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

Assa Bentzur*[1], Shir Ben-Shaanan*[1], Jennifer Benishou[1], Eliezer Costi[1], Amiyaal Ilany[1] and Galit Shoat-Ophir[1,2]

[1]The Mina & Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan, Israel

[2]The Leslie and Susan Gonda Multidisciplinary Brain Research Center, Bar-Ilan University, Ramat-Gan, Israel

*Authors contributed equally to this work

Correspondence should be addressed to G.S.O. (galit.ophir@biu.ac.il) or A.I. (amiyaal.ilany@biu.ac.il)

Summary

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, and the neuronal and molecular mechanisms that mediate it. Here we present a practical 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 are 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 dissect 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 requires visual and pheromonal cues. Particularly, the male specific pheromone cVA and Or65a sensory neurons are necessary for the expression of different aspects of social interaction in a group. 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 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. This paves the path for using simple model organisms to dissect the neurobiology of behavior in complex environments associated with living 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 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 internal motivational state, previous memories and other physiological processes affect their action selection, giving rise to diverse activity levels, behavioral responses, and engagement with others[6]. This results in a highly complex and continuously 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[7,8]. The complex nature of this environment imposes conceptual challenges in the quantification and analysis of group behavior[9].

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 behavior[10,11]. Although much is known about the interplay between social experience, internal motivational states[12–17] and their effects on social interaction in pairs of animals[13,18–22], 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)[23]. Both lines of study progressed substantially with advances in machine vision and machine learning technologies that allow automated tracking and unbiased behavioral analysis[24–30]. 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 structure[23,31–33]. 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 hierarchy[3,31,42–45,34–41]. Although individual behaviors and social network analysis highlight different aspects of social interaction, they are 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 encounters[46–51]. Various studies provided mechanistic understanding of these complex behaviors, demonstrating that their expression require multi-sensory inputs as well as specific neuronal pathways in the brain[50,52–57]. Modulation of behavior by previous social experience was also investigated in flies, revealing that the expression of certain genes in specific neuronal populations can lead to long-lasting behavioral changes[19,58–62]. While *Drosophila* proves to be a useful model organism for mechanistically exploring complex behaviors[63,64], only a small number of studies have

examined social group interaction in flies. These studies demonstrated that flies possess the neuronal ability to recognize different individuals in a group[65], that groups of flies exhibit non-random group structures which depend on certain sensory systems[4,57,66] and that group interaction facilitates collective responses to threat[67,68]. 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 fitness[64].

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 used this framework to characterize the principles and mechanisms that govern social interaction in groups of flies. We show 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, and show that group signatures are strongly influenced by both visual cues and sensing of 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 related to inter-individual synchronization 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)[69]. 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, Fig. 1A)[69–71]. Although Ctrax is successfully used in many behavioral setups its output includes some tracking errors, impeding measurements that require maintenance of identities 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 using the supervised machine learning algorithm JAABA[69] (Fig. 1A; full description in Supplementary Table S1), and calculate six social network features (Box1). 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).

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 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 (Fig. 1C). To resolve this, we added an additional requirement of a minimal gap, 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. When considering all pairs of flies (X and Y) within a group, total interaction time initiated by X was correlated with total interaction time initiated by Y (Fig. 1E), suggesting symmetric interactions over the course of the test, making directed analysis redundant. We therefore decided to use undirected networks in this study.

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 calculated 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, Box 1) 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 socially experienced flies form networks with higher-order structures 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 ones (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 across individuals, 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 not statistically different (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). Therefore, 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 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 experienced and socially isolated flies in the presence or absence of light (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 into 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 two main clusters based on social history; one of conditions in which flies were isolated prior to test and another of conditions in which flies were socially experienced. Interestingly, socially experienced flies that were tested in the dark did not cluster with either groups, reinforcing the notion that visual cues are specifically necessary for the expression of group signatures associated with social experience, but are not sufficient to fully change group signature from that of 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 aggression[59,72]. cVA is perceived via two olfactory receptor neurons (ORNs): Or67d, which mediates acute responses to cVA, and Or65a, which mediates chronic responses to cVA[59,73]. We investigated whether cVA perception impacts the group signature of socially experienced flies. For that, we blocked cVA perception by constitutively expressing the inward 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 the function of Or67d neurons is not necessary for the formation of the behavioral signature associated with social group experience (Fig. 5A). In contrast, the inhibition of Or65a neurons dramatically changed the group signature of socially experienced flies, increasing average velocity and, overall time flies engaged in approach, chase and touch behaviors compared to genetic controls (Fig. 5B). Analysis of network structure revealed increased 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

