**Social interaction and network structure in groups of *Drosophila* are shaped by prior social experience and group composition**

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# Abstract

Living in a group creates a complex and dynamic environment in which the behavior of the individual is influenced by and affects the behavior of others. Although social interactions and group living are fundamental adaptations exhibited by many organisms, relatively little is known about how prior social experience, internal states, and group composition shape behavior in a group, or the neuronal and molecular mechanisms that mediate it. Here we present a practical and conceptual framework for studying the interplay between social experience and group interaction in *Drosophila melanogaster* and show that the structure of social networks and group interactions is sensitive to group composition and individuals’ social history. We simplified the complexity of interaction in a group using a series of experiments in which we controlled the social experience and motivational states of individuals to illuminate patterns that represent distinct structures and behavioral responses of groups under different social conditions. Using high-resolution data capture, machine learning, and graph theory, we analyzed 60 distinct behavioral and social network features, generating a comprehensive representation (“group signature”) for each condition. We show that social enrichment promotes the formation of a distinct group structure that is characterized by high network modularity, high inter-individual and inter-group variance, high inter-individual synchrony, and stable social clusters. Using environmental and genetic manipulations, we show that this structure is dependent on specific sensory information during both experience and test. Finally, we explored social interactions in heterogenous groups containing different ratios of sub-populations and identified clusters of features that are sensitive to increasing ratios of aggressive flies, some of which reveal that inter-individual synchronization depends on group composition. 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, and that group signatures are a sensitive tool for exploring the neuronal and molecular mechanisms that shape social interaction in a group.

# 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 behavior1–5, and can be described by measuring the behavior of individuals, the interaction between two or more individuals, and the resulting social network, defined here as “group behavior”.

When individuals interact in a group, their internal motivational state, previous memories and other physiological processes affect their action selection, giving rise to diverse activity levels, behavioral responses, and engagement with others6. This results in a highly complex and continuously changing environment that is susceptible to various physical and biological factors, 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 conditions7. The complex nature of this environment imposes conceptual challenges in the quantification and analysis of group behavior.

A fundamental question in this respect is how internal and external factors, such as previous social experience, internal motivational state, specific group composition or the existence of available resources, shape group behavior8. Although much is known about how prior social experience affects internal motivational states9–15 and subsequently modulates social interaction in pairs of animals10,16–21, relatively little is known about how these elements shape social group behavior, mainly due to the technical challenges of high-resolution data collection and analysis. Therefore, 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)22. Both lines of study progressed substantially with advances in machine vision and learning technologies that allow automated tracking and unbiased behavioral analysis7,23–28. 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 and thus provide insights into the formation, dynamics, and function of group structure22,29,30. This type of analysis can be employed to investigate transmission processes in groups as a basis for understanding complex phenomena such as disease spreading, social grooming, decision making, and hierarchy3,29,31–42. Although these two approaches highlight different aspects of social interaction, they could be considered complementary for understanding 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 encounters43. Numerous studies have provided a mechanistic understanding of these complex behaviors, demonstrating that their expression requires multi-sensory inputs as well as specific internal neuronal processes44–46. Modulation of behavior by previous social experience was also extensively investigated in flies, revealing connections between expression of certain genes and neuronal processes that led to long-lasting behavioral changes47. While *Drosophila* proves to be a useful model organism for mechanistically exploring complex behaviors48,49, only a small number of studies have examined social group interaction in flies. Flies possess the neuronal ability to recognize different individuals in a group50, groups of flies exhibit non-random group structures that depend on certain sensory systems4,51,52, and group interaction facilitates collective responses to threat53. These and other findings opened the path to understanding the principles and mechanisms that shape social group interaction in *Drosophila* and its potential contribution to fitness49.

