Sexual deprivation modulates social interaction and reproductive physiology

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Abstract

In highly polyandrous species, where 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 paternity rates. 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 affect male-male social interactions in a competitive environment such as the social group. Here we show that repeated failures to mate promote a coordinated physiological and behavioral responses that can serve to increase paternity chances over mating rivals. We exposed male flies to sexual deprivation or successful mating and analyzed the behavioral repertoires of individuals within groups and the structure of their emerging social networks. We discovered that failures to mate and successful mating generate distinct emergent group interactions and structures, where sexually deprived males form low density social networks and actively minimize their encounters with other group members, while increasing their aggressive behavior. In addition, sexually deprived male flies elevate the production of seminal fluid proteins (known to facilitate post-mating responses in females) and extend mating duration upon mating with receptive females, altogether leading to reduced re-mating rates. Our results demonstrate the existence of a flexible mating strategy that may provide a short-term fitness advantage over competing rivals and pave the path for using simple model organisms to dissect the neurobiology of social plasticity as coping strategy to living in a highly dynamic 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[1,2]. 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 physiology in response to changes in their social environment. This includes the ability of male flies to change their aggressive behavior in response to prior fighting experience[21–23], regulate sperm composition and the duration of copulation events in response to perceived competition[24–26], and suppress courtship efforts towards non-receptive female flies[27–29].

Recent studies in *Drosophila* demonstrate that fruit flies generate complex and rich group structures that are sensitive to the density of the group, its composition, as well as to the prior experience of its members[30–33]. We previously showed that sexual experience in male flies can modulate their motivational state and, subsequently, their reward seeking behaviors[34,35]. However less is known about the way by which prior sexual interactions that are experienced as success or failure to mate shape social interaction of male flies in a group context. Furthermore, it is not clear whether sexually deprived male flies exhibit loser-like responses, as in the case of social defeat[23], or rather actively increase their competitive behavior to cope with mating rivals. Here we explored the effects of success or failure to mate on the dynamics of social interaction in groups of male flies. We discovered that sexual deprivation and successful mating generate opposite emergent group interactions and structures, wherein sexually deprived male flies actively minimize their interactions with group members. Moreover, sexual deprivation enhances competitive behaviors and leads to changes in reproductive physiology, possibly to increase paternity chances over mating rivals.

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 sexual deprivation in the form of repeated rejection events by non-receptive female flies, alter internal state and consequently motivational responses[34,35]. The negative valence of rejection, reflected by its capacity to induce courtship suppression and increase the consumption of ethanol, prompted us to ask whether sexually deprived male flies exhibit loser-like responses[23] or rather actively increase their competitive behavior to cope with mating rivals. To this end, we generated two cohorts of male flies that were exposed to repeated encounters with either receptive virgin female flies (mated-isolated) or non-receptive female flies (rejected-isolated), consisting of 1h sessions 3 times a day for 4 days (Fig. 1A). At the end of this experience, their interactions in group context were tested by introducing 10 flies from each cohort into a shallow arena in which 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[32,36,37] (Fig. 1A). The tracking data obtained was used to generate a comprehensive behavioral representation for each cohort composed of 60 distinct features, including kinetic features, eight distinct complex behaviors, and six social network features (Table 1)[32]. The overall differences between the two cohorts across all features are depicted in a scatter plot of normalized differences and are divided into 4 main categories: activity-related features, interaction-related features, coordination between individuals, and features associated with social clustering (Fig. 1B). The two cohorts of male flies exhibited distinct repertoires of behavioral responses upon first encounters with other male flies. Sexually deprived male flies exhibited increased activity manifested as longer overall time spent walking, increased average velocity, and higher number of body-turns (Fig. 1B, highlighted in pink, Supp Fig. 1A-C). When analyzing social-related behaviors, rejected male flies exhibited lower rates of close touch encounters (Fig. 1B, highlighted in blue, Supp Figure 1D), and while they displayed similar levels of active approaches towards other members of the group, the duration of these encounters was significantly shorter (Fig. 1B, highlighted in blue, Supp. Fig. 1E,F). In contrast, mated males exhibited long periods of quiescence (Fig. 2B, highlighted in blue, Supp. Fig. 1B), and formed close-distance social (Fig. 1B, highlighted in blue, Supp Fig. 1G), reflected also by an increase in the number of flies found in close proximity to one another (Fig. 1C).

Failure to mate promotes social avoidance

We next analyzed the properties of emerging social networks in both groups using weighted networks as described by Bentzur el al.,[32] (Fig. 2A). We calculated network weights according to the overall duration of interactions (emphasizing long-lasting interactions) or the overall number of interactions (emphasizing short interactions) between each pair of flies. Analysis by duration revealed that social networks of rejected males are characterized by lower density (Fig. 2B), reduced modularity (Fig. 2C), and reduced variation in individual strength levels across the group (SD strength, Fig. 2D). These findings suggest that rejection promotes the formation of sparser groups containing fewer subgroups and that individuals in those groups are more homogenous in the strength of their interactions. Analysis by number of interactions revealed that, although rejected networks have lower modularity and SD strength, there is no significant differences in the density of their networks, suggesting that they maintain an overall similar number of interactions as mated male flies (Fig. 2E-G). Together, these differences indicate that mated male flies form networks with higher-order structures compared to those formed by rejected male flies. Notably, although rejected male flies participate in a similar number of interactions, their networks are simpler and sparser. The apparent differences in the density of networks measured by duration are consistent with significant differences between the two cohorts in the average distance between the two closest flies in each frame (dcenter), which is considerably higher in rejected males (Fig. 2H). More importantly, while in mated males the average distance between flies decreased along the experiment as flies adapt to the arena, it remained constantly high in groups of rejected male flies (Fig. 2H). Considering that the elevated activity of rejected male flies (Fig. 1B) is expected to increase the opportunity to encounter others, the maintenance of a larger distance throughout the experiment and the reduced density suggest that rejected individuals actively avoid social interactions with other flies. Together, these experiments point to sexual deprivation as the major contributor to the reduced social interaction. To further test the strength of this conclusion, we divided a cohort of rejected-isolated males into two subgroups, one of which was left undisturbed, and the other subgroup was allowed to mate with virgin females for 2.5 hours immediately before testing. The rejected, then mated sub-group exhibited intermediate levels of activity related features such as walk, stop, turn and average velocity when compared to subgroups that had only experienced rejection or successful mating (Fig. 2I). The rejected and then mated subgroup exhibited also intermediate degrees of social interaction related features such as social clustering, number of flies found in close proximity to one another, and the levels of grooming behavior that is tightly associated with social clustering (Fig. 2I). The capacity of mating to partially reverse the effects of sexual deprivation is consistent with sexual deprivation being the major contributor to social avoidance.

