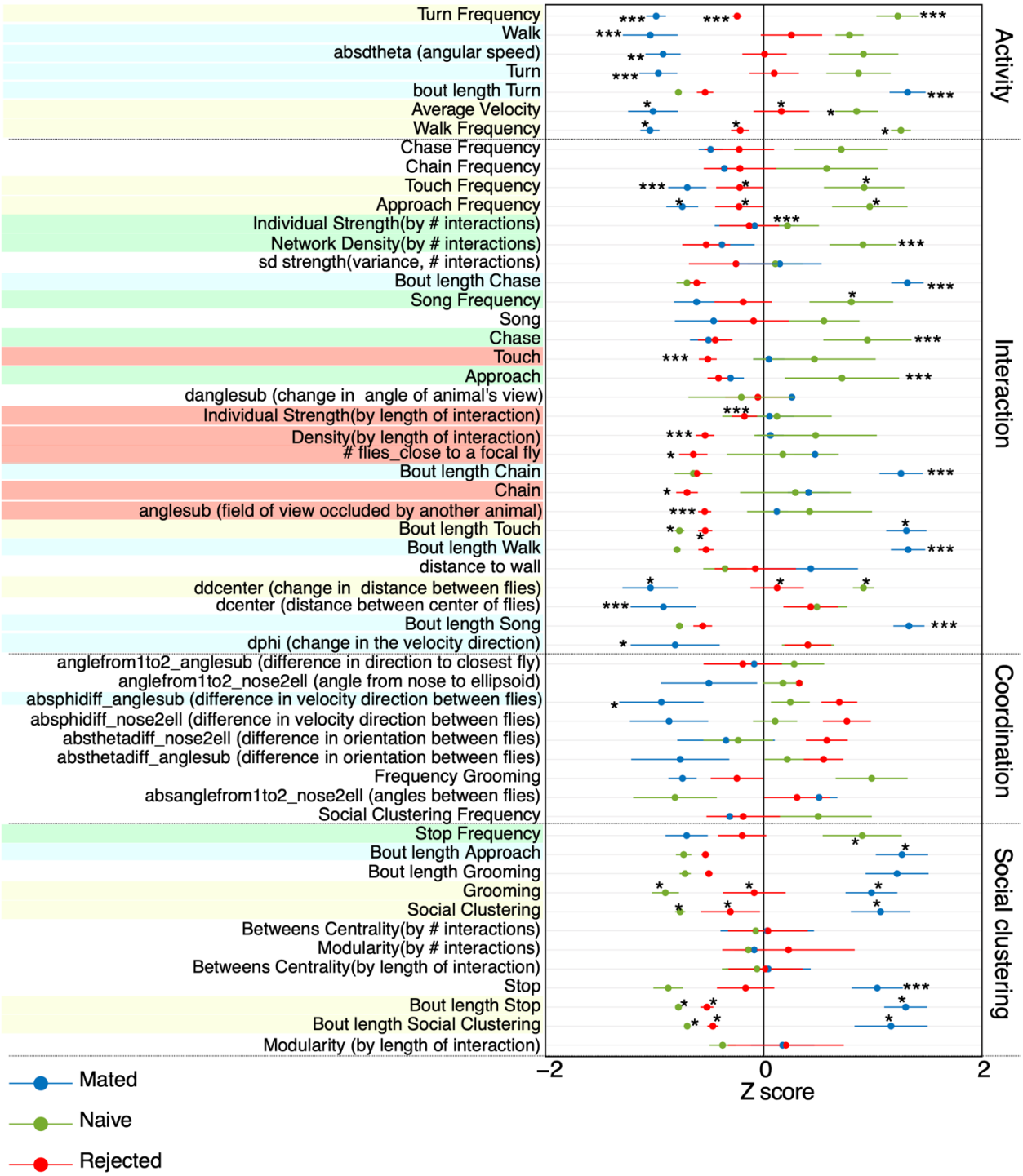



**Figure 3.** Behavioral signatures of mated, rejected and naïve WT male flies. Data is represented as normalized Z scores of 60 behavioral parameters, n = 15, 10, 15 for mated, naïve and rejected respectively. statistical significance determined by one-way ANOVA followed by Tukey's range test for experiments that were distributed normally, and by Kruskal–Wallis test followed by Wilcoxon signed-rank test for experiments that were not distributed normally. FDR correction was used for multiple tests. LOI: calculated according to the length of interactions. NOI: calculated according to the number of interactions. Features marked in yellow exhibit statistically significant differences among 3 cohorts. Features colored in blue are different between mated and the two other conditions. Features colored in red are different between rejected and the two other conditions. Features colored in green are different between naive and the two other conditions.

