Article

Current Biology

Early Life Experience Shapes Male Behavior and Social Networks in *Drosophila*

Highlights

- A new framework enables comprehensive representation of interaction in groups
- Social enrichment promotes distinct social networks and behavioral variability
- Group structure depends on visual and pheromonal cues
- Genetically heterogenic groups show emergent structure beyond the sum of its parts

Authors

Assa Bentzur, Shir Ben-Shaanan, Jennifer I.C. Benichou, Eliezer Costi, Mali Levi, Amiyaal Ilany, Galit Shohat-Ophir

Correspondence

amiyaal.ilany@biu.ac.il (A.I.), galit.ophir@biu.ac.il (G.S.-O.)

In Brief

Bentzur et al. present a new analytical framework for studying the interplay between social experience and the formation of social group interaction in *Drosophila*. They show that groups of *Drosophila* exhibit complex and dynamic social networks shaped by genetic and environmental factors and also by group composition.

Bentzur et al., 2021, Current Biology *31*, 486–501
February 8, 2021
https://doi.org/10.1016/j.cub.2020.10.060

CellPress

CellPress

Current Biology

Article

Early Life Experience Shapes Male Behavior and Social Networks in *Drosophila*

Assa Bentzur,[1,3] Shir Ben-Shaanan,[1,3] Jennifer I.C. Benichou,[1] Eliezer Costi,[1] Mali Levi,[1] Amiyaal Ilany,[1,*] and Galit Shohat-Ophir[1,2,4,*]

[1]The Mina & Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan 5290002, Israel
[2]The Leslie and Susan Gonda Multidisciplinary Brain Research Center, Bar-Ilan University, Ramat-Gan 5290002, Israel
[3]These authors contributed equally
[4]Lead Contact
*Correspondence: amiyaal.ilany@biu.ac.il (A.I.), galit.ophir@biu.ac.il (G.S.-O.)
https://doi.org/10.1016/j.cub.2020.10.060

SUMMARY

Living in a group creates a complex and dynamic environment in which behavior of individuals is influenced by and affects the behavior of others. Although social interaction and group living are fundamental adaptations exhibited by many organisms, little is known about how prior social experience, internal states, and group composition shape behavior in groups. Here, we present an analytical framework for studying the interplay between social experience and group interaction in *Drosophila melanogaster.* We simplified the complexity of interactions in a group using a series of experiments in which we controlled the social experience and motivational states of individuals to compare behavioral patterns and social networks of groups under different conditions. We show that social enrichment promotes the formation of distinct group structure that is characterized by high network modularity, high inter-individual and inter-group variance, high inter-individual coordination, and stable social clusters. Using environmental and genetic manipulations, we show that visual cues and cVA-sensing neurons are necessary for the expression of social interaction and network structure in groups. Finally, we explored the formation of group behavior and structure in heterogenous groups composed of flies with distinct internal states and documented emergent structures that are beyond the sum of the individuals that constitute it. Our results demonstrate that fruit flies exhibit complex and dynamic social structures that are modulated by the experience and composition of different individuals within the group. This paves the path for using simple model organisms to dissect the neurobiology of behavior in complex social environments.

INTRODUCTION

Many species have adapted to living in groups, from simple organisms, such as nematodes, to humans. Group living takes different forms with various levels of complexity, from almost random interactions to fully synchronized collective behavior,[1–5] and can be described by measuring the behavior of individuals, the interaction between individuals, and the resulting social network, altogether defined here as "group behavior." When individuals interact in a group, their previous experience, motivation, and physiological state (termed here as "internal state") affect their action selection, giving rise to diverse activity levels, behavioral responses, and engagement with others.[6–8] This results in a highly complex and ever-changing environment, where each interaction can change the social context of subsequent interactions, leading to a variety of behavioral outcomes from what seem to be identical starting conditions.[7,9] The complex nature of this environment imposes conceptual challenges in the quantification and analysis of group behavior.[10]

A fundamental question in this respect is how internal and external factors, such as previous social experience, specific group composition, or the existence of available resources, shape group behavior.[11,12] Although much is known about the interplay between social experience, internal states,[13–18] and their effects on social interaction in pairs of animals,[18–23] relatively little is known about how these elements shape social behavior in a group. Currently, group behavior is mainly studied at two organizational levels: the behavioral repertoires of individuals within groups and the structure and dynamics of all interactions within a group (social network analysis).[24] Both lines of study progressed substantially with advances in machine vision and machine learning technologies that allow automated tracking and unbiased behavioral analysis.[25–31] Analyzing the behavioral repertoires of individuals within a group can provide a comprehensive description of behavioral responses of all individuals under different conditions, enabling the dissection of mechanisms that shape each behavior, the sensory requirements for a given behavior, and the specific context it is presented in. However, this approach does not provide much information about group structure. By evaluating every interaction between pairs of individuals in a group, network analysis can be used to represent integrated systems such as social groups, providing insights into the formation,

dynamics, and function of group structure.[24,32–34] This type of analysis can be employed to investigate transmission processes in groups as a basis for understanding complex phenomena such as microbe transmission, social grooming, decision-making, and hierarchy.[3,32,35–47] Although analysis of individual behaviors and social networks highlight different aspects of social interaction, they are complementary for understanding complex emergent phenomena such as group behavior.

Studies of social interaction in *Drosophila melanogaster* have mainly focused on understanding the neuronal basis of innate and recognizable behaviors such as male-male aggression and male-female courtship encounters.[48–53] Various studies provided mechanistic understanding of these complex behaviors, demonstrating that their expression requires multi-sensory inputs, as well as specific neuronal pathways in the brain.[52,54–59] Modulation of behavior by previous social experience was also investigated in flies, revealing that gene regulation in specific neuronal populations can lead to long-lasting behavioral changes.[18,20,60–63] The social behavior of *D. melanogaster* in the wild remains largely understudied. Nonetheless, it was shown that wild flies are relatively stationary, moving only a few meters a day, tending to group with conspecifics while avoiding flies of different species.[64] These aggregations seem to be plastic and dynamic and facilitate mating with members of other groups to decrease inbreeding. Aggregations are a substrate for a rich repertoire of social interaction that includes courtship, competition over mating partners, mating, and communal oviposition.[64] Sex-specific adaptations for space use were suggested, possibly driven by avoidance of predators, parasites, or males.[65]

While *Drosophila* proves to be a useful model organism for mechanistic dissection of complex behaviors,[66,67] only a small number of studies examined social interaction in groups of flies. These studies demonstrated that flies possess the neuronal ability to recognize different individuals in a group,[68] that groups of flies exhibit non-random group structures, which depend on certain sensory systems[4,59,69] and group size,[70] and that group interaction facilitates collective responses to threats.[4,71] These findings, together with the existence of dedicated circuits for processing social information, and evidence for the presence of social aggregates in wild flies, support the notion that group living is a fundamental component of *Drosophila* behavior. Still, little is known about how group behavior in *Drosophila* unfolds under different biological and environmental conditions. Specifically, it is not clear whether flies form groups with different structures under various conditions, whether the group is affected by internal properties of its constituting individuals and their composition or by different environmental conditions, and whether individual recognition plays a role in such groups.

To bridge these gaps, we searched for conditions that can facilitate the formation of distinct group behaviors. We hypothesized that groups composed of flies with different social histories, such as flies that were socially raised and flies that were socially isolated, will exhibit distinct emergent group structures that result from differences in motivation, experience, activity level, and/or sensory sensitivity of the interacting flies. To analyze the emergent group properties, we established an experimental framework that clusters various behavioral and social network parameters into behavioral "group signatures." We presumed the group signature of socially raised flies to reflect a snapshot of established relationships between members of the group that developed over the course of the experience phase, while that of solitary flies were presumed to reflect initial interaction of flies that are exposed for the first time to other flies. Additionally, studies from various animal species[21,22,72–74] including *Drosophila* have shown that isolation results in increased activity/arousal, increased aggression, and in some cases social avoidance. Extending these findings to group context, we predicted groups of solitary flies to exhibit increased activity, increased aggression, and reduced social interaction. In contrast, groups of socially raised flies were predicted to show increased social interaction due to reduced aggression.[75,76] Here, we show that social experience can drive the formation of groups with distinct behavior and network structures and that group signature is a useful tool for simplifying the analysis of the multifaceted repertoire of parameters associated with social interaction in groups. Moreover, we show that the group signature of socially raised male flies is strongly influenced by both visual cues and the sensing of the male-specific pheromone 11-cis-vaccenyl acetate (cVA). Finally, we explored social interactions in heterogenous groups and identified clusters of features that are sensitive to increasing ratios of aggressive flies, some of which reveal that inter-individual coordination depends on group composition.

RESULTS

Establishing a Data Capture and Analysis Pipeline for Studying Complex Behavior in Groups

To explore the interplay between social history, internal states, and social group interaction, we exposed male flies to distinct social conditions and recorded their social interactions within circular arenas that are suitable for analyzing complex group behavior (Fly Bowl system).[77] To quantify and analyze the behavioral repertoire of individual flies, group interaction, and the resulting social networks, we adapted the Fly Bowl suite of tracking and behavior analysis tools (Ctrax, JAABA, and JAABA plot; Figure 1A).[77–79] Although Ctrax is successfully used in many behavioral setups, its output includes some tracking errors such as unifying identities and failure to recognize a fly for several frames, impeding analysis that requires accurate and stable identities throughout the experiment. To resolve this, we developed a secondary processing algorithm for Ctrax output data, named FixTRAX. FixTRAX uses a set of rules to find tracking errors, calculates statistical scores that determine which identities to correct per frame, and generates a graphical summary of tracking quality per movie (detailed explanation of the algorithm, error rate, and code are found in the STAR Methods and Data S1, S2, and S3). Corrected output data are used to calculate kinetic features and classify eight distinct complex behaviors using the supervised machine learning algorithm JAABA[77] (Figure 1A; full description in Table S1).

We used the following requirements for an interaction: (1) consistent with basic interaction criteria described by Schneider et al.[69] and based on the fact that 95% of social interactions (approach, touch, and social clustering) occur in the range of 1–8 mm (Figures S1A–S1C), we set the distance threshold for interaction between two flies to be 8 mm or less, which is the average of two body lengths. (2) The visual field of view of the