Sexual deprivation modulates competitive behaviors

Considering the major differences in group behavior displayed by rejected and mated male flies, we hypothesized that the responses exhibited by rejected males reflect behavioral adaptation to coping with high sexual competition over mating partners, where repeated encounters with mated females are indicative of high male to female sex ratio. If so, rejected male flies are expected to increase behaviors that provide them with an adaptive competitive value over rival male flies. This prediction can be tested by measuring their aggressive responses toward other males in the presence of limited food resources or their mating behavior upon opportunities to mate with virgin female flies. Indeed, pairs of rejected male flies exhibited significantly higher aggressive displays in comparison to pairs of mated male flies (Fig. 3A), and that in mixed pairs, rejected males exhibited greater numbers of lunges compared to their mated counterparts (Fig. 3B,C). When allowed to mate with virgin female flies, rejected male flies extended the duration of copulation events by 25% (3.5 minutes longer) compared to naïve males (Fig. 3D). Thus, rejected male flies exhibited an overall increase in behaviors that can provide them with an adaptive competitive value over rival male flies.

Failure to mate induce changes in sperm and seminal fluid composition

The act of mating alone does not guarantee fitness benefits including known strategies that reflect male investment in sperm and non-sperm components, such as fecundity-enhancing seminal fluid proteins[38,39,40]. To determine whether prior rejection affects reproductive physiology in a manner that may improve mating competitiveness, expression levels of genes related to sperm production and reproduction were assessed. First, the expression of DON-JUAN (DJ), a protein that is specifically expressed in mature male sperm cells[32,33], was measured using a GFP-based reporter line in which a GFP sequence was inserted within the coding locus, so that the expression of GFP reflects the expression of the endogenous DJ protein. The reliability of the DJ-GFP reporter as a sensitive measure for changes in sperm production was first confirmed in male flies raised among a high number of rival males (5 flies for 4 days), compared to the flies that were housed in pairs (Supp Fig.2), social conditions known to affect the amount of mature sperm[25,41] (Supp Fig.2). The relative levels of GFP were then measured in rejected and naïve male flies (Fig. 4A-B). Surprisingly, there was a twofold decrease in the levels of GFP in the rejected cohort compared to naïve males (with no prior sexual experience), suggesting that male flies decrease their investment in sperm allocation in response to sexual deprivation (Fig. 4A-B). Next, the relative expression of the following reproductive related genes was directly assessed in fly abdomens by qRT-PCR. We measured the expression of *Sex-Peptide (Acp70A), Acp63, Acp53, Ovulin (Acp26Aa)*, which are responsible for the females' long-term post-mating responses and fertility[38]. We also measured the expression of genes encoding the *Ejaculatory bulb protein (Ebp),* which is responsible for the posterior mating plug formation at the end of mating[42], *don-juan (dj)*[40], the *corazonin (Crz)* neuropeptide, which promotes sperm and seminal fluid ejaculation in males and its receptor *Crz-receptor*[35,43], and finally *Esterase 6 (est-6),* an enzyme that is transferred to females during copulation and presumably functions to degrade the pheromone cVA[44] (Fig. 4C). There was a two-fold increase in the levels of *Acp-70A (Sex-Peptide)* and *Acp-63* in rejected male flies when compared to naïve males, suggesting that rejected male flies increase their investment in the production of seminal fluid proteins that are transferred to females flies during copulation (Fig. 4C). Nevertheless, in agreement with the observed reduction in DJ-GFP reporter levels, there was a drastic decrease in the transcript levels of *don-Juan* in rejected males. The transcript levels of *Ebp, Est-6, Crz* and its receptor were similar in both cohorts (Fig. 4C). Overall, these results suggest that rejected male flies respond to sexual deprivation by elevating seminal fluid protein transcript levels, presumably to maximize their fitness. In addition to proteins associated with the male reproductive system, levels of several genes expressed in the brain and antenna were also assessed. These included the neuropeptides *Crz*, *Neuropeptide F* (*npf*) and its receptor (*npfr*), and two olfactory related genes associated with aggression (the *Odorant binding protein 69a*[45], and *Cyp6a20*[46]*)*. In agreement with previous studies, the levels of *npf* were significantly lower in sexually deprived male flies[34]; we also observed a reduction in *npfr* (Fig. 4D). Interestingly, sexually deprived male flies also exhibited reduced levels of *Cyp6a20* in comparison to naïve male flies (Fig. 4D), consistent with their enhanced aggression (Fig. 3 A-C).

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

The molecular changes associated with the rejected condition support our initial hypothesis that rejected male flies adjust their behavior and physiology to cope with high sexual competition. If this is correct, the changes in seminal fluid composition and the extended copulation are expected to provide rejected male flies with an advantage over rival male flies. To test this prediction, several aspects associated with female fecundity were measured. First, the fertility of female flies was assessed by counting the number of eggs they laid after one mating event with either rejected or naïve male flies. There was no significant difference in the number of eggs laid across five days between the two cohorts (Fig. 5A). The lack of difference in the amount of progeny suggested that lower sperm investment in rejected males (as reflected by reduced DJ levels) does not affect the total offspring number, meaning that there is no link between sperm investment and the number of offspring.