courses[59]. Experimental flies exhibited more touch, approach, chase and chain behaviors than controls, and increased 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 Or65a neuronal inhibition are identical in both socially isolated and experienced flies, including a decrease in 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 was 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 in a group (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)[19], 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, angular speed (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; Table 1). 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 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. 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 cVA[59]. 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 recognition[5], and with the conceptual model proposed by Anderson and Adolphs suggesting that certain emotional behaviors are associated with distinct internal states[10]. 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 animals[74–82], and was shown recently to be under neuromodulation in *C. elegans*, suggesting that behavioral variability is a biologically regulated process[83]. The functional importance of

such variability can be seen in *Drosophila* studies demonstrating that increased behavioral variability can contribute to group fitness [84]. Notably, our results also indicate the existence of increased variability between groups of socially experienced 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[12,66]. 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 structure[66]. This apparent discrepancy between our study and that of Schneider 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 behaviors[1,5,85]. 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[4,53,68,86–92]. 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 studies[93,94]. 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 Bono[95].

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 structures[1]. Importantly, although the groups of mixed populations 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 mechanism underlying this remains to be elucidated.

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 arenas[96], and 15 minutes of video was acquired with Fly Bowl Data Capture (FBDC)[70] and analyzed using CTRAX[71] 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 of that specific error. The subsidiary algorithm unifyies 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.[69] to generate general statistical features as in[71] (Fig. 3A).

Kinetic analysis

Scripts were written in MATLAB to use the JAABA code to generate the statistical features as specified in Kabra et al.[69]. Time series graphs (per frame) were created using JAABA Plot[69].

Quantification of specific behaviors

JAABA Classifiers[69] 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[69].

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 ≤ 8 mm, number of frames for interaction ≥ 60 and number of gap frames ≥ 120. Interaction end is defined as distance or angle conditions are not maintained for 4 seconds.

Networks and their features were generated from the interaction matrix in R using the igraph package. The function that was used to the generate networks is "graph_from_adjacency_matrix" with parameters "mode = undirected" and "weighted = TRUE". Density was calculated on all movies with the formula:

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

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

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 for experiments of ratios of sub populations (Fig. 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.[66]. Scatter plots were created using R.

Sub populations experiment (Fig. 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.

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 from 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 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 or water, for 4 consecutive days as described previously by[97]. 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 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.

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.