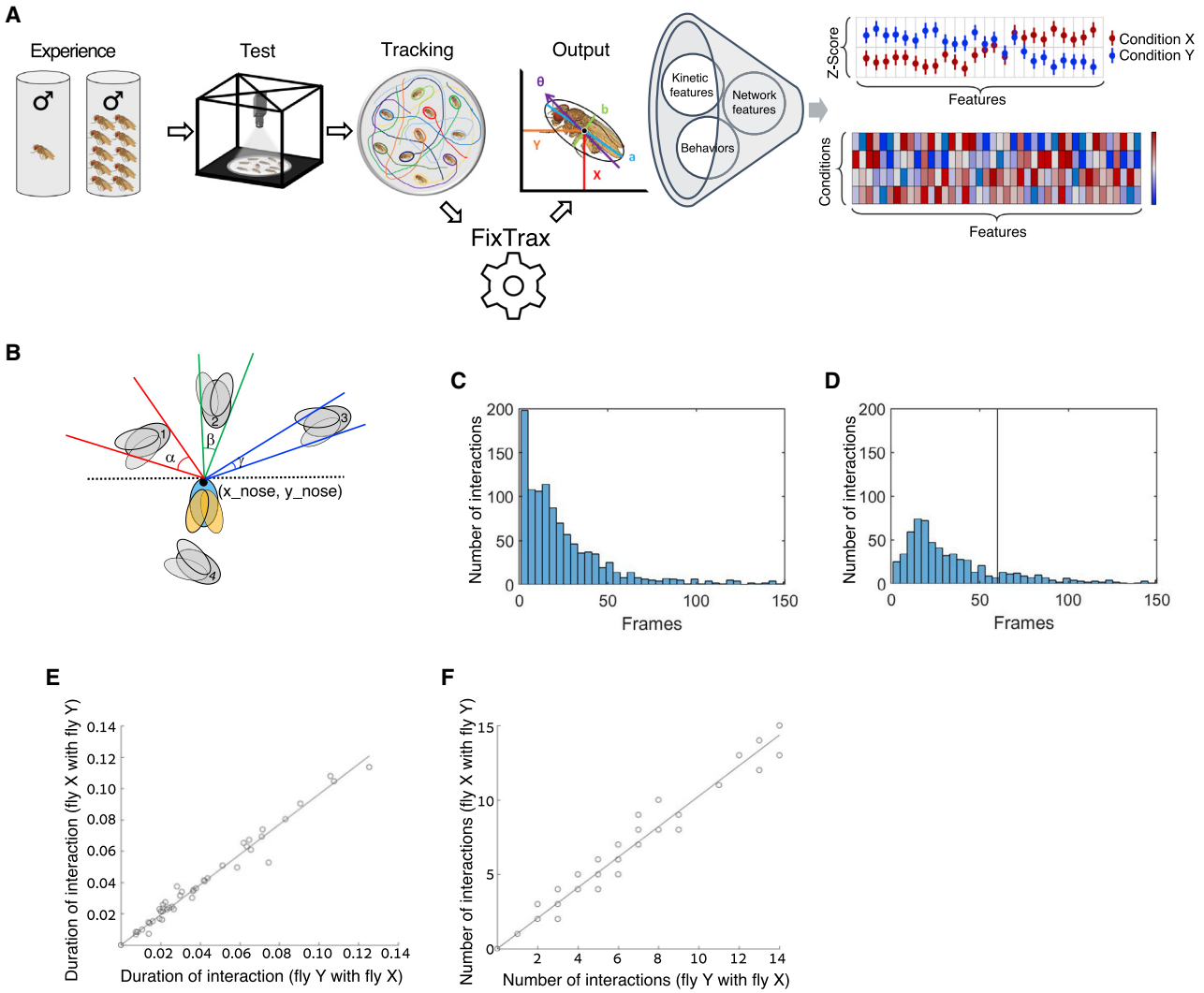

Figure 1. A Conceptual and Experimental Setup for Studying Complex Behavior in Groups of *Drosophila*

(A) Illustration of social conditioning, data capture, and analysis. Naive male flies were housed in groups of 10 flies or in isolation for 3 days and inserted in groups of 10 into Fly Bowl arenas, where their social interaction was recorded for 15 min (at 30 fps). Tracking was performed using Ctrax. Error correction of Ctrax output data was performed using FixTRAX, generating an output file of position, angle, and size per fly per frame. The fixed output file was used to calculate kinetic features, classify specific behaviors using JAABA, and analyze social network structure (see Table S1). Group signature was generated by normalizing all features as a series of *Z* scores per condition (far right upper graph). Hierarchical clustering of conditions (y axis) and features (x axis) was performed using Partek and presented as heatmaps (far right lower graph).

(B) Illustration of the angle criteria used to define an interaction; angle subtended (α, β, or γ) > 0.

(C and D) Total number of encounters as a function of encounter duration in representative movie of socially raised WT flies (C), and when adding a 60-frame gap requirement between interactions (D). Black line represents the threshold (60 frames) under which encounters are not considered interactions for network analysis (see also Figure S1).

(E) Directed interactions quantified as the total duration between each pair of flies.

(F) Directed interactions quantified as the total number of interactions between each pair of flies. See also Figure S1, Table S1, and Data S1, S2, and S3.

focal fly is occupied by the other fly (angle subtended > 0), indicating that the focal fly can see the other fly (Figure 1B). To minimize the number of false positives (random interactions), we required the angle and distance criteria be maintained for at least 2 s (Figure 1C). This resulted in a large number of very short interactions, some of which could actually be long interactions that are mistakenly recognized as separate short interactions, due to small numbers of intermittent frames in which one of the conditions is not met (Figure 1C). To resolve this, we added an additional requirement of a minimal time interval (gap) below which a subsequent interaction is considered an extension of the previous interaction between the same pair of flies. To find the optimal gap length, we tested a series of interaction and gap lengths and eventually selected a gap length of 4 s (120 frames) (Figure S1D), which substantially reduced the number of very short interactions (Figure 1D). We used weighted networks to

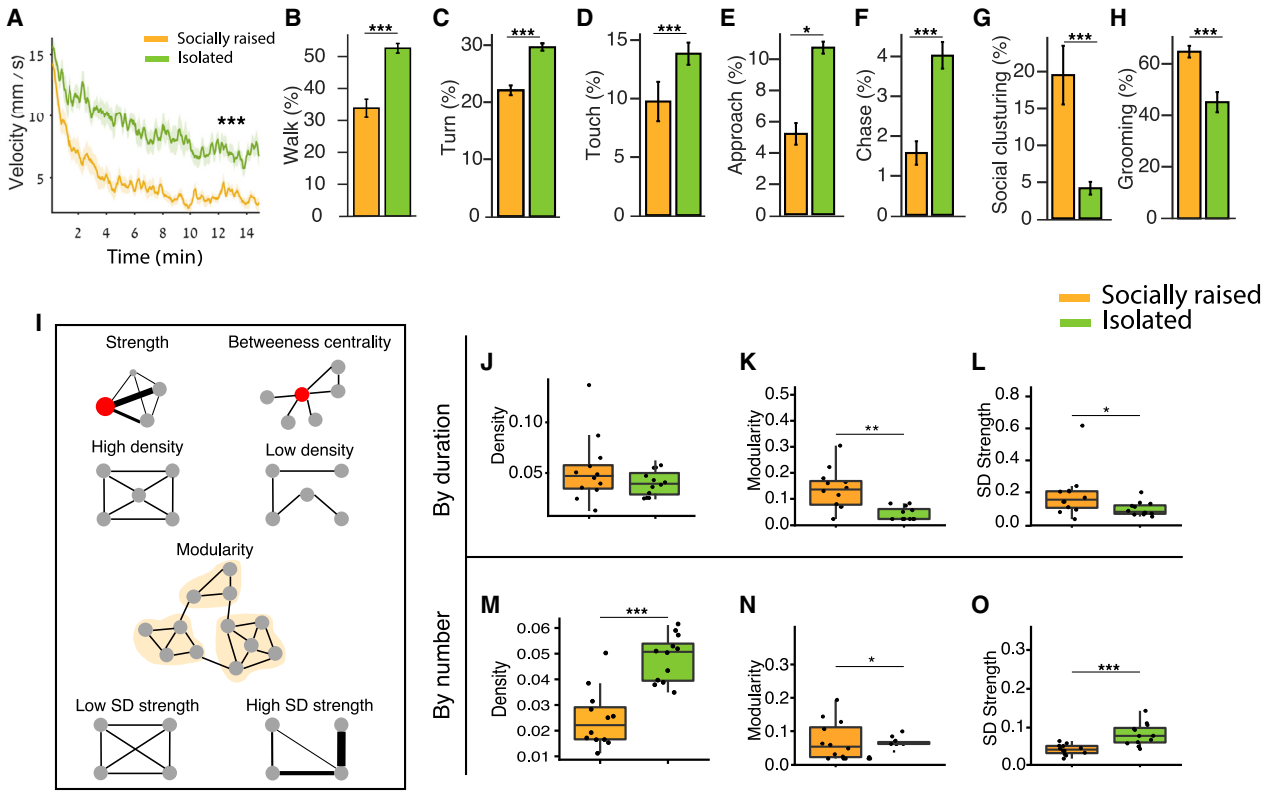

Figure 2. Prior Social Interaction in a Group Facilitates the Formation of Ordered Social Structures

(A) Average velocity per frame of previously isolated male flies (green) versus socially raised male flies (orange) over 15 min.

(B–H) Average percentage of time previously isolated male flies (green) versus socially raised male flies (orange) took to perform walk (B), turn (C), touch (D), approach (E), chase (F), social clustering (G), and grooming (H) behaviors (see also Figure S2).

(I) Illustration of network parameters; strength is proportional to vertex size. Betweenness centrality is a measure of the tendency of the individual to serve as a hub connecting different sub-groups (high in red individual). 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. Standard deviation (SD) strength is a measure of the heterogeneity of the connections between individuals.

(J–O) Network density, modularity, and SD strength calculated by network weights according to duration (J–L, respectively) or number of interactions (M–O, respectively) between previously isolated (green) and socially raised (orange) WT male flies. n = 18, Wilcoxon test and FDR correction for multiple tests *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Error bars signify SEM.

See also Figure S2.

account for the between-dyad variation in total interaction times over each test and to avoid network saturation, an inherent limitation of binary networks. Next, we analyzed the symmetry level between interacting flies by testing whether the total amount of time in which individual X interacts with individual Y correlates with the total amount of time in which individual Y interacts with individual X. Performing this for all pairs of flies within each group resulted in high correlation (Figure 1E), which was also apparent when quantifying total number of interactions between each pair (Figure 1F). This suggests symmetric interactions over the course of the test, making directed analysis redundant in this setup. We used the interaction data to calculate four network features: strength, density, betweenness centrality, and modularity (schematic illustration and explanation of the features are depicted in Figure 2I). In total, our data analysis pipeline generates 60 features that represent the behavioral repertoire of individuals within a group and their corresponding social networks. To process and analyze such rich datasets, we generated a comprehensive representation of all features using normalized *Z* score scatterplots and hierarchical clustering to compare between experimental groups and highlight similarities and differences between them (Figure 1A).

Prior Social Interaction in a Group Facilitates the Formation of Ordered Social Structures

To test whether social experience can drive divergent forms of group behaviors, we generated two cohorts of wild-type (WT) Canton S male flies: one cohort of flies raised for 3 days with 9 other flies (as groups of 10 male flies) and the other cohort raised in complete social isolation upon eclosion. After 3 days, 10 flies from each cohort were introduced into Fly Bowl arenas, and their behavior was recorded for 15 min and analyzed (Figure 1A). The two cohorts exhibited distinct repertoires of behavioral responses upon interaction with other flies in a group; socially

raised flies displayed lower average activity levels, manifested by lower average velocity (Figure 2A), shorter time spent walking (Figure 2B), and fewer body turns than isolated male flies (Figure 2C). Analysis of specific social behaviors revealed that socially raised flies exhibited less touch behavior (Figure 2D), were less engaged in active approach (Figure 2E), and spent less time chasing (Figure 2F). Socially raised flies also spent more time grooming than isolated flies (Figure 2H). Analysis of average duration (bout length) and frequency of specific behaviors revealed that touch, chase, approach, grooming, and social clustering behaviors were significantly different between the two cohorts (Figures 3A and S2A–S2H). Interestingly, average bout duration of approach behavior was similar between the two cohorts, while its frequency was higher in isolated flies (Figures 3A, S2A, and S2E), suggesting the difference in their social experience did not affect the duration of social encounters but rather the frequency at which they occur.

The difference between socially raised and socially isolated flies can result from inherent differences in the kinetic properties of individuals or from an emergent property of flies interacting in a group. To distinguish between these two possibilities, we compared the behavior of socially isolated and raised flies that were tested singly. If the differences between the groups stem from inherent differences in the kinetic properties of individuals, we would expect to identify kinetic differences between the two cohorts of singly tested flies. Remarkably, we did not observe any significant differences between the two cohorts, suggesting that the effects of social experience on behavior are an emergent group property expressed during group interaction (Figures S2N–S2V). Another example for a difference in the emergent properties of socially raised and isolated groups is the tendency of socially raised flies to concentrate in certain zones within the arena, forming semi-stable social clusters consisting of three or more flies (Figures 2G and S2M). This behavior was not apparent in male flies that were raised in social isolation prior to testing, suggesting this behavior emerges from the social experience of flies rather than from the context of the behavioral test itself (Figure 2G).

To investigate how group structure is affected by social history, we analyzed the network structures of groups composed of socially raised or socially isolated individuals. 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 socially raised flies displayed higher modularity (Figure 2K), standard deviation (SD) strength (Figure 2L), and betweenness centrality (Figure S2L), suggesting that prior social experience promotes the formation of subgroups. Network analysis by number of interactions, which assigns equal values to long and short interactions and thus undervalues social clusters (Figures 2J–2L versus Figures 2M–2O), revealed that the social networks of isolated flies are characterized by higher density (Figure 2M), SD strength (Figure 2O), and strength (Figure 3A), suggestive of overall more interactions. In contrast, networks of socially raised flies have higher modularity (Figure 2N) and betweenness centrality (Figure 3A), similar to the results obtained with analysis by duration of interaction. Taken together, these differences indicate that socially isolated flies perform more short interactions compared to socially raised flies, while socially raised flies form networks with higher-order structures compared to those formed by isolated flies. Overall, these results show that the behavioral group signature of socially raised flies differs from that of previously isolated ones (Figure 3A).