In the present study, we established an experimental framework for assaying and analyzing the interplay between social history, sensory information, internal motivational states and group behavior, by computationally reducing social interactions into behavioral “group signatures” composed of hierarchically clustered behavioral and network parameters. We demonstrate that distinct types of social experience result in various levels of structure/order within groups, corresponding to distinct social network structures and specific group signatures. Additionally, we show that group signatures are strongly influenced by both visual cues and the male-specific pheromone 11-cis-vaccenyl acetate (cVA). Finally, we explore the formation of group behavior in heterogenous groups composed of flies with opposing internal states, revealing that specific clusters of behavioral and network features are sensitive, in a dynamic fashion, to ratios of opposing subgroups.

# Results

## Establishing a data capture and analysis pipeline for studying complex behavior in groups

To explore the interplay between social history, internal motivational 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)54. 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 (Fly Bowl Data Capture [FBDC], Ctrax, JAABA, and JAABA plot, Fig. 1A)12,55. Although Ctrax is used in many behavioral setups, its output includes some tracking errors that impede the reliable analysis of certain measurements—those requiring identities to be maintained throughout the experiment, such as social network analysis. To resolve this, we developed a secondary processing algorithm for Ctrax output data, which we named FixTRAX. Briefly, FixTRAX uses a set of rules to find tracking errors and calculates statistical scores, determining which identities to correct per frame (detailed explanation in Methods and Fig. S1). Corrected output data are used to calculate kinetic features, classify eight distinct complex behaviors (Fig. 1A; full description in Supplementary Table S1) using the supervised machine learning algorithm JAABA54, and calculate six network features (Box1).

To analyze the social networks of interacting flies in a group, we first determined the physical criteria that define an interaction between two individuals using two constraints: (1) distance between two flies is less than or equal to 8 mm (average of two body lengths) and (2) angle subtended by each fly is larger than zero (Fig. 1B). Additionally, we required the distance and angle criteria be maintained for at least 2 s to minimize the number of false positives (random interactions) (Fig. 1C). Using these parameters, we identified a large number of very short interactions, some of which could belong to 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 (Fig. 1C). To resolve this, we added the requirement of a minimal gap, which defines a time interval 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) (Fig. S2), which substantially reduced the number of very short interactions (Fig. 1D).

We used weighted networks to account for the between-dyad variation in total interaction times over each test. To test whether directed networks are required, we quantified the number of directed interactions between pairs of flies. This revealed a strong correlation, meaning directions of interactions are symmetric over the course of the test, making directed analysis redundant (Fig. 1E). We therefore decided to use undirected networks in this study. The complex output of all features was combined to generate a comprehensive group behavioral signature per condition, represented by normalized Z-score scatter plots and hierarchically clustered heat maps that highlight similarities and differences across experimental groups (Fig. 1A).

## Prior social interaction in a group facilitates the formation of ordered social structures

To examine how the social experience of individuals shapes social group dynamics, we generated two cohorts of wild-type (WT) Canton S male flies; one cohort of flies experienced 3 days of social interaction with nine other flies (groups of 10 male flies), while the other cohort experienced complete social isolation. After 3 days, 10 flies from each cohort were introduced into Fly Bowl arenas and their behavior was recorded for 15 minutes and analyzed (Fig. 1A). The two cohorts exhibited distinct repertoires of behavioral responses upon interaction with other flies in a group; socially experienced flies displayed lower average activity levels, manifested by lower average velocity (Fig. 2A), shorter time spent walking (Fig. 2B) and fewer body turns than isolated male flies (Fig. 2C). Analysis of specific social behaviors revealed that socially experienced flies exhibited less touch behavior (Fig. 2D), were less engaged in active approach (Fig. 2E) and spent less time chasing (Fig. 2F). Remarkably, socially experienced male flies tended to concentrate in certain zones within the arena, forming semi-stable social clusters consisting of three or more flies (Fig. 2G, Fig. S3M, Movie S1). This behavior was not apparent in male flies that experienced social isolation prior to testing (Fig. 2G). Socially experienced flies also spent more time grooming than isolated flies (Fig. 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 (Fig. 3A, Fig. S3A–H). Interestingly, average bout duration of approach behavior was similar between the two cohorts, while its frequency was higher in isolated flies (Fig. 3A and Fig. S3A, E), suggesting the difference in their social experience did not affect the duration of social encounters, but rather the frequency at which they occur.