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

During copulation, male flies transfer to female flies seminal fluid proteins and also anti-aphrodisiac pheromones such as cVA[28]. The extended copulation observed in rejected male flies may facilitate the transfer of larger amounts of cVA as a means to delay further courtship and copulation events by female flies. As an indirect measure for possible changes in the amount of transferred cVA, we analyzed the courtship behavior of male flies towards females that previously mated with either rejected or naive male flies 1h after the initial mating. No significant difference was observed in the latency to court, i.e. the time it takes male flies to exhibit their first courtship action (wing vibration) following introduction of the pair into the courtship arena (Fig. 5C). However, there was a significant reduction in the number of male flies that courted females previously mated with rejected males than those previously mated with naïve male flies (Fig. 5D), suggesting that mating with rejected male flies results in females that are less attractive courtship targets.

Discussion

In this study we used the FlyBowl[37] as an agnostic tool to explore responses modulated by sexual interaction and discovered that rejected male flies cope with their failures to mate by changing their behavior and physiology to enhance their reproductive success. This is presumably achieved by avoiding interaction with potential rival male flies and competing over mating partners via increased aggression and prolonged copulation; this is known as mate guarding. The latter is strengthened by the increased production of certain seminal fluid proteins that facilitate stronger post-mating responses in female flies.

The behavior of sexually deprived male flies was examined in this study under behavioral contexts that illuminate different aspects of their action selection. Using the FlyBowl system, we analyzed their emergent group interactions and social networks, and discovered that although rejected males are highly active, they exhibit sparse networks and maintain large distance with other members, as if they were actively minimizing or avoiding interaction with rival male flies. When tested in a social context that promotes fighting over limited resources, rejected male flies exhibited enhanced aggression.

The increased aggression displayed by the rejected cohort is associated with a significant decrease in the levels of *Cyp6a20*. This is consistent with a previous study showing that *Cyp6a20* levels are reduced in social conditions that promote aggression and that this reduction is responsible for the observed increase in aggression[46]. Interestingly, exposure to female flies prior to male-male interactions was previously shown to suppress aggression[47]. However, our findings suggest that not all types of interactions with female flies are sufficient for suppressing aggression, but rather that the quality of the interactions (i.e., the male's sexual success) determines the resulting aggression levels when encountering another male fly.

There are two possible explanations for the behavioral responses exhibited by rejected male flies. First, failure to mate could enhance aggression to improve the chances of successful mating and, upon eventual mating, the increased duration of copulation could increase the relative paternity share. Second, repeated rejection experiences could be perceived by male flies as an indication for high density of sexual competition over mating partners, where encountering mated females is suggestive of high male to female sex ratio. Consistent with the second hypothesis, several studies have described a link between pre-exposure to rival male flies and an extension of copulation events[24,48]. One study also demonstrated that male flies use multiple sensory cues such as auditory, olfactory and gustatory signals to estimate the level of mating competition[48]. Although rejected males were not exposed directly to other male flies during the training phase, the observed extension of their copulation events suggests that they can assess the level of competition by evaluating the quality of their sexual interaction with female flies. Studies performed in *Pieris rapae* butterflies, in which virgin males were shown to allocate their sperm investment by assessing not only the mating status of the female, but also her previous mating history[49], are consistent with this hypothesis.

The behavioral responses to sexual deprivation were accompanied by changes in the repertoire of genes expressed in the brain and reproductive system in the form of increased expression of several accessory gland protein genes (*Acps*). This, together with the increased copulation duration, supports the idea that the observed extension in mating duration serves to transfer a higher amount of Acps to intensify the females' post- mating responses[23,79]. Unlike previous studies that demonstrated a link between the presence of rival male flies and an increase in both copulation duration and sperm allocation (measured by increase number of sperm cells)[25], rejected male flies exhibited a significant reduction in the levels of DJ, a protein expressed in mature sperm cells. Although this finding is limited to only one protein, this is surprising in light of sperm competition theory, which predicts that males should strategically increase their investment in sperm allocation when in competition[50]. Furthermore, our findings are different from studies in crickets, sunfish, birds and rats, which showed that the perceived risk of sperm competition, in the form of the presence of rival males or their odors before and during mating, led to an increase in sperm investment[49,51,52]. The unexpected uncoupling between the investment in sperm and non-sperm components and the regulation of investment in copulation time, demonstrates that sexually deprived male flies regulate each of these processes independently.

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

In summary, our results demonstrate a plastic mating strategy by males that experienced repeated events of rejection that gives them a short-term advantage, promoting reproductive fitness when competing with rival male flies. We postulate that rejected males invest more energy in the production of seminal fluid proteins over sperm; these Acps are known to have important roles in modulating different aspects of female mating physiology and behavior. Furthermore, at low population density, the chances to meet a receptive female are low, therefore an investment in sperm ejaculate may be more costly[53]. Further research is needed to dissect the molecular and neuronal mechanisms that mediate these adaptive responses, identify the sensory modalities that perceive failure to mate, which encode this information within the nervous system leading to ejaculate plasticity.

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Materials and methods:

Fly lines and culture

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

Sexual experience paradigm

Male and female flies were anesthetized under CO2 and isolated immediately after eclosion. Flies were reared as single-housed in vials (23 mm by 94 mm) containing 7 ml of medium and were aged separately for 3–4 day. Rejected and mated cohorts were generated as previously described[34]. In the naïve cohort, male flies were isolated for 4 days.

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[36], and their behavior was recorded for 30 minutes and analyzed using CTRAX, FixTrax[32] and JAABA[36]. For kinetic features, scripts were written in MATLAB to use the JAABA code to generate the statistical features as specified in Kabra et al. [36]. Time series graphs (per frame) were created using JAABA Plot[36]. Quantification of complex behavios was done using JAABA Classifiers[36] to identify specific behaviors: Walk, Stop, Turn, Approach, Touch, Chase, Chain, Song, Social Clustering and Grooming. Bar graphs were created using JAABA Plot[36]. Network analysis was performed using an interaction matrix according to the interaction parameters described previously[32]. 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 "graph_from_adjacency_matrix" with parameters "mode = undirected" and "weighted = TRUE". Density was calculated on all movies with the formula:

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

Modularity was calculated using the "modularity" function on output from the "cluster_walktrap" function[54]. Strength was calculated using "strength" function and SD Strength was calculated on all movies using "sd" function on the strength value. Betweenness Centrality was calculated on all flies using the "betweenness" function and SD Betweenness Centrality was calculated on all movies using "sd" function on the Betweenness Centrality value. Box plots were created using R.