Behavioral Signature of Socially Raised Flies Does Not Require Individual Recognition

It is plausible that the observed differences between socially raised and isolated cohorts result from the familiarity of raised flies with the individuals they are tested with. Therefore, we asked whether the distinct features exhibited by socially raised males result from their familiarity with individual members that occurred during housing or from the internal state associated with the general experience of living in a group. To distinguish between these two possibilities, we tested socially raised flies with either familiar or unfamiliar individuals. One cohort was tested with the same flies they were previously housed with (familiar), while the other cohort was tested with socially raised flies from other groups (unfamiliar). Encountering familiar or unfamiliar flies did not result in different behavioral signatures (Figure 3B), suggesting that the dynamics captured during the test result from the general experience of interacting with others rather than by specific previous interactions. We next tested whether other conditions that are known to modulate internal state such as repeated ethanol exposure, starvation, and different circadian time shifts, also affect group interaction. We did not observe any significant difference between these conditions and their controls (Figure S3), implying that not all experiences that modulate internal state affect group dynamics in the context used in our experimental paradigm.

Prior Social Interaction Increases Behavioral Variability

The existence of a complex social structure in groups of socially raised flies suggests that in addition to the observed differences in the means of various behaviors, there may be additional effects on the distribution of certain features. Indeed, when analyzing the behavioral signatures of socially raised and isolated male flies, we observed that socially raised flies exhibited higher variance across several behavioral features (Figures 2 and 3A; compare error bars). To further investigate this, we compared the variance of all behavioral features between groups of socially raised and isolated male flies. We analyzed the variance of each behavioral feature in three ways: (1) average SD of each group (each movie), reflecting variation inside each group (SD within groups); (2) SD of averages between experimental groups per condition, reflecting variation between groups (SD between groups); and (3) SD across all flies per condition, reflecting individual differences between all flies regardless of groups (SD all flies) (Figure 3C). We documented a higher number of behavioral features that displayed significantly higher variance (SD 2-fold higher in one condition + statistically significant) in socially raised flies between groups (18 out of 56 parameters; Figure 3D), within groups (11 out of 56 parameters; Figure 3D) as well as between all flies (21 out of 56 parameters; Figure 3D). This indicates that the behavior of socially raised flies is more diverse than that of isolated flies, possibly reflecting a broader repertoire of behaviors in individuals that is shaped by prior interactions during the experience phase. Increased variability between

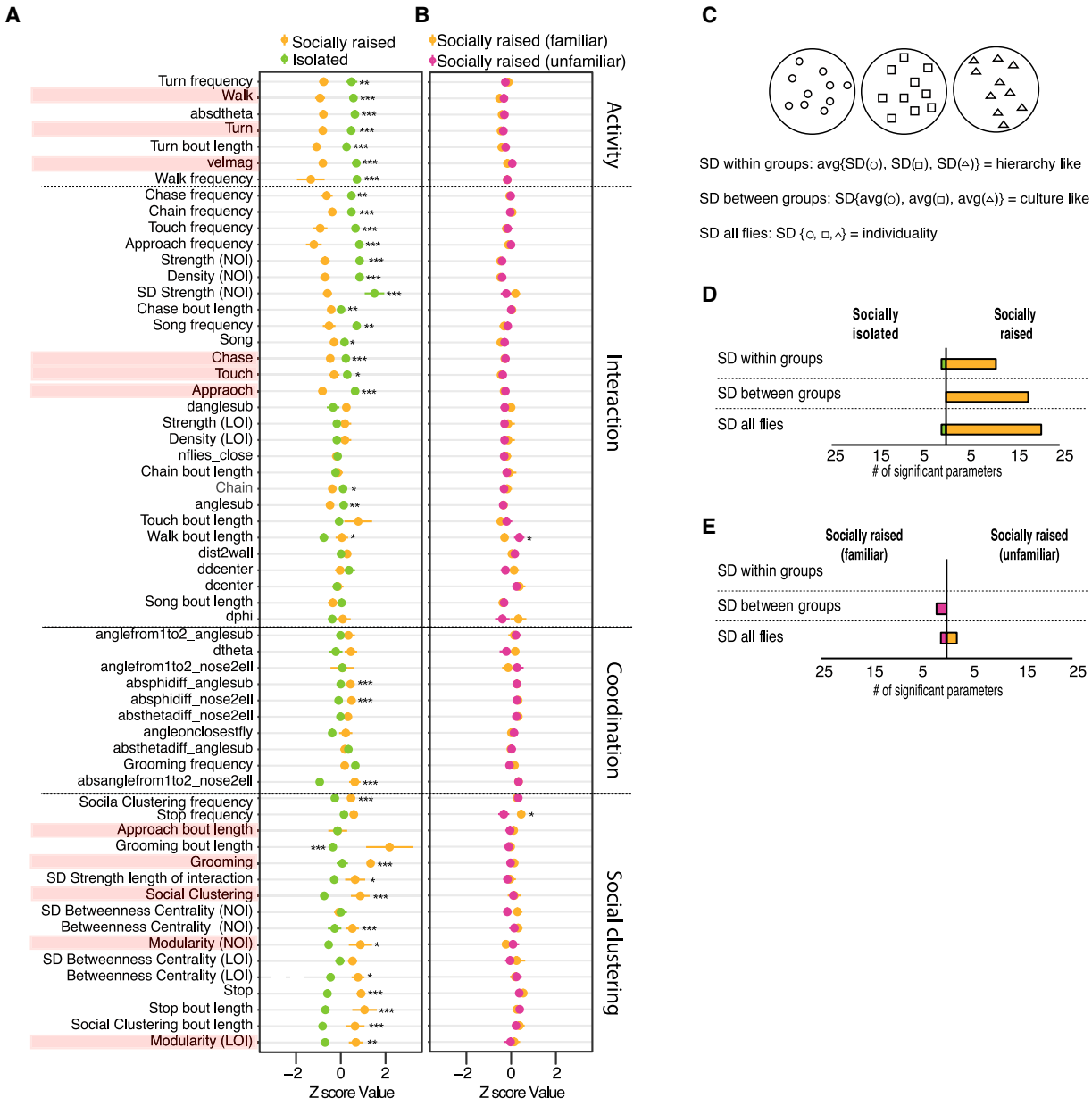

Figure 3. Social Experience Facilitates Distinct Group Signature and Increases Behavioral Variability

(A and B) Behavioral signatures of previously isolated versus socially raised WT male flies (A) and familiar versus unfamiliar socially raised WT flies (B). Data are represented as normalized *Z* scores of 60 behavioral (A: n = 18. B: n = 25 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. p values were corrected using FDR. *p < 0.05, **p < 0.01, ***p < 0.001). Features mentioned in the results section are highlighted in pink.

(C) Graphical illustration of measuring variance within groups, between groups, and across all individuals (all flies) in each condition.

(D and E) Number of behavioral features that display significantly higher variance; their SD is at least 2-fold higher when comparing isolated to socially raised (D) and familiar versus unfamiliar (E). Statistical analysis was performed on SD of the entire population (all flies) (F-test), SD of repetitions in each condition (between groups) (F-test), and average SD within each repetition per condition (inside groups) (t test). p values were corrected using FDR. *p < 0.05, **p < 0.01, ***p < 0.001. Error bars signify SEM.

See also Figure S3.

groups of socially raised males that have presumably had identical experience suggests that each group possesses distinct group characteristics that were shaped during the housing period before the test. To test this hypothesis, we asked whether between-group variance stems from inter-individual recognition or is based on the general experience of living in a group. For that, we performed a similar analysis in male flies that were housed in groups and tested either with the same group members or with flies that were housed in other groups (data taken from the experiment of Figure 3B). We documented very few

parameters that were distributed differently between flies tested with familiar or unfamiliar flies, implying that the general experience of living in a group also shapes the variance of behavioral responses, and that individual recognition has little to no effect on behavioral variance in a group (Figure 3E).

Visual Cues Are Necessary for Expressing the Behavioral Signature of Socially Raised Flies

So far, we have shown that different types of social history can form divergent group dynamics and structure. Next, we set out to dissect the sensory elements required for the expression of such differences. We started by assessing the role of visual cues in forming specific behavioral signatures during the test. For that, we analyzed the behavior of socially raised flies in light or dark conditions (this did not interfere with tracking since recording is performed using IR backlight). Socially raised flies that were tested in the dark displayed more walk, turn, and touch behaviors than those tested in the light and spent a larger fraction of time in chase and approach behaviors while showing less social clustering and grooming behaviors (Figure 4A). Moreover, approach behavior in the dark was significantly longer and more frequent than that in the light (Figure 4A), whereas frequency and duration of social clustering was lower in the dark. Interestingly, although the average velocity of flies in the presence or absence of light was not statistically different (Figure 4A), flies tested in the light reduced their velocity over time, while flies tested in the dark maintained a constant velocity for the duration of the experiment. This was also evident in several other behavioral features, such as walk and turn behaviors, suggesting that flies habituate to environmental conditions in the light but not in the dark (Figures S4A–S4L). Network analysis revealed lower SD strength and betweenness centrality in groups tested in the dark by analysis of duration of interactions (Figure 4A), while analysis by number of interactions revealed that flies in the dark display higher density, strength, and SD strength than flies in the light (Figure 4A). Therefore, we postulate that light is required for the group signature of socially raised male flies.

We next aimed to uncouple the behavioral changes observed during light deprivation: those that result from the role of visual cues in a typical social interaction in a group from those that specifically depend on prior social experience. For that, we tested groups of socially raised and socially isolated flies in the presence or absence of light (Figures 4A and 4B). Behavioral features that are affected equally by light in both groups represent features that are light-dependent but not sensitive to social experience, while features that are only affected in one group are those that turn into light-dependent by previous social experience. To visualize this, we plotted distinct features that are influenced by visual cues in each condition. We identified 22 unique features that are sensitive to visual cues only in socially raised flies, and only 7 in isolated flies, suggesting that the experience of an enriched social environment requires light to be fully expressed (Figure S4M). Most features unique to the socially raised group are associated with social clustering (reduced in the absence of light) and interaction (increased in the absence of light). The opposite regulation of these two types of features suggests that in the absence of light, socially raised flies undergo a shift from a quiescent state to a more active state, characterized by more approach, chase, and touch behaviors. In contrast, groups of previously isolated flies displayed a decrease in a few interaction-related parameters and an increase in a class of parameters that reflect changes in angle and speed between two close individuals in the absence of light (absanglefrom1to2, absphidiff, absthetadiff, and angleonclosestfly; see Table S1 for more details) (Figures 4A–4C). This may signify an increase in coordination between pairs of flies and suggest that isolated flies in the dark generally tend to be less mobile but more engaged with others when interacting (Figures 4B and S4G–S4L).