To investigate the effect of social clustering on group structure, we explored the structure and features of the social networks in two ways: by calculating network weights according to the overall duration of interactions (emphasizing long-lasting interactions) or the overall number of interactions (emphasizing short and acute interactions) between each pair of flies. Analysis by duration revealed that socially experienced flies displayed higher modularity (Fig. 2J), SD strength (Fig. 2K) and betweenness centrality (Fig. S3L), 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 (Fig. 2I–K vs. L–N), revealed that the social networks of isolated flies are characterized by higher density (Fig. 2L), SD strength (Fig. 2L, N) and strength (Fig. 3A), while networks of socially experienced flies have higher modularity (Fig. 2M) and betweenness centrality (Fig. 3A). Together, these differences indicate that networks formed by socially experienced flies possess a higher-order structure compared to those formed by isolated flies. Overall, these results indicate that the behavioral group signature of socially experienced flies differs dramatically from that of previously isolated flies that experience social interaction for the first time during the test (Fig. 3A).

## Behavioral signature of socially experienced flies does not require individual recognition

It is plausible that the observed differences between socially experienced and isolated cohorts are simply a result of the familiarity of experienced flies with the individuals they are tested with. Therefore, we asked whether the distinct features exhibited by socially experienced males result from their familiarity with individual members that occurred during housing, or stem from the internal motivational state associated with the general experience of living in a group. To differentiate between these two possibilities, we tested socially experienced flies with familiar and unfamiliar individuals. One cohort was tested with the same flies they were housed with (familiar), while the other cohort was tested with socially experienced flies from other groups (unfamiliar). Encountering familiar or unfamiliar flies did not result in different behavioral signatures (Fig. 3B, 4D), suggesting that the dynamics captured during the test resulted from the general experience of interacting with others in a group rather than by previous interactions with specific individuals. To explore this further, we asked whether group signatures are only affected by changes in internal motivational states that are social in nature or if they can also be affected by other conditions known to modulate internal motivational states. To test this, we assayed conditions known to affect internal motivational state but that are not social in nature: repeated ethanol exposure, starvation, and different circadian time shifts. We did not observe any significant difference between these conditions and their controls (Fig. S4), implying that not all experiences that modulate internal motivational state affect group dynamics in the context used in our experimental paradigm.

## Prior social interaction increases behavioral variability

The existence of a specific structure in groups of socially experienced flies suggests that behavioral effects in these groups might manifest in additional ways that are not evident when reducing analysis to behavioral means alone. Indeed, when analyzing the behavioral signatures of socially experienced and isolated male flies, we observed that socially experienced flies exhibited higher variance across several different behavioral features (Fig. 2, 3A; compare error bars). To verify this observation, we compared the variance of all behavioral features between groups of socially experienced and isolated male flies. We analyzed the variance of each behavioral feature in three ways: (a) average standard deviation (SD) of each group/repetition per condition, reflecting variation inside each group (SD within groups, Fig. 3C); (b) SD of the averages of all experimental groups per condition, reflecting variation between groups (SD between groups, Fig. 3C); and (c) SD across all flies per condition, reflecting individual differences between all flies regardless of groups (SD all flies, Fig. 3C). We documented a higher number of behavioral features that displayed significantly higher variance (SD two-fold higher in one condition + statistically significant) in socially experienced flies when comparing variance between different groups (18 out of 56 parameters; Fig. 3D), within groups (11 out of 56 parameters; Fig. 3D) as well as between all individuals in the same group (21 out of 56 parameters; Fig. 3D). This indicates that the behavior of socially experienced flies is more diverse than that of isolated flies, possibly reflecting a broader repertoire of behaviors in individuals shaped by prior interactions during the experience phase. Increased variability between groups of socially experienced 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 Fig. 3B). We documented very few parameters that were distributed differently between flies tested with familiar or unfamiliar flies, suggesting 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 (Fig. 3E).