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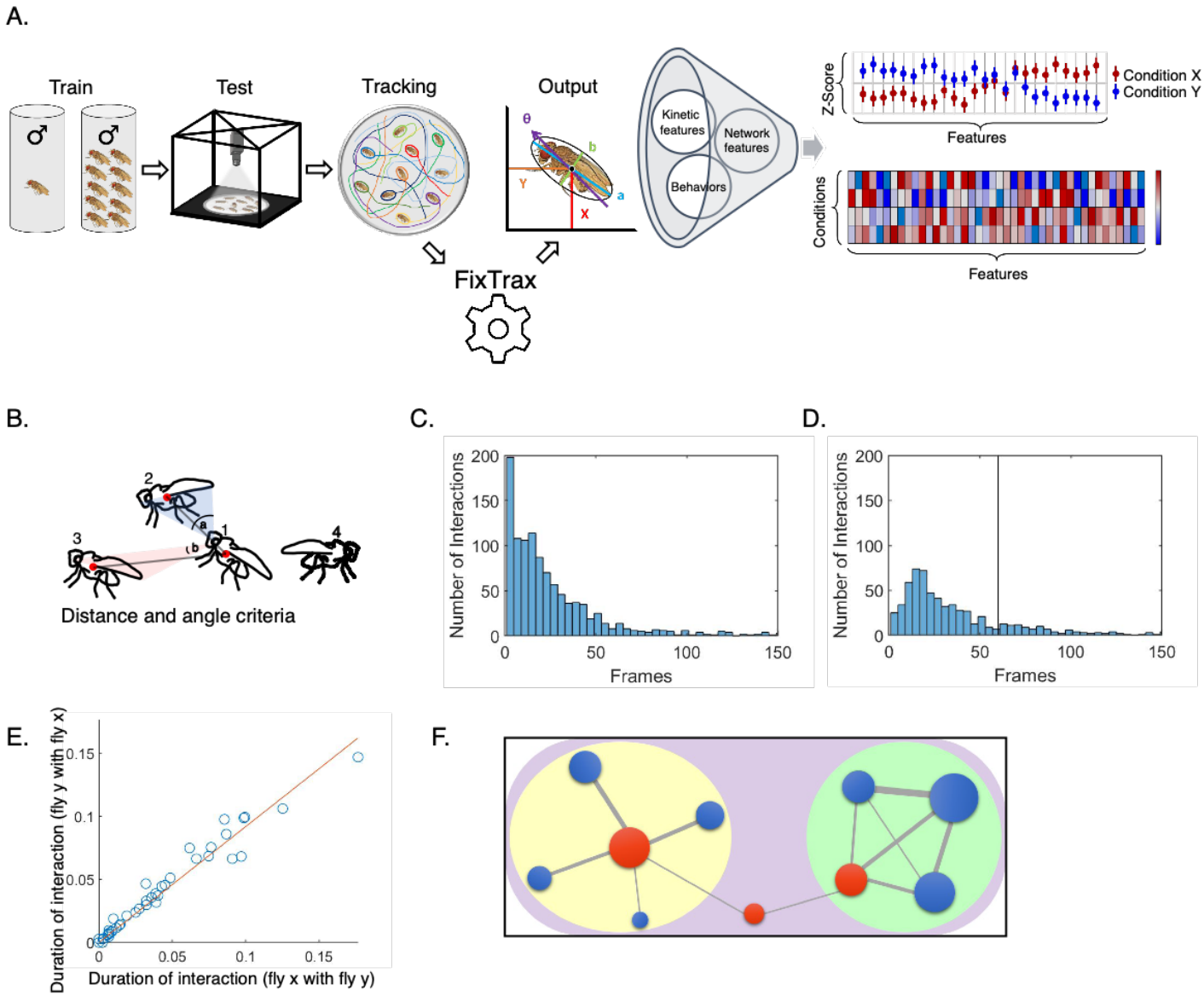

Figure 1. A conceptual and experimental setup for studying complex behavior in groups of *Drosophila*. A. Illustration of social conditioning, data capture and analysis. Naïve male flies were housed in groups of 10 flies or in isolation for 3 days and inserted in groups of 10 into the Fly Bowl arenas, where their social interaction was recorded for 15 minutes (30fps). 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. Fixed output file was used to calculate kinetic features, to classify specific behaviors using JAABA and to analyze social network structure. 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 criteria used to define an interaction; distance between flies <8mm and angle subtended (α or β) >0. Total number of encounters as a function of encounter duration in representative movie of socially experienced WT flies (C), and when adding a 60-frames gap requirement between interactions (D). Black line represents the threshold (60 frames) under which encounters are not considered interactions for network analysis. E. Directed interactions between each pair of flies from (C, D) reveal a high correlation. F: Illustration of network parameters; Strength is proportional to vertex size. Betweenness centrality is represented by vertex color (high for red and low for blue). Density of subnetworks is represented by color; green for high and yellow for low). Modularity; low in green and yellow subnetworks and high in purple (whole) network.