To assess whether the group signatures of these conditions reveal an underlying similarity, we performed hierarchical clustering analysis on group signatures of all conditions (Figure 4C, list of features in Figure S4N). This analysis revealed two main clusters based on social history; one with conditions in which flies were isolated prior to the test and another with conditions in which flies were socially raised. Interestingly, socially raised flies that were tested in the dark did not cluster with either group, reinforcing the notion that specific visual cues are necessary for the expression of group signatures associated with social experience but are not sufficient to fully shift group signature from that of socially raised to that of isolated.

cVA Perception via Or65a Neurons Shapes Social Group Interaction

In addition to visual cues, a central element in social interaction of flies is pheromone-based communication. The male-specific pheromone cVA is known to mediate experience-dependent changes in aggressive behavior, whereas chronic exposure to cVA found on conspecifics during group housing is known to reduce male-male aggression.[60,80] cVA is perceived via two olfactory receptor neurons (ORNs): Or67d, which mediates acute responses to cVA, and Or65a, which mediates chronic responses to cVA.[60,81] We investigated whether cVA perception impacts the group signature of socially raised flies. For that, we blocked cVA perception by constitutively expressing the inward rectifying potassium channel Kir2.1 in Or65a- or Or67d-expressing neurons of socially raised flies and then analyzed their group behavior. Inhibition of Or67d neurons did not lead to major differences between experimental flies and genetic controls, suggesting that the function of Or67d neurons is not necessary for the formation of the behavioral signature associated with social group experience (Figure 5A). In contrast, inhibition of Or65a neurons dramatically changed the group signature of socially raised flies, increasing average velocity and overall time flies engaged in approach, chase, and touch behaviors compared to genetic controls (Figure 5B). Network analysis revealed higher strength and lower betweenness centrality in the Or65a experimental group compared to genetic controls by both duration and number of interactions (Figure 5B). Overall, this suggests that Or65a- but not Or67d-expressing neurons function in shaping the group behavior of socially raised flies.

This experimental design does not distinguish between the role of Or65a neurons during experience and test phases due to the constitutive nature of this neuronal inhibition. To test the role of Or65a neurons during the test phase, we performed a similar experiment in isolated male flies, which are expected to be exposed to cVA only during the test. If Or65a-expressing neurons function only to shape the group signature of socially raised flies via exposure to cVA during the experience phase and before

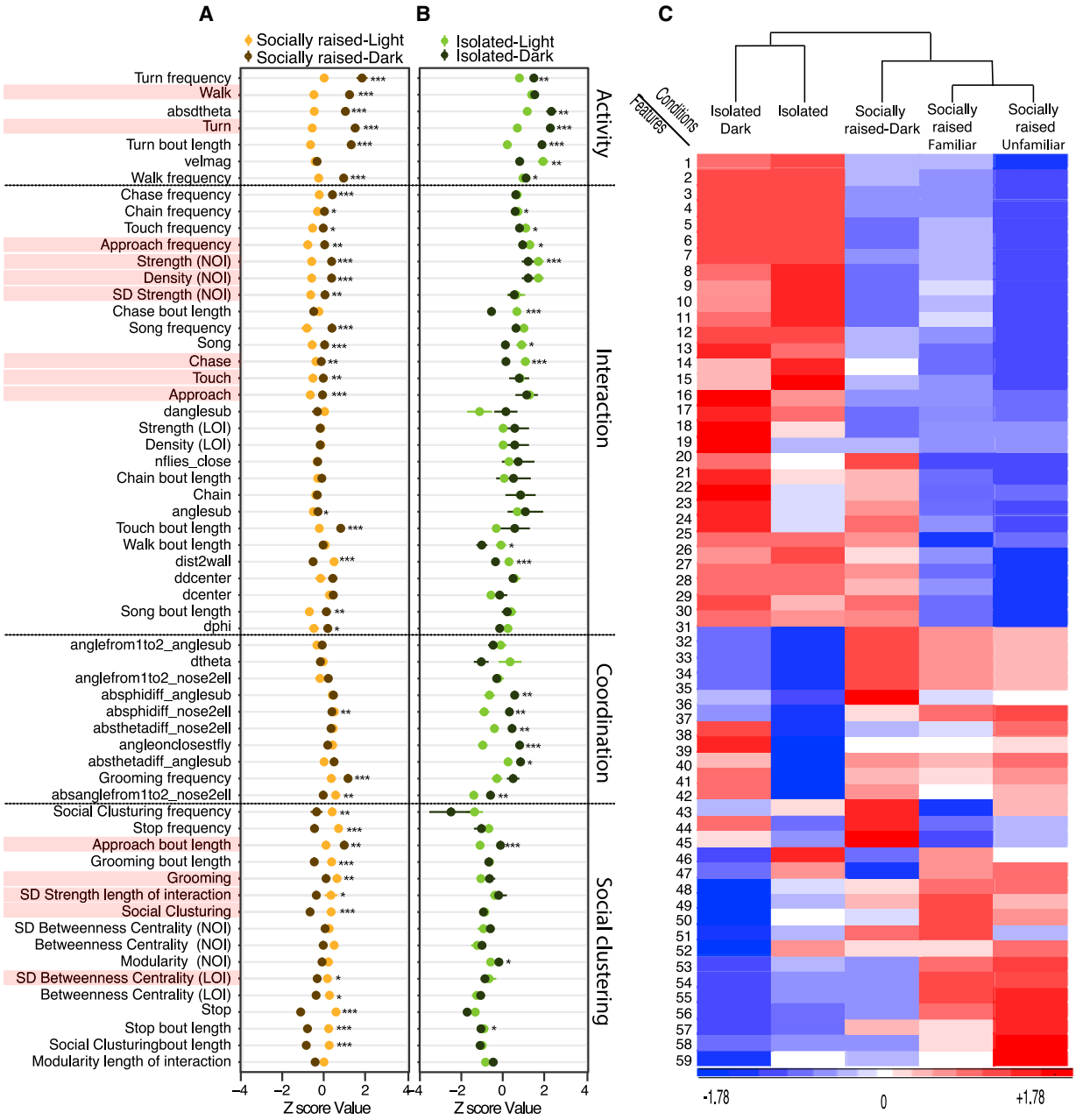

Figure 4. Visual Cues Are Necessary for Expressing the Behavioral Signature of Socially Raised Flies

(A and B) Behavioral group signatures (represented as normalized *Z* scores) of socially raised (A) or previously isolated (B) WT male flies tested in normal lighting conditions (light) versus light deprivation (dark). Length of interactions (LOI): calculated according to the length of interactions. Number of interactions (NOI): calculated according to the number of interactions. n = 18 and 10, respectively. t test for normally distributed parameters or Wilcoxon test for non-normally distributed parameters. p values were corrected using FDR. *p < 0.05, **p < 0.01, ***p < 0.001. Error bars signify SEM. Features mentioned in the results section are highlighted in pink.

(C) Hierarchical clustering (dendrogram) of group signatures of the following experimental conditions: socially raised (raised familiar), unfamiliar socially raised (raised unfamiliar), socially raised tested in dark (raised dark), socially isolated tested in light (isolated), and socially isolated tested in dark (isolated dark). List of numbers represent behavioral features. Full list in Figure S4N.

See also Figure S4.

test, we expect the inhibition of these neurons not to affect the behavioral signature of isolated flies. Surprisingly, inhibition of Or65a neurons in isolated male flies resulted in changes of several behavioral features, although Or65a neurons are thought to only mediate chronic responses to cVA over long time courses.[60] Experimental flies (*Or65a* > *Kir*) exhibited more touch, approach, chase, and chain behaviors than genetic controls and increased network strength as measured by duration of interaction

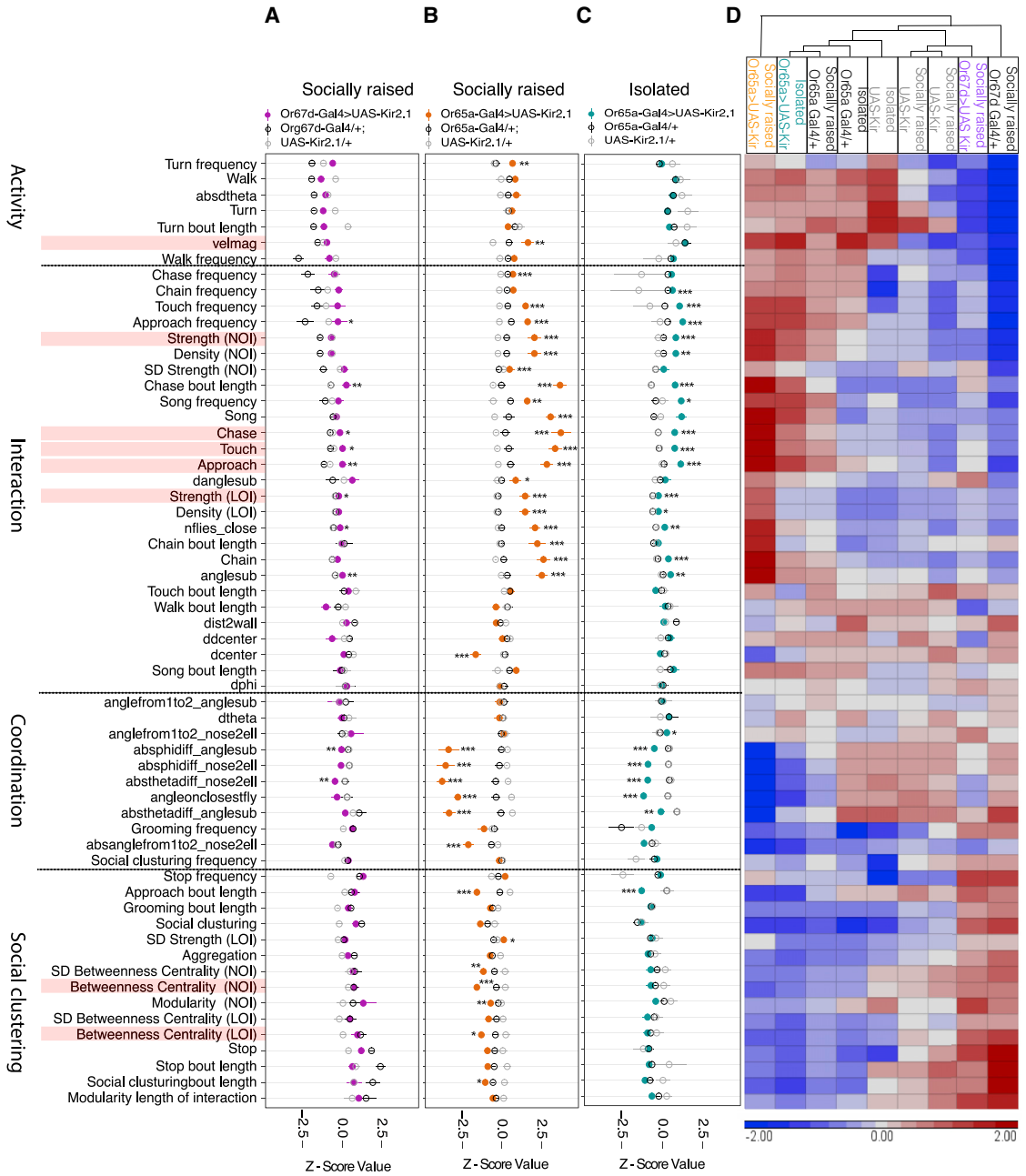

Figure 5. cVA Sensing via Or65a Neurons Shapes Social Group Interaction

(A–C) Behavioral group signatures (as normalized *Z* scores) of socially raised Or67d-Gal4/+; UAS-Kir2.1/+ flies compared to genetic controls (A), socially raised Or65a-Gal4/+; UAS-Kir2.1/+ flies compared to genetic controls (B), and previously isolated Or65a-Gal4/+; UAS-Kir2.1/+ flies compared to genetic controls (C) (see also Figure S5). LOI: calculated according to the length of interactions. NOI: calculated according to the number of interactions. n = 7, 13, and 8, respectively. One-way ANOVA with Tukey's range test for normally distributed features or Kruskal Wallis followed by Wilcoxon signed-rank test for non-normally distributed features. p values were corrected using FDR. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Error bars signify SEM. Features mentioned in the results section are highlighted in pink.

(D) Hierarchical clustering (dendrogram) of behavioral group signatures of all experimental conditions in (A–C).

See also Figure S5.

(Figure 5C). However, these effects were less extreme than those displayed by socially raised male flies (Figure 5B versus Figure 5C). This unexpected result suggests that Or65a neurons mediate acute as well as chronic responses to cVA.