## Visual cues are necessary for expressing the behavioral signature of socially experienced flies

So far, we have shown that different social conditions associated with distinct internal motivational states affect the structure and dynamics of group interactions to create specific behavioral signatures. Next, we dissected the elements required for the formation of these state-dependent differences during group behavior. We started by assessing the role of visual cues in forming specific behavioral signatures during the test by analyzing the behavior of socially experienced male flies in light and dark conditions. Socially experienced flies that were tested in the dark displayed more walk, turn and touch behaviors than those tested in the light (Fig. 4A), and spent a larger fraction of time in chase and approach behaviors, while showing less social clustering and grooming behaviors (Fig. 4A). Moreover, approach behavior in the dark was significantly longer and more frequent than that in the light (Fig. 4A), while 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 similar (Fig. 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 (Fig. S5). Network analysis revealed lower SD strength and betweenness centrality in groups tested in the dark, by analysis of duration of interactions (Fig. 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 (Fig. 4B). We postulate that light is required for the group signature of socially experienced male flies.

We next aimed to uncouple the behavioral changes observed during light deprivation: those that result from the general role of visual cues in group behavior, and those that are only modulated by light after exposure to a specific social experience. For that, we searched for features modulated by the presence or absence of light in groups of socially experienced and socially isolated flies (Fig. 4A, B). Behavioral features that are equally affected by the presence of light in both groups represent features that are light-dependent but not sensitive to social experience, while features that are only modulated in one group are those that social experience turns light-dependent. To visualize this, we plotted distinct features that are influenced by visual cues in each condition (Fig. 4B, C). We identified 22 unique features that are sensitive to visual cues only in socially experienced flies, and only seven in isolated flies, suggesting that the group signature of socially experienced flies is highly dependent on visual cues (Fig. 4C). Most features unique to the socially experienced group are part of two main groups: features associated with social clustering (which reduce in the absence of light), and features associated with interaction (which increase in the absence of light). The opposite regulation of these two types of features suggests that, in the absence of light, socially experienced 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 (absanglefrom1to2, absphidiff, absthetadiff and angleonclosestfly; see Table S1 for more details) in the absence of light (Fig. 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 (Fig. 4B, C).

To assess whether the group signatures of these conditions reveal an underlying similarity, we performed hierarchical clustering on the group signatures of all tested conditions (Fig. 4D). This analysis revealed a clear division based on social history, such that behavioral parameters in conditions in which flies were isolated prior to test were clustered together, whereas those of socially experienced flies were also clustered together. Interestingly, socially experienced flies that were tested in the dark did not cluster with groups of either previously isolated or socially experienced flies tested in the light, reinforcing the notion that visual cues are specifically necessary for the expression of group signatures associated with previous social experience, but are not sufficient to change group signature from experienced to isolated.

## cVA perception via Or65a neurons shapes social group interaction

In addition to visual cues, another central element in social interaction is pheromone-based communication. The male-specific pheromone cVA is known to mediate experience-dependent changes in aggressive behavior, where chronic exposure to cVA found on conspecifics during group housing is known to reduce male–male aggression56,57. cVA is perceived via two olfactory receptor neurons (ORNs): Or67d, which mediates acute responses to cVA, and Or65a, which mediates chronic responses to cVA57,58. We investigated whether cVA perception impacts the group signature of socially experienced flies. For that, we blocked cVA perception by constitutively expressing the inwardly rectifying potassium channel Kir2.1 in Or65a- and Or67d-expressing neurons of socially experienced flies and analyzed their group behavior. Inhibition of Or67d neurons did not lead to significant differences between experimental flies and genetic controls, suggesting that functional Or67d neurons are not necessary for the formation of the behavioral signature associated with social group experience (Fig. 5A). In contrast, Or65a neuron inhibition dramatically changed the group signature of socially experienced flies, increasing average velocity and overall time engaged in approach, chase and touch behaviors compared to genetic controls (Fig. 5B). Analysis of network structure revealed greater strength in the experimental group and lower betweenness centrality than genetic controls, by both duration and number of interactions (Fig. 5B). Overall, this suggests that Or65a- but not Or67d-expressing neurons function in shaping group behavior of socially experienced 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. 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 courses57. Experimental flies exhibited more touch, approach, chase and chain behaviors than controls, and greater network strength as measured by duration of interaction. However, these effects were less extreme than those displayed by socially experienced male flies (Fig. 6B vs. 6C). This unexpected result suggests that Or65a neurons mediate acute as well as chronic responses to cVA.