Each feature of the FlyBwol experiment was standardized according to all values calculated in our experiments for that feature to generate a z-score. Scatter plots were created using R.

Aggression

Pairs of rejected or mated male flies were introduced into aggression arenas (circular chambers, about 0.08 cm3 in volume), which contained a mixture of agarose and apple juice (1% agarose, 50% apple juice) that was placed in arenas to enhance aggressive behavior. Flies were filmed for 30 min with Point-Grey Flea3 (1080×720 pixels) at 60 fps. Aggressive behavior was later quantified by counting the number of lunges for each pair using CADABRA software (ref). 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 is significantly different from 0.

Copulation duration

Rejected and naïve male flies were put into courtship arenas (circular chambers, about 0.04 cm3 in volume) with virgin females and were allowed to mate for 1 hour. They were recorded for the whole experiment using a Point-Grey firefly camera. Courtship arenas consist of 25 flat arenas each arena containing only one pair of male-female flies. The copulation duration was measured from the moment the mating began until it ended. We calculated the time in seconds for each fly and the average for each group.

Egg laying assay

Egg production was determined for females that had been allowed to copulate with rejected or naive males for 1 hour at the end of the conditioning (as described above). Every female was put in a glass vial containing fresh food every day for 5 days in total and was kept in the incubator. Days 3 and 4 have received approximated values since day 3 was Saturday and we couldn't replace the vail that day; therefore, we tried to divide the number of eggs equally. Eggs can be spotted easily as circular white dots on the surface of the medium. The sum of the number of eggs in the vials of each female was used for analysis.

Receptivity assay

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

Latency to court assay

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

Courtship Index

Courtship index for a given male is the fraction of time a male fly spent in courtship activity in the 10 min observation period (600 sec). It is calculated by dividing the number of seconds the male courted over the total observation time and is been exhibit in percentage (CI = courtship behavior [sec] · 100 / total observation [sec]).

Molecular methods

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

Quantitative Real-Time PCR analysis

Frozen flies were placed on ice and decapitated using a scalpel. Total RNA was extracted from ~15 frozen heads and bodies (separately), using TRIZOL reagent according to the manufacturer's protocol. mRNA was reverse transcribed using BIORAD cDNA synthesis kit. cDNA was analyzed by quantitative real-time PCR (BIORAD CFX96) using specific primers for the head and for the body. Relative expression was quantified by ΔΔCT method using RPL32[55] as a loading control. We run each sample in triplicates. Each experiment was repeated four times using independent sets of experimental flies.

Statistical analysis

For each experiment, Shapiro–Wilk test was done on each experiment to test for normal distribution. Statistical significance was determined by t-test for experiments that were distributed normally, and by Wilcoxon test for experiments that were not distributed normally. For experiments with three or four conditions: 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.

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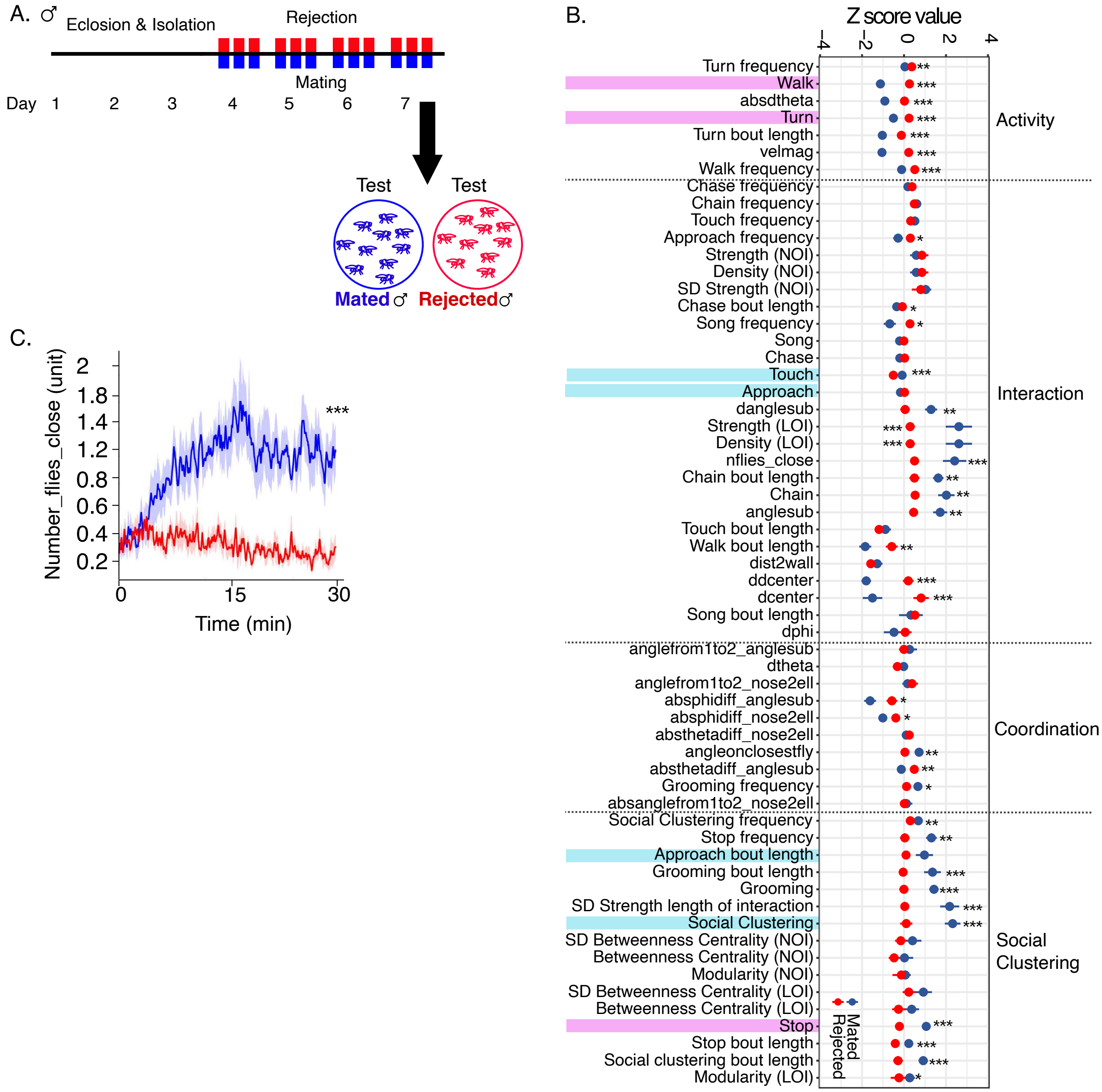


Figure 1. Failure to mate modifies action selection upon encounters with rival male flies. A. Schematic representation of the behavioral paradigm. **B.** Behavioral signatures of mated versus rejected WT male flies. Data is represented as normalized Z scores of 60 behavioral parameters, n = 18. Statistical 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.