Interestingly, some effects of Or65a neuronal inhibition are identical between socially isolated and socially raised flies, including a decrease in three coordination-related parameters (Figures S5A–S5C) and a significant increase in chain, chase,

chase bout length, touch, and approach behaviors (Figure S5D–S5H). Moreover, both experimental groups displayed higher network strength (measured by duration of interaction; Figure S5I), suggesting that inhibition of Or65a neuronal activity facilitates behaviors that are associated with social isolation. Overall, although these two conditions share similarities, the effect of Or65a inhibition was more profound in socially raised flies than in socially isolated flies, reflected by the higher number of behavioral features affected (35 versus 22 out of 60; Figures 5B and 5C). Hierarchical clustering analysis between conditions revealed that flies in which Or67d neurons were inhibited are similar to their corresponding genetic controls, supporting the conclusion that Or67d neurons do not mediate behavioral responses of socially raised male flies in a group (Figure 5D). In contrast, socially raised male flies in which Or65a neurons were inhibited are clustered apart from their genetic controls and all other tested conditions, indicating that cVA perception though Or65a-sensing neurons is necessary for the formation of a certain internal motivational state via the experience of group housing, leading to a specific group signature (Figure 5D).

Heterogenous Groups of Flies Exhibit Dynamic Social Interaction that Is Shaped by Group Composition

So far, we have used homogeneous groups of flies that were subjected to environmental or genetic manipulation as a tool to investigate the interplay between social experience and the resulting group behavior. This approach eliminates the inherent contribution of inter-individual differences to group structure, which proved valuable in dissecting the elements that shape social group behavior. Next, we asked how the dynamics inside the group are shaped by different individuals. For this, we generated groups that contain varying ratios of male flies with two distinct states: socially raised flies and hyper-aggressive isolated flies. Hyper-aggressive male flies were generated by knocking down (k.d) *Cyp6a20* (a manipulation known to induce aggression)[20] and keeping these flies isolated from eclosion. We postulated that highly aggressive k.d flies would disrupt collective-like group behaviors such as social clustering and thus change the behavioral signature of the group.

To verify that these flies indeed behave as expected, we tested their social interaction in groups of flies and compared it to *Cyp6a20* k.d flies that were socially raised before the test and to that of socially raised WT control flies (Figures S6A–S6H). We did not document any difference between the two cohorts of *Cyp6a20* k.d flies. However, compared to the WT control group, both *Cyp6a20* k.d cohorts displayed more walk, turn, and chase behaviors (Figures S6B, S6C, and S6F), while exhibiting lower social clustering and grooming behaviors, as expected (Figures S6G and S6H). This suggests that the genetic manipulation in this case eliminates the effects of previous social experience on group signature.

Next, we introduced increasing numbers of hyper-aggressive flies into groups of socially raised WT male flies (10%–50% of the total number of individuals) and measured their group behavior. The behavior of each experimental group was normalized to a control group of 100% socially raised WT flies that was tested at the same time, enabling statistical comparison of all behavioral features across all experimental groups (0%–50%), unlike previous experiments in this work, which can only be compared to their controls. To gain a general overview of the patterns associated with gradual changes in group composition, we examined the normalized behavioral signatures using hierarchal clustering (Figure 6A). Overall, the conditions are clustered into two main branches: one containing the homogeneous WT group with the 10%–30% mixed ratio groups and a separate branch containing groups of 40%–50% mixed ratios, suggesting a behavioral transition from homogeneous to 50% mixed ratio groups. The differences between these two extremes resemble those of socially raised versus socially isolated flies, suggesting that the addition of 50% aggressive flies is sufficient to convert group behavior into that of a social isolation-like state (Figures 3A versus Figure 6A). Overall, clustering of features suggests a somewhat gradual transition from 0% to 50%. This trend is best demonstrated by the increase in the number of features that exhibit a significant difference compared to 100% WT flies (Figure 6B). We identified a suit of features associated with an increasing number of *Cyp6a20*-knockdown (KD) flies: a cluster of decreasing features and a cluster of increasing features (Figure 6A). Some decreasing features corresponded to social clustering and network structure, whereas increasing features were related to activity and interaction (Figure 6A). Some of these features exhibited a gradual change as the number of *Cyp6a20*-KD flies in a group increased. These included a gradual decrease in social clustering, grooming, stop, and stop bout length (Figure S6I–S6L), and a gradual increase in walk, angular speed (absdtheta), turn, and turn bout length (Figure S6M–S6P). Interestingly, some behavioral features showed parabolic-like changes across increasing ratios of *Cyp6a20*-KD flies, with maximal or minimal values at 20%–30%, including touch behavior and several other features expected to be associated with coordination between two individuals (absphidiff_nose2ell, absphidiff_anglesub; Table S1). Some behavioral features were more sensitive than others to changes in group composition, such as grooming, approach, and turn behaviors, which were significantly different from controls even at 20% mixed ratio, while other features such as social clustering exhibit a significant change only at 40%–50%. This suggests that changes in the level of approach behavior within a group precede changes in more collective-like behaviors such as social clustering (Figure 6A).

It could be argued that the behavioral pattern exhibited by mixed groups represents an average of two distinct subgroups and not an integrated structure of all individuals within the group. If so, the differences observed at the group level would result from the existence of *Cyp6a20*-KD flies having higher values of approach behavior and lower values of social clustering, which would drastically affect the group average, depending on their relative ratio within the group. To test this, we analyzed the per-fly distribution of each condition. If each group is composed of two distinct subgroups (WT and *Cyp6a20*-KD flies), we would expect this to be reflected in a bi-modal distribution, which would become more pronounced as the ratio of *Cyp6a20* k.d flies increases. Single-fly analysis of features that exhibit changes with an increased number of mutant flies, such as walk, approach, and social clustering, did not show a bi-modal distribution, making it impossible to identify subgroups that correspond to mutant or WT flies (Figure 6C, F-test). To further analyze the distribution of group members in these mixed-ratio

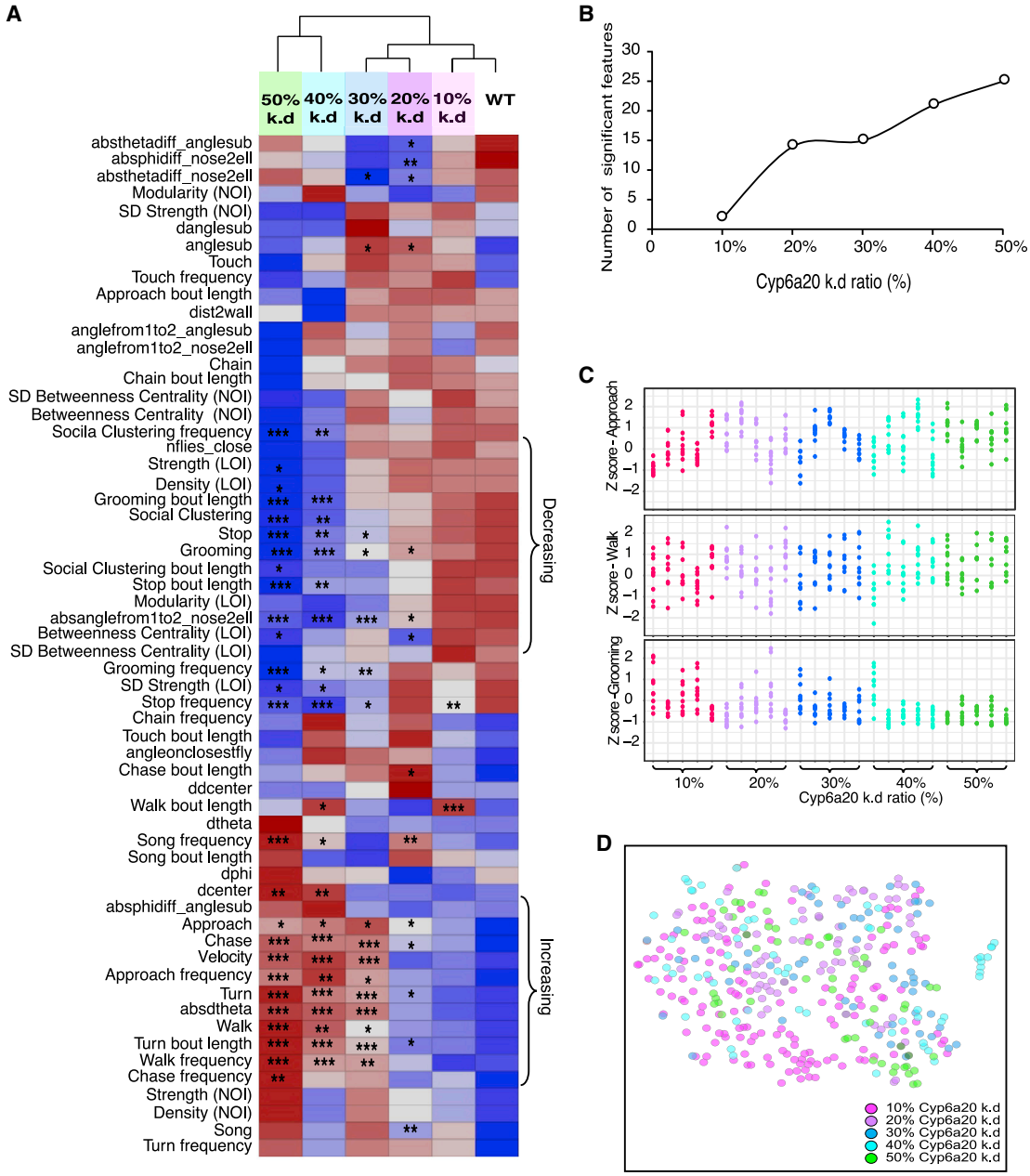

Figure 6. Subpopulations of Aggressive Flies in a Group Affect Different Features of Group Behavior

(A) Hierarchical clustering of behavioral signatures of groups composed of different ratios of socially isolated Cyp6a20-Gal4/+; UAS-Cyp6a20-RNAi/+ and socially raised WT flies (0%–50%). LOI: calculated according to the length of interactions. NOI: calculated according to the number of interactions. Data of each experimental group was normalized to a WT control group that was tested at the same time. To compare log-ratios of means (test/control), all values were log2-transformed and statistically tested as mean log-values. The effect of treatment and mutant number on the fraction of each parameter was tested with a linear regression and a two-way ANOVA was performed on the resulting model. Log-ratios between different number of mutants were compared in terms of differences defined by linear contrasts; FDR correction was applied to all comparisons. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ n = 14, 8, and 6 for groups of 10%, 20%–30%, and 40%–50%, respectively (see also Figure S6).

(B) Number of significantly different behavioral features compared to controls as a function of the ratio of isolated *Cyp6a20* k.d to raised WT flies in a group (10%–50%).

(C) Per-fly distribution of three normalized behavioral features (interaction, walk, grooming) in groups containing increasing ratios (0%–50%) of isolated *Cyp6a20* k.d to socially raised WT flies. Each column represents individuals as dots in one movie. Analysis of the distribution inside each group is not significantly different between conditions (F-test, n.s.).

(D) t-SNE analysis of all individuals in 10%–50% groups across all behavioral features.

See also Figure S6.

groups, we use t-SNE, a dimensionality reduction technique, to analyze all individuals across all features, which failed to depict any clear existence of subgroups (Figure 6D). This finding suggests that both WT and mutant flies change their behavioral responses when interacting in a group to generate a single behavioral signature, implying that group structure and dynamics reflect a level of complexity that cannot be explained as a simple average of the individuals that constitute it.

DISCUSSION

Understanding the principles underlying the complex nature of social group interaction is conceptually and computationally challenging. In this work, we simplified this complex phenomenon to a series of experiments in which we controlled the social experience and internal states of individuals within a group to illuminate patterns representing distinct structures and behavioral responses of groups under different social conditions. Each condition was represented by a group signature containing a collection of 60 distinct social network and behavioral features. This comprehensive analysis provided a broad examination of behavioral states, highlighting similarities and differences between groups, confirming our initial hypothesis that different social histories give rise to the formation of distinct and robust group signatures, that are indicative of specific social group structures. We showed that groups composed of socially raised male flies exhibit social clusters and high network modularity, indicating the existence of stable subgroups and ordered social structure that are not apparent in groups of isolated flies. Some of the observed differences between the groups of socially raised and socially isolated flies satisfied our initial predictions, such as the increased activity in isolated flies and increased social interaction, as well as the formation of social clusters in the socially raised group due to reduced aggression. On the other hand, the prediction that isolated flies will exhibit social avoidance was not supported. In fact, socially isolated flies displayed higher number of interactions, approaches, and network density.