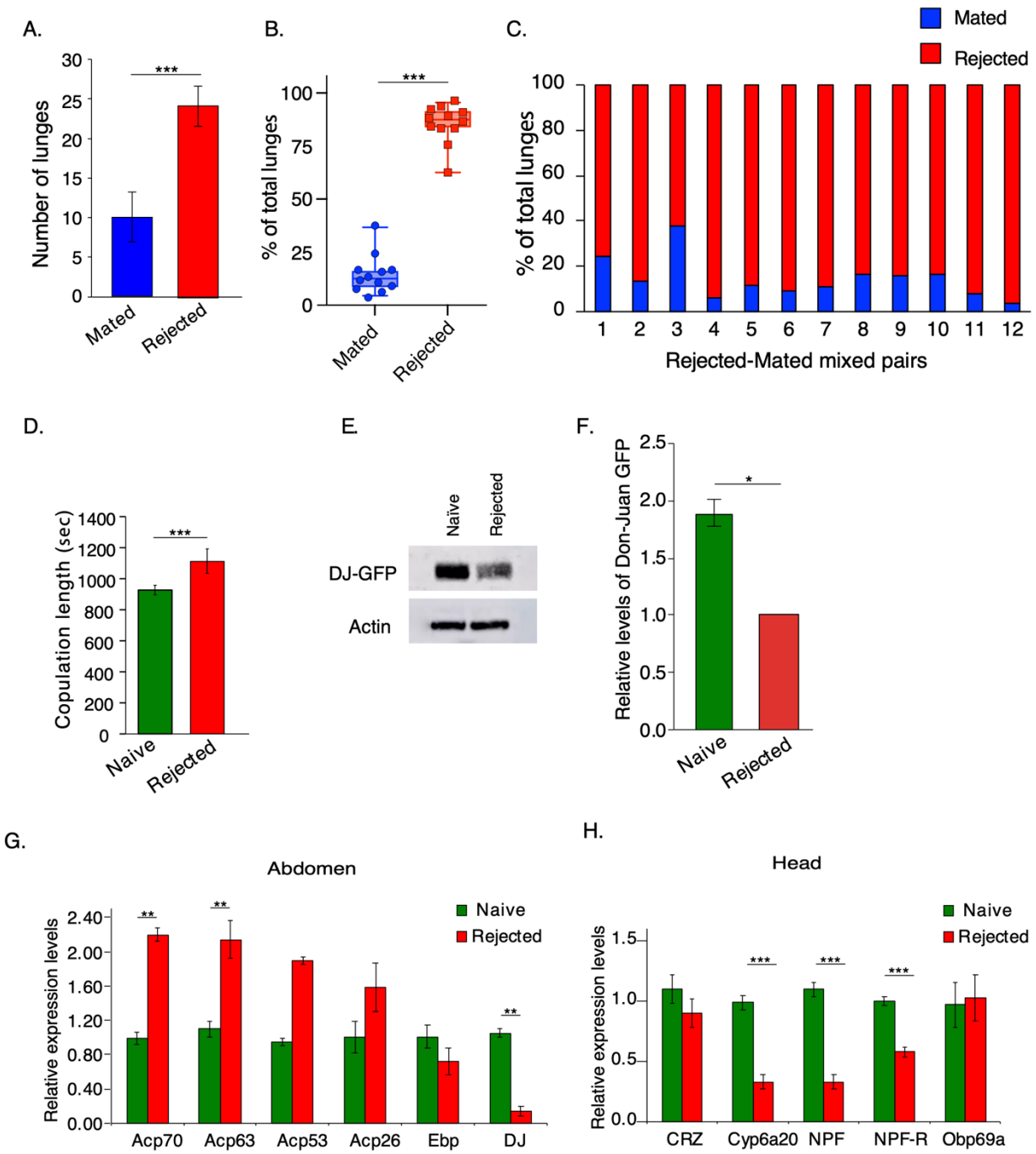



**Figure 4. Sexual rejection modulates competitive behaviors and ejaculate composition. A.-C.** Aggression display (number of lunges) was compared between pairs of rejected and mated male flies (n=16, statistical significance determined by T-test, $p < 0.005$ **(A),** and mixed pairs (n=12) **(B-C).** The $\log_2$ 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. **E-F.** Protein lysates prepared from abdomen of rejected and naïve male flies and were analyzed for the relative levels of Don-Juan-GFP using western blot, actin was used as a loading control. Expression levels of Don-Juan-GFP protein were quantified and normalized to actin levels (n=3), Statistical significance was determined by T-test, $p<0.05$ (F). **G-H.** Relative transcript levels of candidate genes expressed in abdomen (G) and heads (H) of rejected and naïve male flies were quantified by qRT-PCR, n = 6 independent experiments of 15–20 fly heads and abdomen. Statistical significance was determined by Student's T-test with Bonferroni correction for multiple comparisons. **, $p < 0.01$ ***, $p < 0.005$.