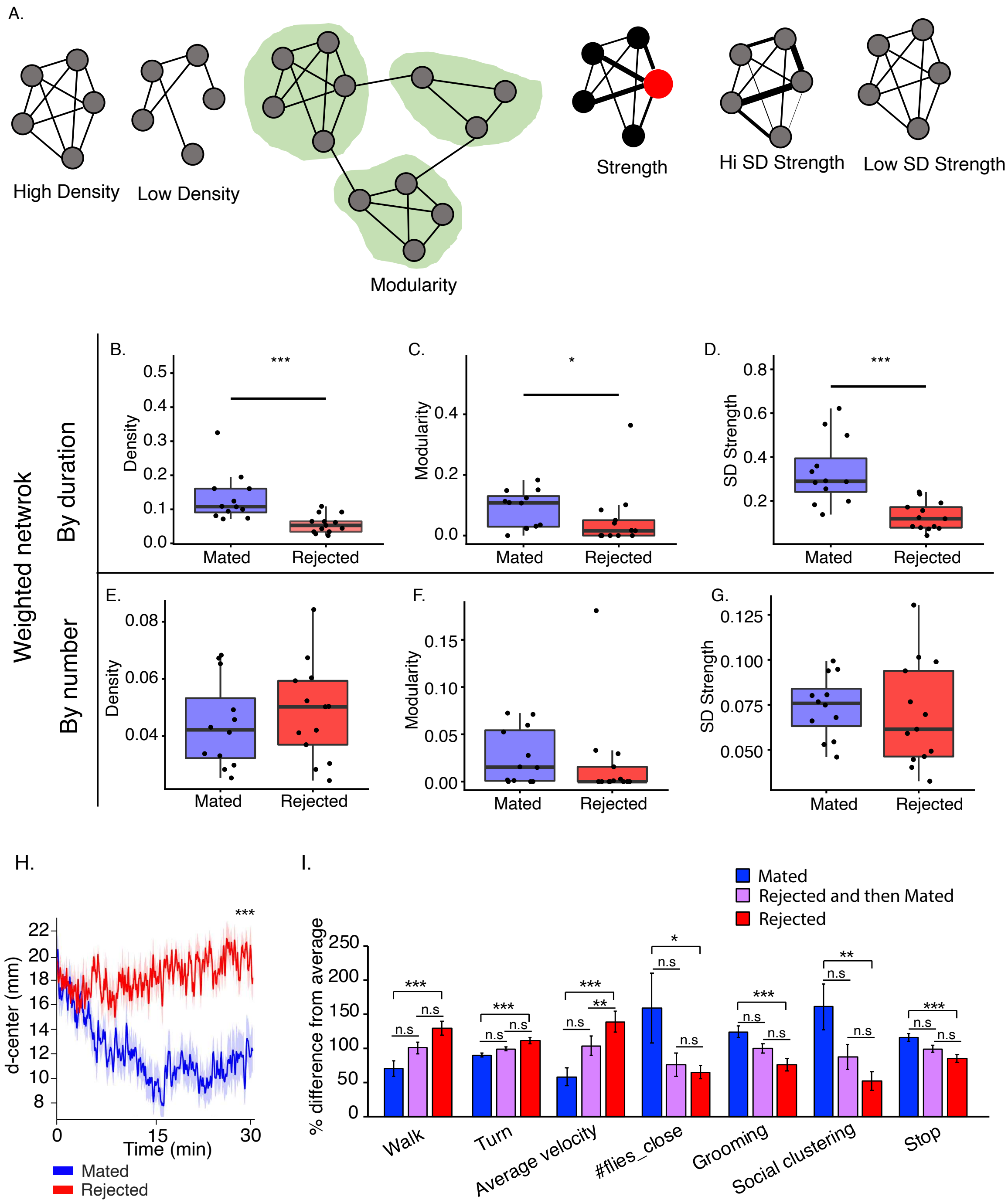


Figure 2. Sexual deprivation promotes social avoidance. A. Illustration of network parameters. Density of networks represents how saturated they are compared to the maximum possible. Modularity is a measure of the division of a network into sub-networks. Strength is proportional to vertex size (high 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.