Using hierarchical clustering to compare between group signatures allowed us to identify specific elements that are shared across conditions. For instance, clustering of socially raised flies tested in the dark with that of previously isolated flies highlights the contribution of visual cues to the expression of group signatures, whereas clustering analysis of flies in which cVA sensing neurons were inhibited suggests that cVA perception shapes group structure during the experience phase and during the test. Moreover, the analysis of group signatures revealed two aspects relevant to the connection between sensory information and behavior: (1) existence of behavioral features that are "primed" by social experience to become light dependent (i.e., social experience affects their light dependence) and (2) an emerging role for *Or65a* expressing neurons in regulating acute male-male interactions in addition to its well-established role in suppressing aggression upon long exposure to cVA[60] or possibly a cVA-independent role. Accordingly, hierarchical clustering indicated that inhibition of Or65a neurons affected many features in socially raised flies, some of which were also changed in isolated flies and are associated with increased activity in both cohorts. These common features are higher in isolated experimental flies when compared to their corresponding genetic controls, suggesting a role for Or65a neurons in reducing activity levels during the test.

Based on evidence suggesting that inter-individual recognition plays a role in male-male aggression encounters,[82] we expected recognition to also shape social interaction of flies. We found no evidence for a role of inter-individual recognition in the formation of groups composed from socially raised flies, suggesting that although recognition is valuable in the context of aggression over limited resources, the context used in our study is not sufficient to measure its importance. This finding is consistent with studies in social insects demonstrating that collective group behaviors do not require individual recognition.[5] Another example for the role of context to the expression of behavior is seen in the emergent differences in group behavior between groups of socially raised and isolated flies that are only evident in group context and not when the flies are tested alone. This fits well the conceptual model proposed by Anderson and Adolphs for the interplay between emotional behaviors and distinct internal states,[11] suggesting that group signatures integrate the expression of internal states, shaped by experience, with the specific context in which group behavior is measured.

The differences in variance between socially raised and isolated flies indicate that early life experiences can modulate behavioral variability within and between groups. Inter-individual variability is a broad phenomenon documented in many species[83–91] and was shown recently to be under neuromodulation in *C. elegans*, suggesting that behavioral variability is a biologically regulated process.[92] The functional importance of such variability can be seen in *Drosophila* studies demonstrating that increased behavioral variability can contribute to fitness.[93] Notably, our results also reveal increased variability between groups of socially raised flies, suggesting that social experience increases the repertoire of possible group phenotypes, the functional outcome of which remains to be studied.

Using network analysis as a tool to quantify social structures, we show that certain aspects of group structure are modulated by the social history of individuals that compose the group. Previous studies in *Drosophila* used social network analysis to dissect the principles that shape social interaction.[13,69] Interestingly, although the presence of visual cues affected several network features in our behavioral setup, Schneider et al.[69] reported no effects of the absence of light on network structure. This apparent discrepancy between our study and that of Schneider et al.[69] could result from different approaches when measuring network structure (binary versus weighted); while both studies documented shorter interactions in the absence of light, the effect on network structure is only evident when using weighted networks.

Studies of collective behaviors in various animals including honeybees, ants, birds, and fish exemplify synchronization as a key component of collective behavior.[1,5,94] Although *Drosophila* do not display such a degree of collective/coordinated behaviors as these organisms, they do exhibit behavioral responses that involve collective features, such as different responses to threat when in a group, changes in memory retrieval that depend on social experience, cooperation in feeding behavior, and even aggregation, suggesting the existence of a collective response that can increase survival or reproductive success.[4,55,71,95–101] Adding to this, our results demonstrate

the presence of social clusters, characterized with increased coordination between individuals, stable distances between individuals, long-lasting interactions, which are correlated with increased grooming, all of which are suggestive of a semi-collective state, in agreement with previous studies.[102,103] We show that the degree of this highly social state strongly depends on prior social experience, and its expression requires cVA perception and visual cues. The existence of such an ancient form of coordinated behavior may serve to explore the neuronal and genetic mechanisms underlying collective behaviors, as suggested by de Bono.[104] Yet the relevance of these findings to our ability to understand group behavior in natural settings remains to be explored.

Lastly, we demonstrate that group behavior and its corresponding structure depends on its composition. Hierarchical clustering of groups composed of different ratios of super-aggressive flies identified a cluster of features that is highly sensitive to changes in group composition. This cluster contains features associated with coordination between individuals and features associated with social clustering, implying that specific clusters of behavioral parameters within a behavioral signature may reflect changes in the ability of the group to form semi-collective structures.[1] Importantly, although the groups of mixed populations consist of two types of individuals that form distinct signatures when tested separately, their combination does not result in two distinct populations but rather a single close-to-normal distribution of all individuals within the group, as supported by Philippe et al.[105] This raises questions about the interactions and mechanism that facilitate the formation of unimodal distribution in groups composed of individuals with highly different internal properties.

The finding of state-dependent group signatures hints at the existence of distinct and consistent behavioral responses of groups to specific social conditions, which give rise to distinct group structures. These structures and their dependency on specific sensory information raise questions about the kinetics of their formation and the neuronal mechanisms that shape interactions that sustain such structures. These complex multi-sensory requirements also raise general questions about the ability of semi-natural social interactions such as technology-based social communication platforms to fully mimic the complex repertoire of experiences associated with face-to-face interaction, as a prerequisite for the full expression of social group interactions.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead Contact
  - Materials Availability
  - Data and Code Availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
- METHOD DETAILS
  - Behavioral setup
  - Tracking
  - FixTRAX
  - Kinetic analysis
  - Quantification of specific behaviors
  - Network analysis
  - Variance analysis
  - Standardization and normalization
  - Hierarchical clustering
  - Statistical analysis

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2020.10.060.

ACKNOWLEDGMENTS

We thank all members of the Shohat-Ophir and Ilany labs 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 and Karla Kaun (Brown University) for her productive suggestions. We specially thank Moshe Ben Shaanan for his mentorship and support in FixTRAX design. This work was supported by the Israel Science Foundation grant 384/14, Israel Science Foundation grant 174/19, and Israel Science Foundation grant 244/19.

AUTHOR CONTRIBUTIONS

Conceptualization, A.B., S.B.-S., A.I., and G.S.-O.; Methodology, A.B., S.B.-S., and E.C.; Investigation and Software, S.B.-S.; Writing, A.B., A.I., M.L., and G.S.-O.; Statistical Analysis, J.I.C.B., Funding Acquisition, G.S.-O. and A.I.; Supervision, G.S.-O. and A.I.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: March 16, 2020
Revised: August 20, 2020
Accepted: October 20, 2020
Published: November 12, 2020; corrected online: November 18, 2020

REFERENCES

1. Couzin, I.D. (2018). Synchronization: the key to effective communication in animal collectives. Trends Cogn. Sci. *22*, 844–846.
2. Dyer, J.R.G., Johansson, A., Helbing, D., Couzin, I.D., and Krause, J. (2009). Leadership, consensus decision making and collective behaviour in humans. Philos. Trans. R. Soc. Lond. B Biol. Sci. *364*, 781–789.
3. Falcón-Cortés, A., Boyer, D., and Ramos-Fernández, G. (2019). Collective learning from individual experiences and information transfer during group foraging. J. R. Soc. Interface *16*, 20180803.
4. Ramdya, P., Lichocki, P., Cruchet, S., Frisch, L., Tse, W., Floreano, D., and Benton, R. (2015). Mechanosensory interactions drive collective behaviour in Drosophila. Nature *519*, 233–236.
5. Feinerman, O., and Korman, A. (2017). Individual versus collective cognition in social insects. J. Exp. Biol. *220*, 73–82.
6. Forkosh, O., Karamihalev, S., Roeh, S., Alon, U., Anpilov, S., Touma, C., Nussbaumer, M., Flachskamm, C., Kaplick, P.M., Shemesh, Y., and Chen, A. (2019). Identity domains capture individual differences from across the behavioral repertoire. Nat. Neurosci. *22*, 2023–2028.
7. Aureli, F., and Schino, G. (2019). Social complexity from within: how individuals experience the structure and organization of their groups. Behav. Ecol. Sociobiol. *73*, https://doi.org/10.1007/s00265-018-2604-5.