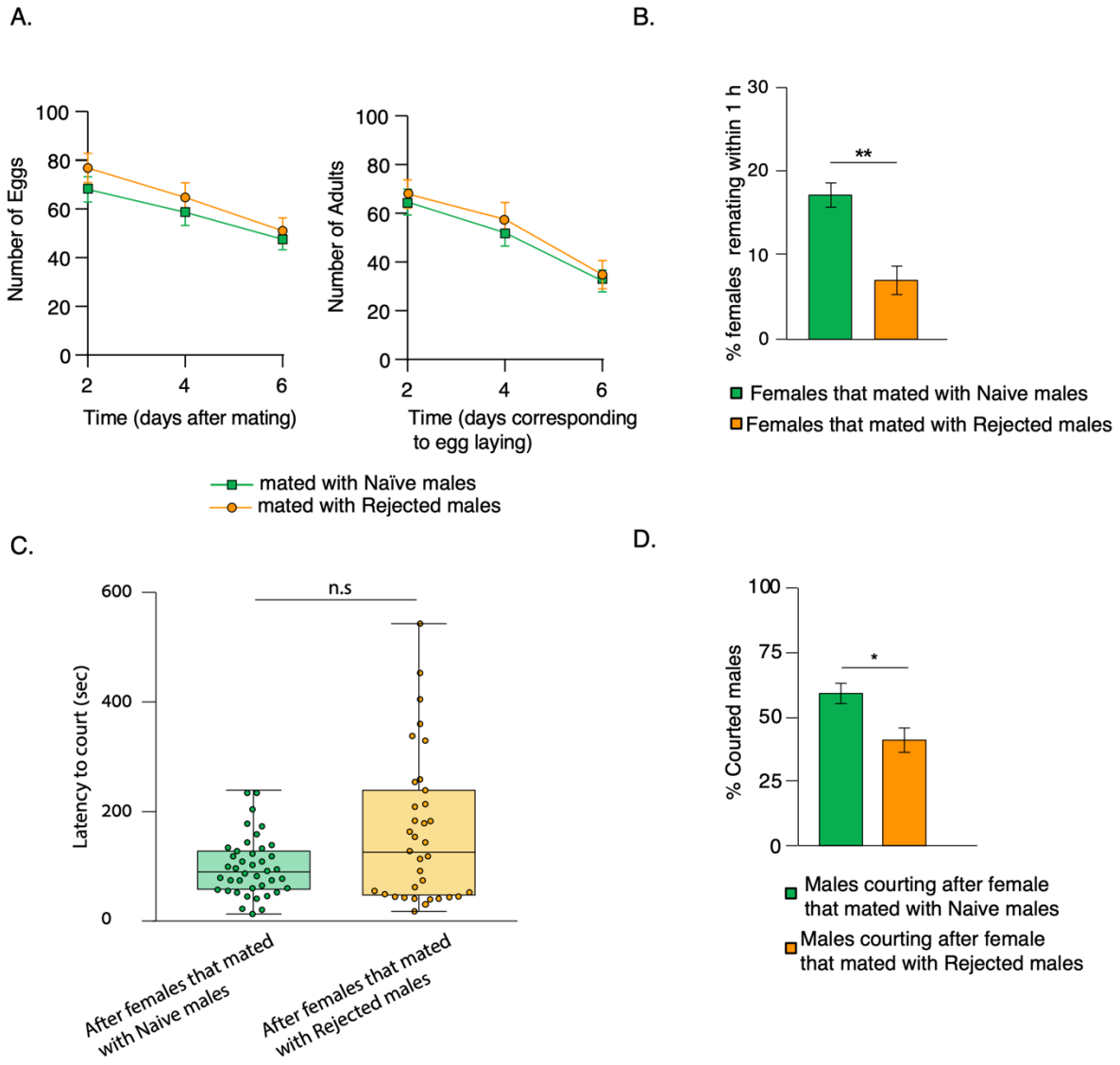
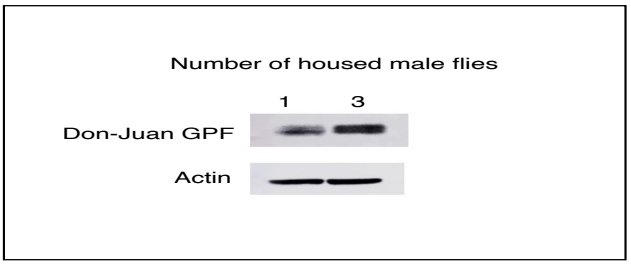



**Figure 5. Effect of male rejection on female's fertility and remating tendencies**. **A.** Number of eggs laid by females that copulated with rejected or naïve male flies over the course of 6 days (left panel) and emerging progeny (right panel). Statistical significance was determined using two-way ANOVA repeated measure, n=30 naive, n=25 rejected $p>0.05$. **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, $p< 0.05$.




**Supplementary Figure 1. 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.