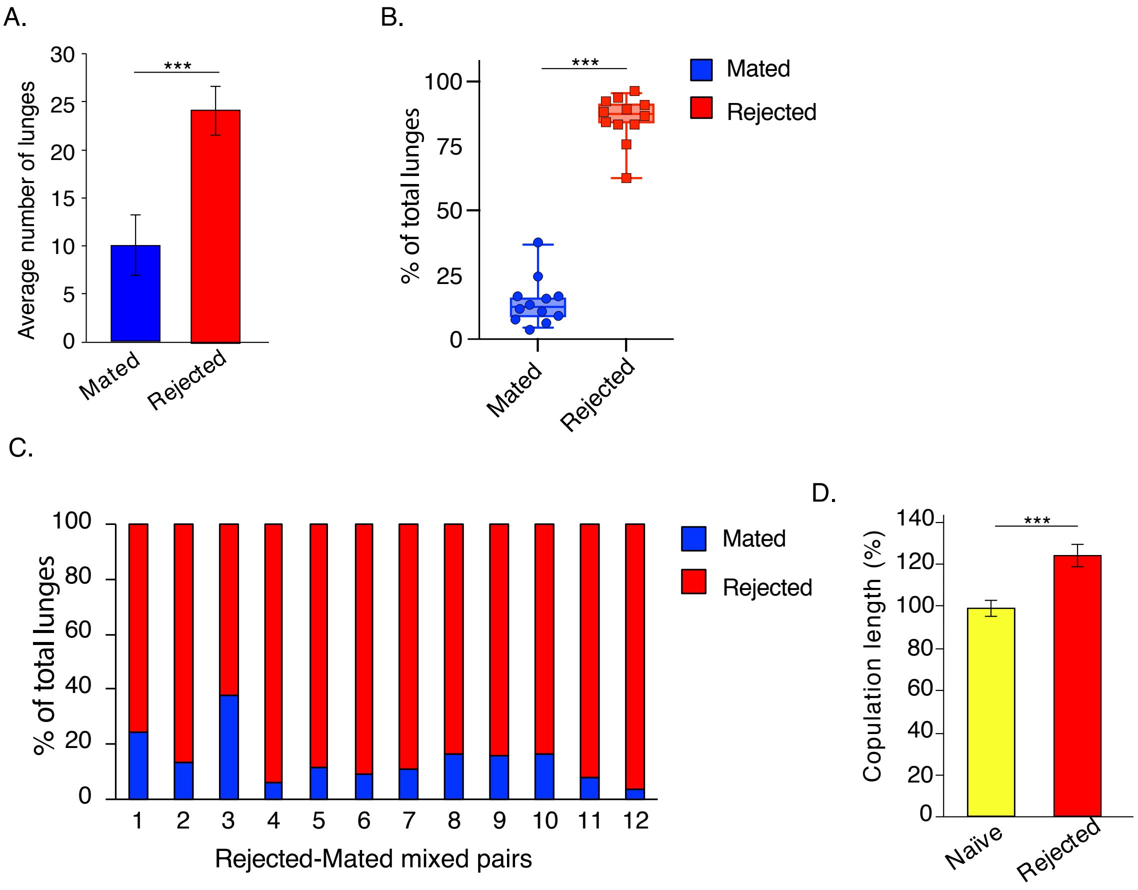


Figure 3. Sexual deprivation modulates competitive behaviors. A.-C. Aggression display (number of lunges) was compared between pairs of rejected and mated male flies (n=16, statistical significance determined by T-test, $p< 0.005$ **(A),** and mixed pairs (n=12) **(B-C).** The log2 ratio between the 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.

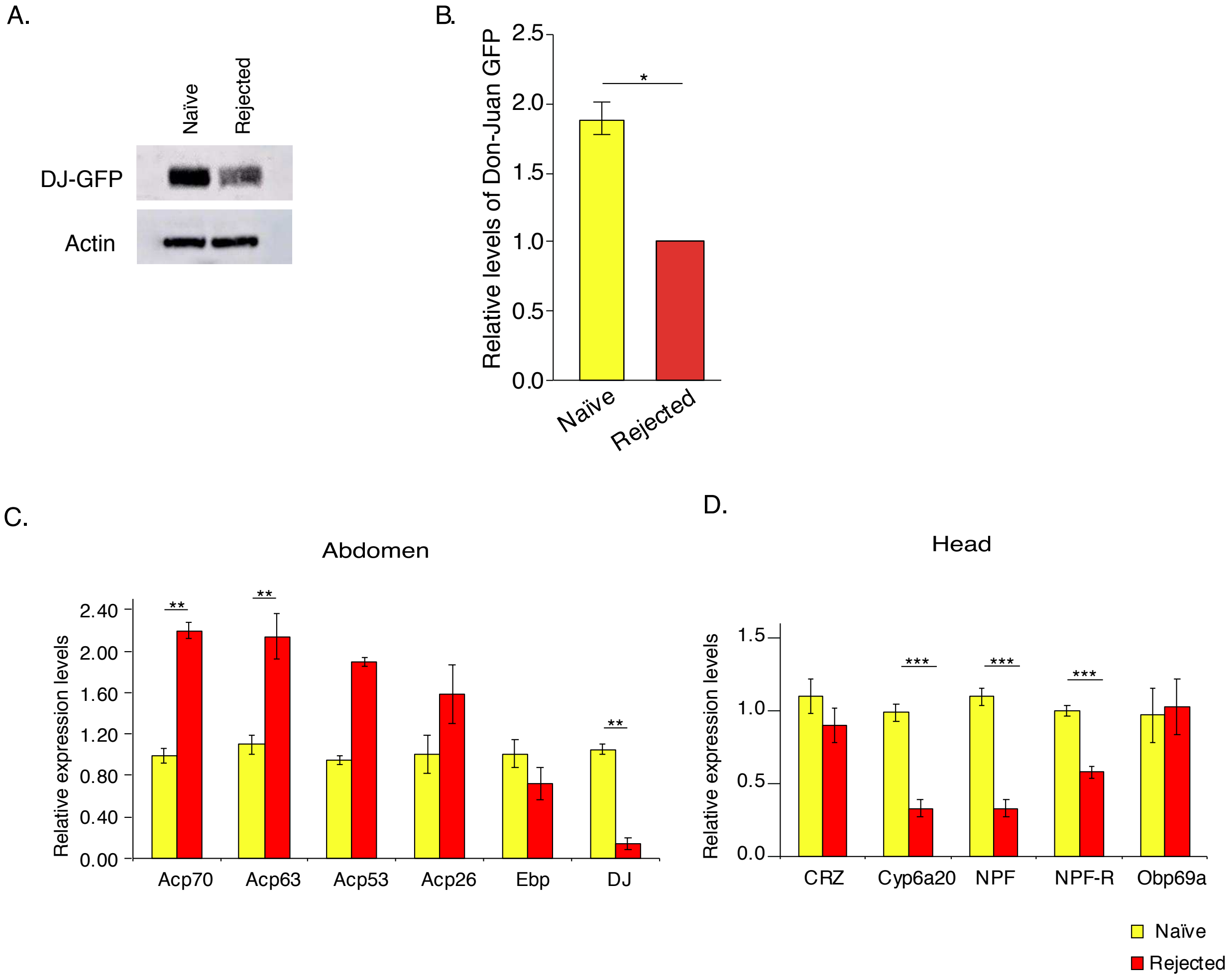


Figure 4. Failure to mate modulate sperm and seminal fluid composition. A,B. Protein lysates prepared from abdomen of rejected and naïve male flies and were analyzed for the relative levels of Don-Juan-GFP using western blot, actin was used as a loading control. Expression levels of Don-Juan-GFP protein were quantified and normalized to actin levels (n=3), Statistical significance was determined by T-test, p<0.05 (F). **C.D.** 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.