8. Shemesh, Y., Sztainberg, Y., Forkosh, O., Shlapobersky, T., Chen, A., and Schneidman, E. (2013). High-order social interactions in groups of mice. eLife *2*, e00759.
9. Hobson, E.A., Ferdinand, V., Kolchinsky, A., and Garland, J. (2019). Rethinking animal social complexity measures with the help of complex systems concepts. Anim. Behav. *155*, 287–296.
10. Datta, S.R., Anderson, D.J., Branson, K., Perona, P., and Leifer, A. (2019). Computational neuroethology: a call to action. Neuron *104*, 11–24.
11. Anderson, D.J., and Adolphs, R. (2014). A framework for studying emotions across species. Cell *157*, 187–200.
12. Geiger, A.P., and Saltz, J.B. (2020). Strong and weak cross-sex correlations govern the quantitative-genetic architecture of social group choice in Drosophila melanogaster. Evolution *74*, 145–155.
13. Liu, G., Nath, T., Linneweber, G.A., Claeys, A., Guo, Z., Li, J., Bengochea, M., De Backer, S., Weyn, B., Sneyders, M., et al. (2018). A simple computer vision pipeline reveals the effects of isolation on social interaction dynamics in *Drosophila*. PLoS Comput. Biol. *14*, e1006410.
14. 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.
15. Wang, X., Fang, X., Yang, P., Jiang, X., Jiang, F., Zhao, D., Li, B., Cui, F., Wei, J., Ma, C., et al. (2014). The locust genome provides insight into swarm formation and long-distance flight. Nat. Commun. *5*, 2957.
16. Zernig, G., and Pinheiro, B.S. (2015). Dyadic social interaction inhibits cocaine-conditioned place preference and the associated activation of the accumbens corridor. Behav. Pharmacol. *26*, 580–594.
17. Agrawal, P., Chung, P., Heberlein, U., and Kent, C. (2019). Enabling cell-type-specific behavioral epigenetics in Drosophila: a modified high-yield INTACT method reveals the impact of social environment on the epigenetic landscape in dopaminergic neurons. BMC Biol. *17*, 30.
18. 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.
19. 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. *28*, 1445–1452.e3.
20. 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. USA *105*, 5657–5663.
21. Zelikowsky, M., Hui, M., Karigo, T., Choe, A., Yang, B., Blanco, M.R., Beadle, K., Gradinaru, V., Deverman, B.E., and Anderson, D.J. (2018). The neuropeptide Tac2 controls a distributed brain state induced by chronic social isolation stress. Cell *173*, 1265–1279.e19.
22. Pinna, G. (2019). Animal models of PTSD: the socially isolated mouse and the biomarker role of allopregnanolone. Front. Behav. Neurosci. *13*, 114.
23. de Bono, M., and Bargmann, C.I. (1998). Natural variation in a neuropeptide Y receptor homolog modifies social behavior and food response in C. elegans. Cell *94*, 679–689.
24. Coleing, A. (2009). The application of social network theory to animal behaviour. Biosci. Horiz. *2*, 32–43.
25. Robie, A.A., Seagraves, K.M., Egnor, S.E.R., and Branson, K. (2017). Machine vision methods for analyzing social interactions. J. Exp. Biol. *220*, 25–34.
26. Kwok, R. (2019). Deep learning powers a motion-tracking revolution. Nature *574*, 137–138.
27. Brewster, L.R., Dale, J.J., Guttridge, T.L., Gruber, S.H., Hansell, A.C., Elliott, M., Cowx, I.G., Whitney, N.M., and Gleiss, A.C. (2018). Development and application of a machine learning algorithm for classification of elasmobranch behaviour from accelerometry data. Mar. Biol. *165*, 62.
28. Valletta, J.J., Torney, C., Kings, M., Thornton, A., and Madden, J. (2017). Applications of machine learning in animal behaviour studies. Anim. Behav. *124*, 203–220.
29. Wang, G. (2019). Machine learning for inferring animal behavior from location and movement data. Ecol. Inform. *49*, 69–76.
30. Weinstein, B.G. (2018). A computer vision for animal ecology. J. Anim. Ecol. *87*, 533–545.
31. Anderson, D.J., and Perona, P. (2014). Toward a science of computational ethology. Neuron *84*, 18–31.
32. Farine, D.R., and Whitehead, H. (2015). Constructing, conducting and interpreting animal social network analysis. J. Anim. Ecol. *84*, 1144–1163.
33. Finn, K.R., Silk, M.J., Porter, M.A., and Pinter-Wollman, N. (2019). The use of multilayer network analysis in animal behaviour. Anim. Behav. *149*, 7–22.
34. Pasquaretta, C., Battesti, M., Klenschi, E., Bousquet, C.A.H., Sueur, C., and Mery, F. (2016). How social network structure affects decision-making in Drosophila melanogaster. Proc. Biol. Sci. *283*, 20152954.
35. Carter, G.G., Schino, G., and Farine, D. (2019). Challenges in assessing the roles of nepotism and reciprocity in cooperation networks. Anim. Behav. *150*, 255–271.
36. Balasubramaniam, K.N., Beisner, B.A., Hubbard, J.A., Vandeleest, J.J., Atwill, E.R., and McCowan, B. (2019). Affiliation and disease risk: social networks mediate gut microbial transmission among rhesus macaques. Anim. Behav. *151*, 131–143.
37. Webber, Q.M.R., and Vander Wal, E. (2019). Trends and perspectives on the use of animal social network analysis in behavioural ecology: a bibliometric approach. Anim. Behav. *149*, 77–87.
38. Sih, A., Spiegel, O., Godfrey, S., Leu, S., and Bull, C.M. (2018). Integrating social networks, animal personalities, movement ecology and parasites: a framework with examples from a lizard. Anim. Behav. *136*, 195–205.
39. Gilbertson, M.L.J., Fountain-Jones, N.M., and Craft, M.E. (2018). Incorporating genomic methods into contact networks to reveal new insights into animal behavior and infectious disease dynamics. Behaviour *155*, 759–791.
40. Kulahci, I.G., Ghazanfar, A.A., and Rubenstein, D.I. (2018). Consistent individual variation across interaction networks indicates social personalities in lemurs. Anim. Behav. *136*, 217–226.
41. Sah, P., Mann, J., and Bansal, S. (2018). Disease implications of animal social network structure: a synthesis across social systems. J. Anim. Ecol. *87*, 546–558.
42. Larson, S.M., Ruiz-Lambides, A., Platt, M.L., and Brent, L.J.N. (2018). Social network dynamics precede a mass eviction in group-living rhesus macaques. Anim. Behav. *136*, 185–193.
43. Lopes, P.C., Block, P., and König, B. (2016). Infection-induced behavioural changes reduce connectivity and the potential for disease spread in wild mice contact networks. Sci. Rep. *6*, 31790.
44. Kulahci, I.G., Rubenstein, D.I., and Ghazanfar, A.A. (2015). Lemurs groom-at-a-distance through vocal networks. Anim. Behav. *110*, 179–186.
45. Brent, L.J.N. (2015). Friends of friends: are indirect connections in social networks important to animal behaviour? Anim. Behav. *103*, 211–222.
46. Wey, T., Blumstein, D.T., Shen, W., and Jordán, F. (2008). Social network analysis of animal behaviour: a promising tool for the study of sociality. Anim. Behav. *75*, 333–344.
47. Sarkar, A., Harty, S., Johnson, K.V.A., Moeller, A.H., Archie, E.A., Schell, L.D., Carmody, R.N., Clutton-Brock, T.H., Dunbar, R.I.M., and Burnet, P.W.J. (2020). Microbial transmission in animal social networks and the social microbiome. Nat. Ecol. Evol. *4*, 1020–1035.
48. LeBoeuf, A.C., Benton, R., and Keller, L. (2013). The molecular basis of social behavior: models, methods and advances. Curr. Opin. Neurobiol. *23*, 3–10.
49. Asahina, K. (2018). Sex differences in *Drosophila* behavior: qualitative and quantitative dimorphism. Curr Opin Physiol *6*, 35–45.

50. Hoopfer, E.D. (2016). Neural control of aggression in *Drosophila*. Curr. Opin. Neurobiol. *38*, 109–118.

51. Aranha, M.M., and Vasconcelos, M.L. (2018). Deciphering Drosophila female innate behaviors. Curr. Opin. Neurobiol. *52*, 139–148.

52. Auer, T.O., and Benton, R. (2016). Sexual circuitry in *Drosophila*. Curr. Opin. Neurobiol. *38*, 18–26.

53. Dulac, C., and Dickson, B.J. (2016). Editorial overview: neurobiology of sex. Curr. Opin. Neurobiol. *38*, A1–A3.

54. Hoopfer, E.D., Jung, Y., Inagaki, H.K., Rubin, G.M., and Anderson, D.J. (2015). P1 interneurons promote a persistent internal state that enhances inter-male aggression in Drosophila. eLife *4*, e11346.

55. Lihoreau, M., Clarke, I.M., Buhl, J., Sumpter, D.J.T., and Simpson, S.J. (2016). Collective selection of food patches in Drosophila. J. Exp. Biol. *219*, 668–675.

56. von Philipsborn, A.C., Liu, T., Yu, J.Y., Masser, C., Bidaye, S.S., and Dickson, B.J. (2011). Neuronal control of Drosophila courtship song. Neuron *69*, 509–522.

57. Koganezawa, M., Kimura, K., and Yamamoto, D. (2016). The neural circuitry that functions as a switch for courtship versus aggression in Drosophila males. Curr. Biol. *26*, 1395–1403.

58. Cohn, R., Morantte, I., and Ruta, V. (2015). Coordinated and compartmentalized neuromodulation shapes sensory processing in Drosophila. Cell *163*, 1742–1755.

59. Ribeiro, I.M.A., Drews, M., Bahl, A., Machacek, C., Borst, A., and Dickson, B.J. (2018). Visual projection neurons mediating directed courtship in *Drosophila*. Cell *174*, 607–621.e18.

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

61. Andrews, J.C., Fernández, M.P., Yu, Q., Leary, G.P., Leung, A.K.W., Kavanaugh, M.P., Kravitz, E.A., and Certel, S.J. (2014). Octopamine neuromodulation regulates Gr32a-linked aggression and courtship pathways in Drosophila males. PLoS Genet. *10*, e1004356.

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

63. Zacarias, R., Namiki, S., Card, G.M., Vasconcelos, M.L., and Moita, M.A. (2018). Speed dependent descending control of freezing behavior in Drosophila melanogaster. Nat. Commun. *9*, 3697.

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

65. Stamps, J.A., and Blozis, S.A. (2006). Effects of natal experience on habitat selection when individuals make choices in groups: a multilevel analysis. Anim. Behav. *71*, 663–672.

66. Mohr, S.E., and Perrimon, N. (2019). *Drosophila melanogaster*: a simple system for understanding complexity. Dis. Model. Mech. *12*, dmm041871.

67. Schneider, J., Atallah, J., and Levine, J.D. (2017). Social structure and indirect genetic effects: genetics of social behaviour. Biol. Rev. Camb. Philos. Soc. *92*, 1027–1038.

68. Schneider, J., Murali, N., Taylor, G.W., and Levine, J.D. (2018). Can Drosophila melanogaster tell who's who? PLoS ONE *13*, e0205043.

69. 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. USA *109* (*Suppl 2*), 17174–17179.

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

71. Gibson, W.T., Gonzalez, C.R., Fernandez, C., Ramasamy, L., Tabachnik, T., Du, R.R., Felsen, P.D., Maire, M.R., Perona, P., and Anderson, D.J. (2015). Behavioral responses to a repetitive visual threat stimulus express a persistent state of defensive arousal in Drosophila. Curr. Biol. *25*, 1401–1415.

72. Arcego, D.M., Toniazzo, A.P., Krolow, R., Lampert, C., Berlitz, C., Dos Santos Garcia, E., do Couto Nicola, F., Hoppe, J.B., Gaelzer, M.M., Klein, C.P., et al. (2018). Impact of high-fat diet and early stress on depressive-like behavior and hippocampal plasticity in adult male rats. Mol. Neurobiol. *55*, 2740–2753.

73. Barrett, C.E., Arambula, S.E., and Young, L.J. (2015). The oxytocin system promotes resilience to the effects of neonatal isolation on adult social attachment in female prairie voles. Transl. Psychiatry *5*, e606.

74. Leser, N., and Wagner, S. (2015). The effects of acute social isolation on long-term social recognition memory. Neurobiol. Learn. Mem. *124*, 97–103.

75. Haller, J., Harold, G., Sandi, C., and Neumann, I.D. (2014). Effects of adverse early-life events on aggression and anti-social behaviours in animals and humans. J. Neuroendocrinol. *26*, 724–738.

76. Holekamp, K.E., and Strauss, E.D. (2016). Aggression and dominance: an interdisciplinary overview. Curr. Opin. Behav. Sci. *12*, 44–51.

77. Kabra, M., Robie, A.A., Rivera-Alba, M., Branson, S., and Branson, K. (2013). JAABA: interactive machine learning for automatic annotation of animal behavior. Nat. Methods *10*, 64–67.

78. Robie, A.A., Hirokawa, J., Edwards, A.W., Umayam, L.A., Lee, A., Phillips, M.L., Card, G.M., Korff, W., Rubin, G.M., Simpson, J.H., et al. (2017). Mapping the neural substrates of behavior. Cell *170*, 393–406.e28.

79. 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.

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

81. Kurtovic, A., Widmer, A., and Dickson, B.J. (2007). A single class of olfactory neurons mediates behavioural responses to a Drosophila sex pheromone. Nature *446*, 542–546.

82. Trannoy, S., and Kravitz, E.A. (2015). Learning and memory during aggression in *Drosophila*: handling affects aggression and the formation of a "loser" effect. J. Nat. Sci. *1*, e56.

83. Honegger, K., and de Bivort, B. (2018). Stochasticity, individuality and behavior. Curr. Biol. *28*, R8–R12.

84. Beever, E.A., Hall, L.E., Varner, J., Loosen, A.E., Dunham, J.B., Gahl, M.K., Smith, F.A., and Lawler, J.J. (2017). Behavioral flexibility as a mechanism for coping with climate change. Front. Ecol. Environ. *15*, 299–308.

85. Stamps, J.A., and Biro, P.A. (2016). Personality and individual differences in plasticity. Curr. Opin. Behav. Sci. *12*, 18–23.

86. Vogt, G., Huber, M., Thiemann, M., van den Boogaart, G., Schmitz, O.J., and Schubart, C.D. (2008). Production of different phenotypes from the same genotype in the same environment by developmental variation. J. Exp. Biol. *211*, 510–523.

87. Hadfield, M.G., and Strathmann, M.F. (1996). Variability, flexibility and plasticity in life histories of marine invertebrates. Oceanol. Acta *19*, 323–334.

88. Jeanson, R., and Weidenmüller, A. (2014). Interindividual variability in social insects - proximate causes and ultimate consequences. Biol. Rev. Camb. Philos. Soc. *89*, 671–687.

89. Körholz, J.C., Zocher, S., Grzyb, A.N., Morisse, B., Poetzsch, A., Ehret, F., Schmied, C., and Kempermann, G. (2018). Selective increases in inter-individual variability in response to environmental enrichment in female mice. eLife *7*, e35690.

90. Gärtner, K. (1990). A third component causing random variability beside environment and genotype. A reason for the limited success of a 30 year long effort to standardize laboratory animals? Lab. Anim. *24*, 71–77.

91. Tervo, D.G.R., Proskurin, M., Manakov, M., Kabra, M., Vollmer, A., Branson, K., and Karpova, A.Y. (2014). Behavioral variability through stochastic choice and its gating by anterior cingulate cortex. Cell *159*, 21–32.

92. Stern, S., Kirst, C., and Bargmann, C.I. (2017). Neuromodulatory control of long-term behavioral patterns and individuality across development. Cell *171*, 1649–1662.e10.