Interestingly, some effects of inhibiting Or65a neurons are shown in both socially isolated and socially experienced flies, including decreasing three coordination-related parameters (Fig. S6A–C) and a significant increase in chain, chase, chase bout length, touch and approach behaviors (Fig. S6D–H). Moreover, both experimental groups displayed higher network strength (measured by duration of interaction, Fig. S6I), suggesting that inhibition of Or65a neuronal activity leads to disinhibition of behaviors associated with social isolation. Overall, although the two types of experimental flies shared these similarities, inhibition of Or65a is more profound in socially experienced flies than in socially isolated flies, reflected by a higher number of behavioral features affected (35 vs. 22 out of 60, Fig. 5B, C). Hierarchical clustering analysis between conditions revealed that flies in which Or67d neurons were inhibited are similar to their corresponding genetic controls, reinforcing the conclusion that Or67d neurons do not mediate behavioral responses of socially experienced male flies (Fig. 5D). In contrast, socially experienced 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 both the formation of the internal motivational state associated with group housing and its expression as a specific group signature (Fig. 5D).

## Sub-populations of flies in a group reveal specific social rules

So far,we have used homogenous groups of flies subjected to environmental or genetic manipulation as a tool to investigate the interplay between social experience, internal motivational state and the resulting group behavior. Although this approach eliminates the inherent contribution of inter-individual differences to group structure, it proved valuable in dissecting the elements that shape social group behavior. To investigate how inter-individual differences regulate group structure and signatures, we generated groups that contain varying ratios of male flies with two distinct motivational states: socially experienced flies and hyper-aggressive isolated flies. Hyper-aggressive male flies were generated by knocking down *Cyp6a20* (a manipulation known to induce aggression)18, and keeping these flies isolated upon eclosion. We introduced increasing numbers of hyper-aggressive flies into groups of socially experienced WT male flies (10%–50% of the total number of individuals) and measured their group behavior. We postulated that highly aggressive flies would disrupt collective-like group behaviors such as social clustering and thus change the behavioral signature of the group.

The behavior of each experimental group was normalized to a control group of 100% socially experienced WT flies tested at the same time, enabling statistical comparison of all behavioral features between all experimental groups. To gain a general overview of the patterns associated with gradual changes in group composition, we examined the normalized behavioral signatures using hierarchal clustering (Fig. 6A). Overall, the conditions are clustered into two main branches: one containing the homogenous 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 homogenous to 50% mixed ratio groups. The differences between these two extremes resemble those of socially experienced vs. socially isolated flies, suggesting that the addition of 50% aggressive flies is sufficient to convert group behavior into a social isolation-like state (Fig. 3D vs. Fig. 6A). Overall, clustering of features suggests a somewhat gradual transition from 0 to 50%. This apparent trend is best demonstrated by the increase in the number of features that exhibit a significant difference compared to 100% WT flies (Fig. 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 (Fig. 6A). Some decreasing features corresponded to social clustering and network structure, while increasing features were related to activity and interaction (Fig. 6A). Some of these features exhibited a gradual change as the number of *Cyp6a20*-KD flies themselves increased. These included a gradual decrease in social clustering, grooming, stop, and stop bout length behaviors (Fig. S7A–D), and a gradual increase in walk, absdtheta, turn, and turn bout length behaviors (Fig. S7E–H). 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 synchrony between two individuals (absphidiff\_nose2ell, absphidiff\_anglesub and absthetadiff\_nose2ell). Some behavioral features are more sensitive than others to changes in group composition, such as grooming, approach and turn behaviors, which are significantly different from control even at 20% mixed ratio, while other features such as social clustering exhibit a significant change only at 40% or higher. This suggests that changes in the level of approach behavior within a group precede changes in more collective-like behaviors such as social clustering (Fig. 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 distinguish between these two hypotheses, 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. Single-fly analysis of features that exhibit changes with an increased number of mutant flies, such as walk, approach and social clustering, showed a normal distribution, making it impossible to identify subgroups that correspond to mutant or WT flies (Fig. 6C). This finding confirms the notion that both WT and mutant flies change their behavioral responses when interacting in a group to generate one behavioral signature, suggesting that group structure and dynamics reflect a layer 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 motivational 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 and revealing that different social histories give rise to the formation of distinct and robust group signatures indicative of discrete social group structures. Groups composed of socially experienced male flies exhibit social clusters and high network modularity, indicating the existence of stable subgroups and substantially higher behavioral variance between both individuals and groups, all of which suggest the existence of an ordered social structure. Using hierarchical clustering to compare group signatures allowed us to identify the elements necessary for the formation and expression of group structures during experience and test phases. For instance, clustering of socially experienced flies tested in the dark with that of isolated flies highlights the contribution of visual cues for the expression of group signature, whereas clustering analysis of flies in which cVA sensing neurons were inhibited demonstrates that cVA perception regulates group structure during both experience and test.