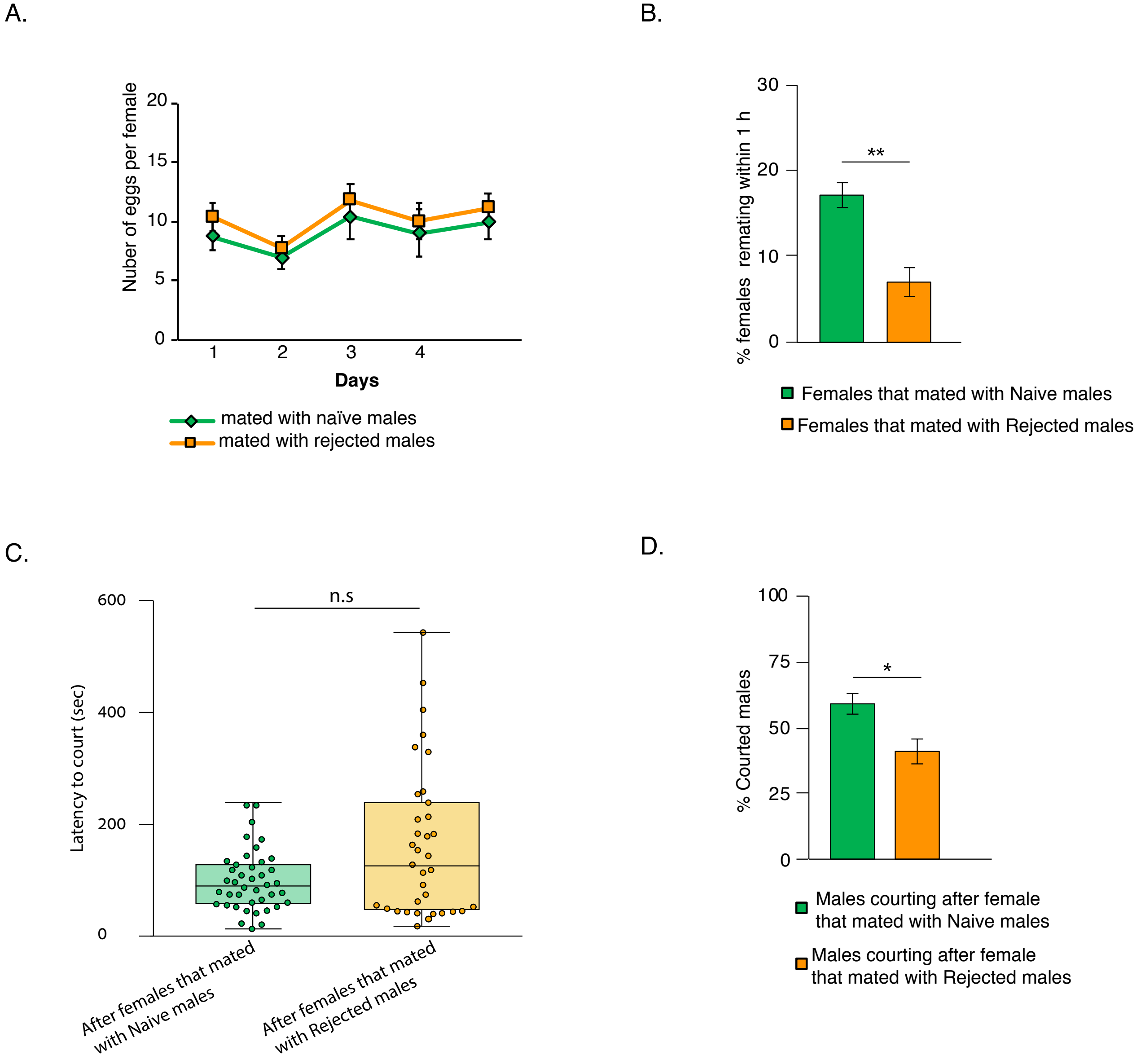
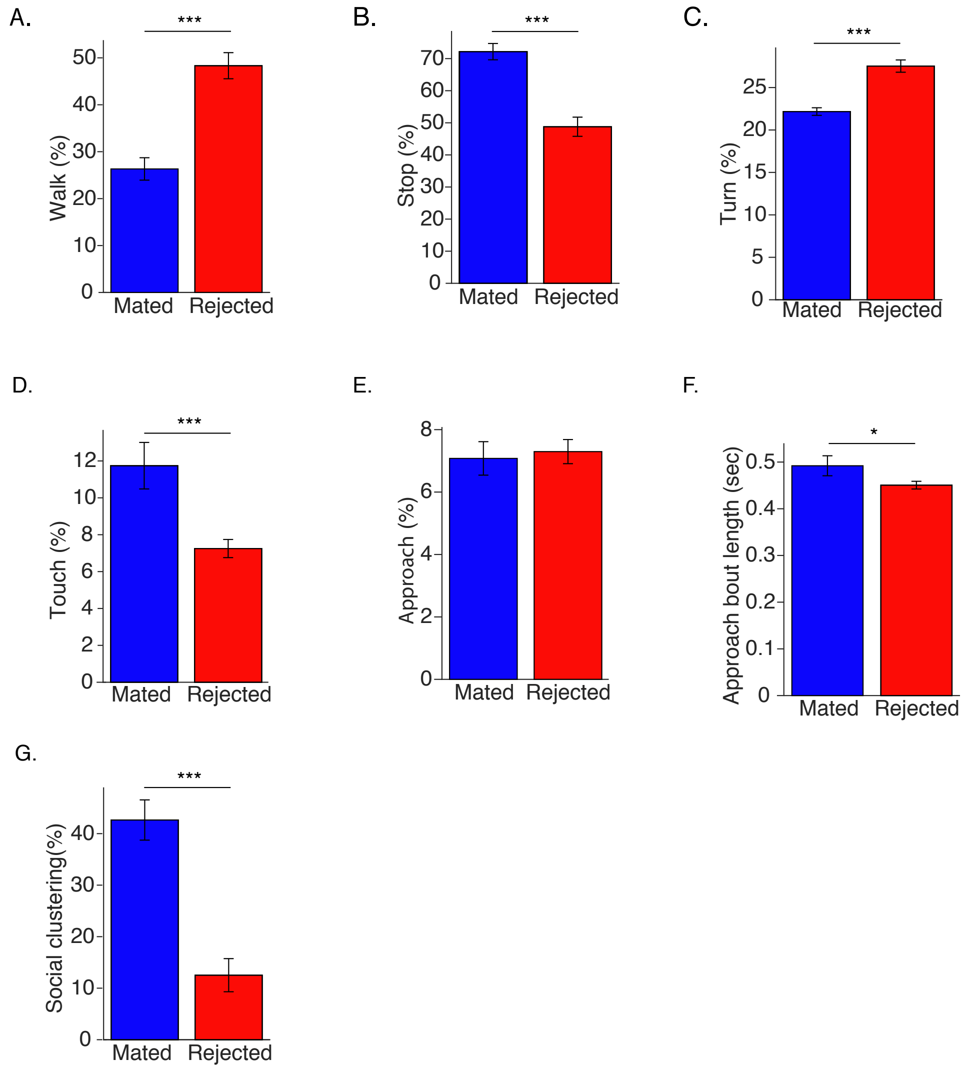