93. Kain, J.S., Zhang, S., Akhund-Zade, J., Samuel, A.D.T., Klein, M., and de Bivort, B.L. (2015). Variability in thermal and phototactic preferences in Drosophila may reflect an adaptive bet-hedging strategy. Evolution *69*, 3171–3185.

94. Copenhagen, K., Quint, D.A., and Gopinathan, A. (2016). Self-organized sorting limits behavioral variability in swarms. Sci. Rep. *6*, 31808.

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

96. Sehdev, A., Mohammed, Y.G., Tafrali, C., and Szyszka, P. (2019). Social foraging extends associative odor-food memory expression in an automated learning assay for *Drosophila melanogaster*. J. Exp. Biol. *222*, jeb207241.

97. Ilany, A., Barocas, A., Koren, L., Kam, M., and Geffen, E. (2013). Structural balance in the social networks of a wild mammal. Anim. Behav. *85*, 1397–1405.

98. Ilany, A., Booms, A.S., and Holekamp, K.E. (2015). Topological effects of network structure on long-term social network dynamics in a wild mammal. Ecol. Lett. *18*, 687–695.

99. Barocas, A., Ilany, A., Koren, L., Kam, M., and Geffen, E. (2011). Variance in centrality within rock hyrax social networks predicts adult longevity. PLoS ONE *6*, e22375.

100. Chabaud, M.A., Isabel, G., Kaiser, L., and Preat, T. (2009). Social facilitation of long-lasting memory retrieval in Drosophila. Curr. Biol. *19*, 1654–1659.

101. Dombrovski, M., Poussard, L., Moalem, K., Kmecova, L., Hogan, N., Schott, E., Vaccari, A., Acton, S., and Condron, B. (2017). Cooperative behavior emerges among Drosophila larvae. Curr. Biol. *27*, 2821–2826.e2.

102. Burg, E.D., Langan, S.T., and Nash, H.A. (2013). Drosophila social clustering is disrupted by anesthetics and in narrow abdomen ion channel mutants. Genes Brain Behav. *12*, 338–347.

103. Jiang, L., Cheng, Y., Gao, S., Zhong, Y., Ma, C., Wang, T., and Zhu, Y. (2020). Emergence of social cluster by collective pairwise encounters in *Drosophila*. eLife *9*, e51921.

104. de Bono, M. (2003). Molecular approaches to aggregation behavior and social attachment. J. Neurobiol. *54*, 78–92.

105. Philippe, A.-S., Jeanson, R., Pasquaretta, C., Rebaudo, F., Sueur, C., and Mery, F. (2016). Genetic variation in aggregation behaviour and interacting phenotypes in Drosophila. Proc. Biol. Sci. *283*, 20152967.

106. 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. J. Vis. Exp. (118)

107. Csardi, G., and Nepusz, T. (2006). The igraph software package for complex network research. InterJournal 1695. http://www.interjournal.org/manuscript_abstract.php?361100992.

108. Pons, P., and Latapy, M. (2005). Computing communities in large networks using random walks. In Lecture Notes in Computer Science, *Volume 3733*, P. Yolum, T. Güngör, F. Gürgen, and C. Özturan, eds. (Springer), pp. 284–293.

109. van der Maaten, L., and Hinton, G. (2008). Visualizing data using t-SNE. J. Mach. Learn. Res. *9*, 2579–2605.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Yeast extract	Merck	Cat# JD6JF66093
Ethanol	Sigma-Aldrich	Cat# SZBE1610V
Sucrose, Molecular biology grade	Calbiochem	Cat# 573113
Experimental Models: Organisms/Strains		
Or65a-Gal4	HHMI Janelia Research Campus	N/A
Or67d-Gal4	HHMI Janelia Research Campus	N/A
UAS-Kir2.1	HHMI Janelia Research Campus	N/A
Cyp6a20-Gal4	Heberlein GAL-4 collection	N/A
UAS-Cyp6a20-RNAi	VDRC	VDRC ID 3313
Canton S	HHMI Janelia Research Campus	N/A
Software and Algorithms		
Leica Application Suite	Leica Microsystems	https://www.leica-microsystems.com/products/microscope-software/details/product/leica-las-x-ls/
Microsoft Excel	Microsoft	https://www.microsoft.com/en-us/download/details.aspx?id=10
Graphpad Prism (v 7.02)	Graphpad Software	https://www.graphpad.com/
JAABA	Branson lab HHMI Janelia Research Campus	https://github.com/kristinbranson/JAABA
CTRAX	Branson lab HHMI Janelia Research Campus	http://ctrax.sourceforge.net/
Fly Bowl Data Capture (FBDC)	Branson lab HHMI Janelia Research Campus	N/A
MATLAB	MathWorks	https://www.mathworks.com/products/matlab.html
Partek	Partek Inc.	https://www.partek.com/partek-genomics-suite/
R	R project	https://www.r-project.org/
Other		
Point Grey camera FL3	Point Grey Research Inc.	N/A

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Galit Shohat-Ophir (galit.ophir@biu.ac.il).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

The code generated during this study are available at as a supplementary zip file (Run FixTrax files).

The raw data supporting the current study have not been deposited in a public repository due to their size, but are available from the corresponding author on request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

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. All transgenic fly lines were backcrossed at least 5 generations into a white Canton S background. Or67d-GAL4, Or65a-GAL4 and UAS-Kir2.1 fly lines were obtained from HHMI Janelia Research Campus. Cyp6a20-GAL4 was obtained from the Heberlein GAL-4 collection and Cyp6a20-RNAi was obtained from VDRC.

METHOD DETAILS

Behavioral setup

Socially raised versus Isolated: flies were lightly anesthetized with CO2 and collected shortly after hatching. Flies were then inserted into food vials, either alone (isolated) or as a group of 10 (raised) for 3 days, in a light/dark cycle of 12/12. The isolated flies were inserted into a food vial in a group of 10 and then loaded into the test arenas, same as experienced flies. All flies experienced similar habituation to the arena of about 1 min.

Light versus dark: flies were collected as before and housed in groups of 10 or in isolation as before. During the behavioral test, light was off (dark) or on (light).

Ethanol exposure: flies were housed in groups of 10 for 3 days as described above. Flies were then exposed to either ethanol (test) or water (control), for 4 consecutive days as described previously by Zer et al.[106] Flies were then inserted into Fly Bowl arenas for video recording, as described above.

Circadian time shift: flies were housed in groups of 10 for 3 days as described above, or with a two h time shift (late wake). Flies were then inserted into FlyBowl arenas as housed or as a mixed group of 5 flies from each condition (mixed).

Starvation: flies were collected in groups of 10 as described above. 24 h before the behavioral test, flies were either moved into vials containing agar (starved) or kept in vials with food (controls). Flies were then inserted into Fly Bowl arenas for video recording, as described above.

Ratios of sub populations within a group: WT flies were housed in groups of 10 as described above. Cyp6a20-Gal-4/+; UAS-Cyp6a20-RNAi/+ flies were collected and housed in isolation, as described above for WT isolated flies. Flies were then inserted into FlyBowl arenas in groups of 10, composed of varying amounts of knock-down flies (1 to 5) and WT flies (9 to 5) for video recording. Video recording was performed as described above.

QUANTIFICATION AND STATISTICAL ANALYSIS

Tracking

Flies where inserted in groups of 10 into Fly Bowl arenas,[79] and 15 min of video was acquired with Fly Bowl Data Capture (FBDC)[78] and analyzed using CTRAX80 to obtain flies' orientation, position, and trajectories.

FixTRAX

We programmed this additional software in MATLAB in order to fix CTRAX tracking errors. FixTRAX uses a set of assumptions to fix CTRAX output based on 4 types of errors we observed in our CTRAX output data, which mostly happen when flies are relatively immobile for long time periods and require correction prior to further analysis. The errors are: (a) unifying two or more identities when flies are close, (b) mistakenly identifying a dark spot as a fly, (c) not recognizing a fly for several frames and (d) not maintaining the same identities over the entire movie. FixTRAX uses two fix algorithms; a main algorithm and a subsidiary control algorithm (Supp FixTRAX code and user instructions). The main algorithm is based on finding a sequence of incorrect frames that represent one mistake, then creating a table from that sequence with statistical scores for every pair of identities: one that disappeared and another that appeared. This score represents the probability that the two identities represent the same fly. Based on their score, the algorithm decides which identities to unify and which identities are false and can be deleted. After unifying two identities, data for missing frames is computed according to the fly's approximate location, calculated as the shortest path between start and end positions of that specific error. The subsidiary algorithm unifies each identity that disappeared with the first identity that appeared. Both algorithms stop when all identities are unified, and the number of identities matches the number of flies the user stated are in the video. FixTRAX selects the fix algorithm that was able to maintain the identities of all flies in the movie with minimal insertions or deletions of identities to the original tracking file. Finally, FixTRAX plots a graph of the number of identities that were added and deleted for per frame, which can help the user adjust CTRAX's tracking parameters and the fix algorithm parameters to minimize tracking errors. Experiments which were not tracked correctly were discarded. Finally, FixTRAX output is converted into JAABA compatible output using the algorithm specified in Kabra et al.[77] to generate general statistical features as in Branson et al.[79] (Figure 3A). FixTRAX error rate is presented in FixTRAX error rate supplementary file.

Kinetic analysis

Scripts were written in MATLAB to use the JAABA code to generate the statistical features as specified in Kabra et al.[77] Time series graphs (per frame) were created using JAABA Plot.[77]

Quantification of specific behaviors

JAABA Classifiers[77] were trained on various movies to identify specific behaviors: Walk, Stop, Turn, Approach, Touch, Chase, Chain, Song, Social Clustering and Grooming. Bar graphs were created using JAABA Plot.[77]

Network analysis

An Interaction matrix was created in MATLAB (using the interaction parameters stated below) and saved as a text file. 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 $\leq$ 8 mm, number of frames for interaction $\geq$60 and number of gap frames $\geq$ 120. Interaction end is defined when distance or angle conditions are not maintained for 4 s.

Networks and their features were generated from the interaction matrix in R using the igraph package.[107] 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.[108] 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. Boxplots were created using R.

Variance analysis

Standard deviation (SD) of all flies was calculated as standard deviation of all per-fly data (all experimental repetitions together) for each feature per condition. SD between groups was calculated as standard deviation of all per-movie (experimental repetitions) averages for each feature per condition. SD within groups was calculated as the average of all per-movie standard deviations (variance within each experimental repetition) for each feature in each condition.

Standardization and normalization

For all experiments except those of ratios of sub populations (Figure 6), each feature was standardized according to all values calculated in our experiments for that feature to generate a z-score, as was done by Schneider et al.[69] Scatterplots were created using R.

Sub populations experiment (Figure 6): Each feature in every experimental group was first normalized to a control condition of 10 WT flies. Features were then standardized according to all normalized values of all other experimental groups to generate z-scores.

Hierarchical clustering

Hierarchical clustering and heatmaps were created using Partek® software (Copyright, Partek Inc. Partek and all other Partek Inc. product or service names are registered trademarks or trademarks of Partek Inc., St. Louis, MO, USA). Each condition (heatmaps y axis) represents average standardized values of all repetitions.

Statistical analysis

For each experiment except experiments with Cyp6a20 RNAi flies, Shapiro–Wilk test was done on each experiment to test for normal distribution. For experiments with two-conditions: 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.

Variance: F-test of the equality of two variances was used for all-flies analysis and between-group analysis. Students t test was used for averages of within groups analysis. FDR correction for multiple testing was performed for all analyses. Ratios of sub populations normalized to controls: To compare log-ratios of means (test/control), all values were log2-transformed and differences between mean log-values were tested. Specifically, the effect of treatment and mutant number on the fraction of each parameter was tested with a linear regression and a 2-way ANOVA was performed on the resulting model. Log-ratios between different number of mutants were compared in terms of difference of differences defined by linear contrasts and FDR correction was applied to all comparisons.

t-Stochastic Neighbor Embedding (t-SNE) analysis and visualization was done using an implementation of the Barnes-Hut algorithm (https://github.com/karpathy/tsnejs and van der Maaten and Hinton[109]). We performed several types of analysis with varying Perplexity levels, learning rate and max iterations, and chose to present the results using perplexity of 30, learning rate of 10 and max iterations of 1000.