Interestingly, analysis of group signatures revealed two aspects relevant to the connection between sensory information and behavior: (a) existence of behavioral features that are “primed” by social experience to become light-dependent (i.e. social experience affects their light-dependence); (b) an emerging role for Or65a in regulating acute male–male interactions in addition to its well-established role in suppressing aggression upon long exposure to cVA57. Accordingly, hierarchical clustering indicated that the inhibition of Or65a neurons affected many features in socially experienced flies, some of which were also changed in isolated flies and are associated in both cohorts with increased activity. 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.

Interestingly, we show that the group signature of socially experienced flies does not depend on prior recognition between individuals, but rather on a general state resulting from the experience of living in a group. This is consistent with studies in social insects demonstrating that collective group behaviors do not require individual recognition5, and with the conceptual model proposed by Anderson and Adolphs suggesting that certain emotional behaviors are associated with distinct internal states8. Interpretation of our results in light of these studies reinforces the notion that group signatures integrate the expression of internal motivational states, shaped by experience, with the specific context in which the group behavior is measured.

Identification of differences in variance between socially experienced and isolated flies indicates that early-life experiences can modulate behavioral variability within and between groups. Inter-individual variability is a broad phenomenon documented in many different animals59–67, and was shown recently to be under neuromodulation in *C. elegans*, suggesting that behavioral variability is a biologically regulated process68. The functional importance of such variability can be seen in *Drosophila* studies demonstrating that increased behavioral variability can contribute to group fitness, a strategy known as bet hedging69. Notably, our results also indicate the existence of increased variability between groups of socially experienced flies. This suggests 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 interaction9,51. Interestingly, although the presence of visual cues affected several network features in our behavioral setup, Schneider et al. reported no effects of the absence of light on network structure51. This apparent discrepancy between our study and that of Scheider et al. could result from different approaches when measuring network structure (binary vs. 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 behaviors1,5,70. 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 survival4,46,71–78. Adding to this, our results demonstrate the presence of social clusters, characterized with increased synchrony between individuals, stable distances between individuals, long-lasting interactions, and seemingly synchronized grooming behavior, all of which are suggestive of a semi-collective state, in agreement with previous studies79,80. 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 synchronized behavior may serve to explore the neuronal and genetic mechanisms underlying collective behaviors, as suggested by de Bono81.

Lastly, we demonstrate that synchronization between individuals in a group 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 synchronization 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 structures1. Importantly, although the groups of mixed ratios consist of two types of individuals, they present a normal distribution of behaviors within the group, suggesting that the group outcome is more than the sum of its parts.