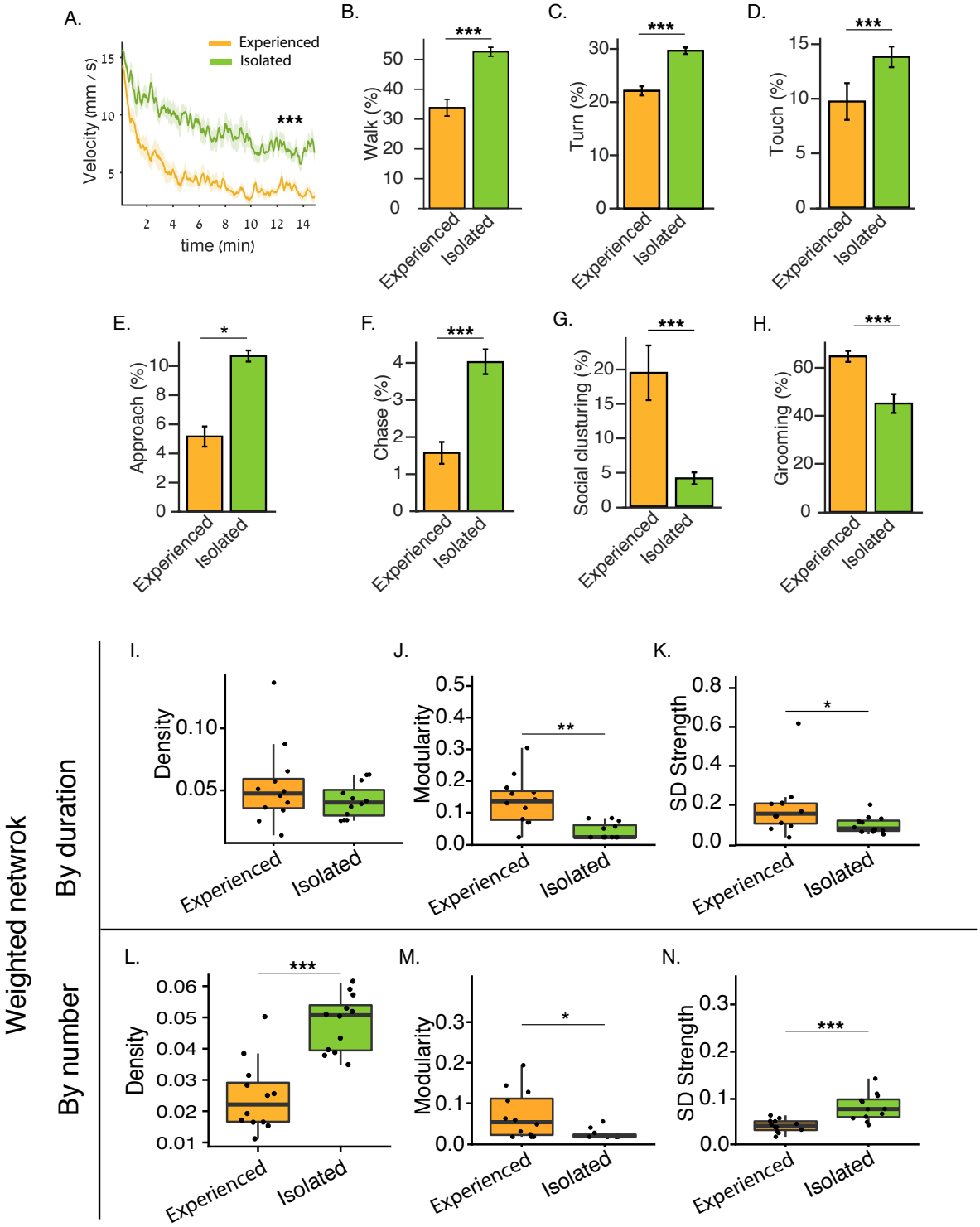

Figure 2: Prior social interaction in a group facilitates the formation of ordered social structures. A. Average velocity per-frame of previously isolated male flies (green) vs. socially experienced male flies (orange) over 15 minutes. B. Average percentage of time flies perform walk behavior. C. Average percentage of time flies perform turn behavior. D. Average percentage of time flies perform touch behavior. E. Average percentage of time flies perform approach behavior. F. Average percentage of time flies exhibit chase behavior. G. Average percentage of time flies exhibit social clustering. H. Average percentage of time flies perform grooming behavior. I-N: Network density, modularity and SD strength calculated by network weights according to duration (I-K respectively) or number of interactions (L-N respectively) between previously isolated (green) and socially experienced (yellow) WT male flies. N=18, Wilcoxon test, * P<0.05, ** P<0.01, *** P<0.001. Error bars signify SEM.