Figure 5. Effect of male rejection on female's fertility and remating tendencies. **A.** Number of eggs laid by females that copulated with rejected or naïve male flies over the course of 5 days. Statistical significance was determined using two-way ANOVA repeated measure, n=28 $p>0.05$. **B.** Female 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, $p< 0.05$.

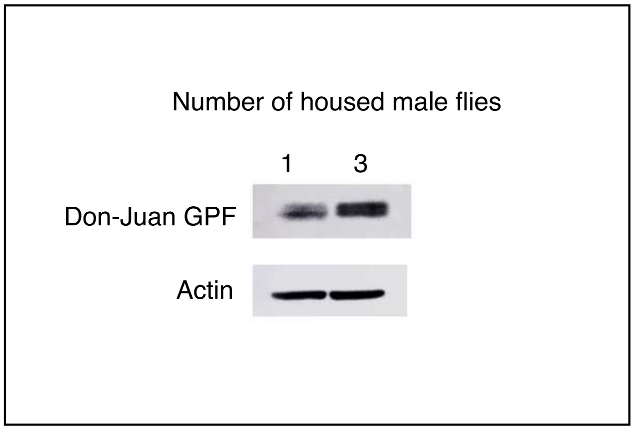
Definition	Description
dnose2ell	Minimum distance from any point of this animal nose to the ellipse of other flies.
absanglefrom1to2 nose2ell	Absolute difference between direction to closest animal based on dnose2ell and current animal's orientation (rad).
absdtheta	Angular speed (rad/s).
absphidiff anglesub	Absolute difference in velocity direction between current animal and closest animal based on anglesub (rad).
absphidiff nose2ell	Absolute difference in velocity direction between current animal and closest animal based on dnose2ell (rad).
absthetadiff anglesub	Absolute difference in orientation between current animal and closest animal based on anglesub (rad).
absthetadiff nose2ell	Absolute difference in orientation between this animal and closest animal based on dnose2ell (rad).
anglefrom1to2 anglesub	Angle to closest (based on angle subtended) animal's centroid in current animal's coordinate system (rad).
anglefrom1to2 nose2ell	Angle to closest (based on distance from nose to ellipse) animal's centroid in current animal's coordinate system (rad).
angleonclosestfly	Angle of the current animal's centroid in the closest (based on distance from nose to ellipse) animal's coordinate system (rad).
anglesub	Maximum total angle of animal's field of view (fov) occluded by another animal (rad).
danglesub	Change in maximum total angle of animal's view occluded by another animal (rad/s).
dcenter	Minimum distance from this animal's center to other animal's center (mm).
ddcenter	Change in minimum distance between this animal's center and other flies' centers (mm/s).
dist2wall	Distance to the arena wall from the animal's center (mm).
dphi	Change in the velocity direction (rad/s).
dtheta	Angular velocity (rad/s).
nflies_close	Number of flies within 2 body lengths (4a).
velmag	Speed of the center of rotation (mm/s).

Definition	Description
Walk	Fly moves.
Stop	Fly is still.
Turn	Changes in fly's direction.
Touch	Fly actively touches another fly.
Approach	Fly approaches another fly and perform interaction (active or passive).
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.
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.
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.
Density SD by length of interactions (LOI)	Accumulated interactions' length relative to the maximum interactions' length possible.
Modularity by length of interactions (LOI)	Representation of how much the network is divided into modules according to interactions' length.
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.
Betweenness Centrality by length of interactions (LOI)	A measure of centrality of a certain fly based on shortest paths according to interactions' length.
SD Betweenness Centrality by length of interactions (LOI)	Standard deviation of the betweenness centralities according to interactions' length of flies from the same movie.
Density by number of interactions (NOI)	Interactions' number relative to the maximum interactions' number possible.
Modularity Strength by number of interactions (NOI)	Representation of how much the network is divided into modules according to interactions' number.
Strength by number of interactions (NOI)	Number of interactions of a certain fly.
SD Strength by number of interactions (NOI)	Standard deviation of the strengths according to interactions' number of flies from the same movie.
Betweenness Centrality by number of interactions (NOI)	A measure of centrality of a certain fly based on shortest paths according to interactions' number.
SD Betweenness centrality (by number of interactions (NOI)	Variance of the betweenness centralities according to interactions' number of flies from the same movie.

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.



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.