The finding of state-dependent group signatures hints at the existence of distinct, consistent, and robust social 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 the interactions that can sustain such structures. These complex multi-sensory requirements also raise questions about the ability of simple semi-natural social interactions 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.

# Methods

**Tracking**

Flies where inserted in groups of 10 into Fly Bowl arenas82, and 15 minutes of video was acquired with Fly Bowl Data Capture (FBDC)12 and analyzed using CTRAX55 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 axioms and assumptions to fix CTRAX output based on 4 types of errors we observed within 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 (Fig. S1). 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 chance 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. The subsidiary algorithm works by unifying 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 the flies in the movie with the minimal insertions or deletions of identities to the original tracking file. Finally, FixTRAX plots a graph of the number of flies that were added and deleted for each frame, which can help the user adjust CTRAX’s tracking parameters and the fix errors in the algorithm’s parameters to minimize tracking errors. Experiments that were not tracked correctly were discarded. Finally, FixTRAX output is converted into JAABA compatible output using the algorithm specified in Kabra et al.54 and generates general statistical features as in55 (Fig. 3A).

**Kinetic analysis**

Scripts were written in MATLAB to use JAABA code to generate the statistical features specified in Kabra et al.54. Time series graphs were created using JAABA Plot54.

**Behavioral analysis**

JAABA Classifiers54 were trained on various movies to identify the behaviors: Walk, Stop, Turn, Approach, Touch, Chase, Chain, Song, Aggregation, and Grooming. Bar graphs were created using JAABA Plot54.

**Network analysis**

An Interaction matrix was created in MATLAB 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 one with the number of separate interactions between each pair of flies divided by the max number of interactions possible that was calculated as:

The parameters to define interaction are: angle subtended by the other fly > 0, distance between the nose of current fly to any point on the other fly ≤ 8 mm, number of frames for interaction ≥ 60 and number of gap frames ≥ 120 (detailed explanation in the first part of the results). Networks and their features were generated from the interaction matrix in R using the igraph package. The function that was used to the generate networks is “graph\_from\_adjacency\_matrix” with parameters “mode = undirected” and “weighted = TRUE”. Density was calculated on all movies with the formula:

Modularity was calculated on all movies using the “modularity” function on the output received from the “cluster\_walktrap” function. Strength was calculated on all flies 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 received. Box plots were created using R. The distance and angle conditions are maintained for at least two seconds, (60 frames) (Fig. 2 B-C and Fig. 2S). Optimally and theoretically, we should include all instances of meeting of two flies. However, our data shows that when doing that, most interactions are random, which is undesirable. Therefore, to avoid increasing randomness in our data, we decided on a minimal limit for a significant interaction.

If the distance or angle condition are not maintained for at least four seconds, we define the end of an interaction (Fig. 2 B-C and Fig. 2S). Undirected networks were used based on high correlation observed between interactions of paired flies (Fig. 2D). We decided to use weighted networks because this lets us create a single network for each experiment which is not saturated and allows for analysis of the variation in interaction strength among dyads.

**Variance analysis**

Standard deviation (SD) of all flies was calculated as standard deviation of all per-fly data (all experimental repetition 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 each experiment except for experiments of ratios of sub populations, 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.51. Scatter plots were created using R.

Each ratio of sub populations experiment was first normalized to a control condition of 10 WT flies from the same experiment, then features were standardized according to all normalized values from ratios of sub population experiments 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 consists of average standardized values of all movies for that condition (from all experiments).

**Fly lines**

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. Canton S flies were used as the wild-type strain. 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 form VDRC.

**Behavioral setup**

Socially experienced vs. 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 (experienced) for 3 days, in a light/dark cycle of 12/12. Flies were then inserted into Fly Bowl arenas for video recording, as described above.

Light vs dark: flies were collected as before and housed in groups of 10 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 or water, for 4 consecutive days as described previously83. Flies were then inserted into Fly Bowl arenas for video recording, as described above.

Starvation: flies were collected in groups of 10 as described above. 24 hrs 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 single housed 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.

**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 two-conditions experiments: 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. Error bars signify SEM.

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