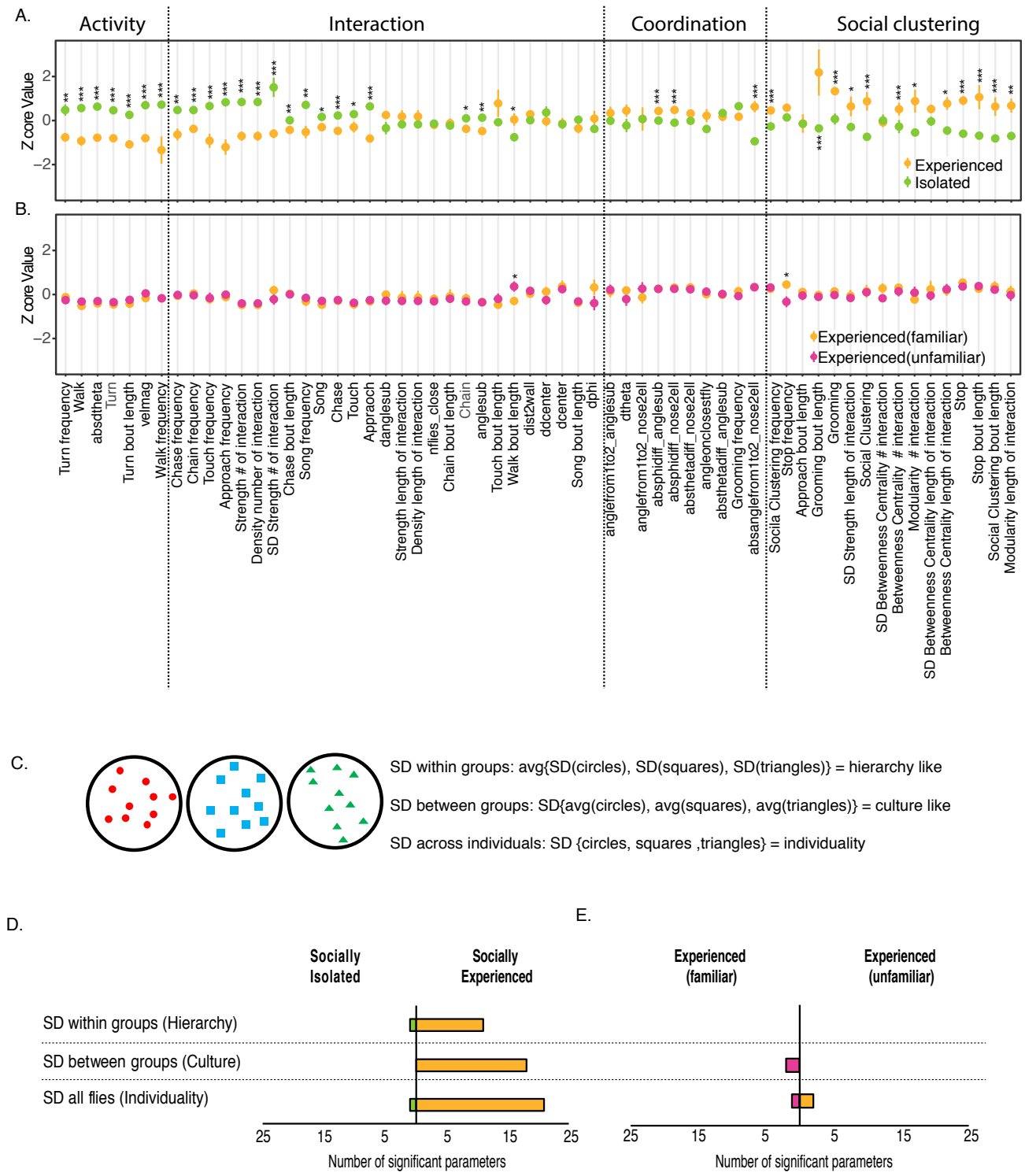

Figure 3. Social experience facilitates distinct group signature and increases behavioral variability. Behavioral signatures of previously isolated vs. socially experienced WT male flies (A) and familiar vs. unfamiliar experienced WT flies (B). Data is represented as normalized Z scores of 60 behavioral (A: N=18. B: N=25 t-test for normally distributed parameter or Wilcoxon test for non-normally distributed parameters. P-values were corrected using FDR. * P<0.05, ** P<0.01, *** P<0.001). C. Graphical illustration of measuring variance within groups, between groups and across all individuals in each condition. Number of behavioral features that display significantly higher variance and that their SD is at least two-fold higher when comparing isolated to experienced (D) and familiar vs unfamiliar (E). Statistical analysis was performed on WT 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.

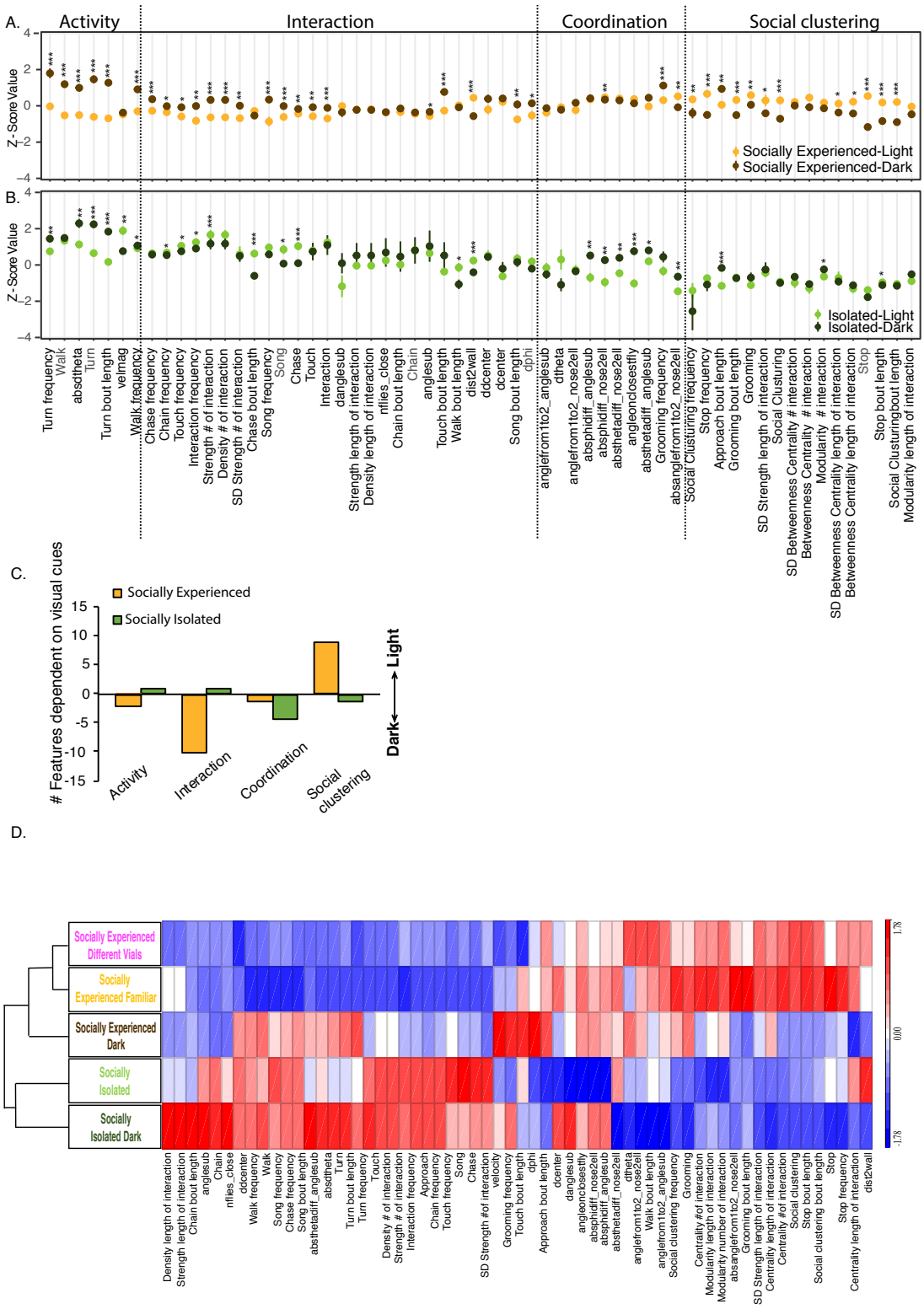

Figure 4: Visual cues are necessary for expressing the behavioral signature of socially experienced flies. Behavioral group signatures (represented as normalized z-scores) of socially experienced (A) or previously isolated (B) WT male flies tested in normal lighting conditions (light) vs. light deprivation (dark), N=18 and 10, respectively. t-test for normally distributed parameters or Wilcoxon test for non-normally distributed parameters. *P<0.05, **P<0.01, ***P<0.001. Error bars signify SEM. C. Number of behavioral features that display significantly higher scores in either dark (negative y axis) or light (positive y axis) per condition (isolated or experienced), divided into 4 categories; activity, interaction, coordination and social clustering related features. D. Hierarchical clustering of group signatures of the following experimental conditions: socially experienced, unfamiliar socially experienced, socially experienced dark, socially isolated and socially isolated dark. Hierarchical clustering was performed using Partek.

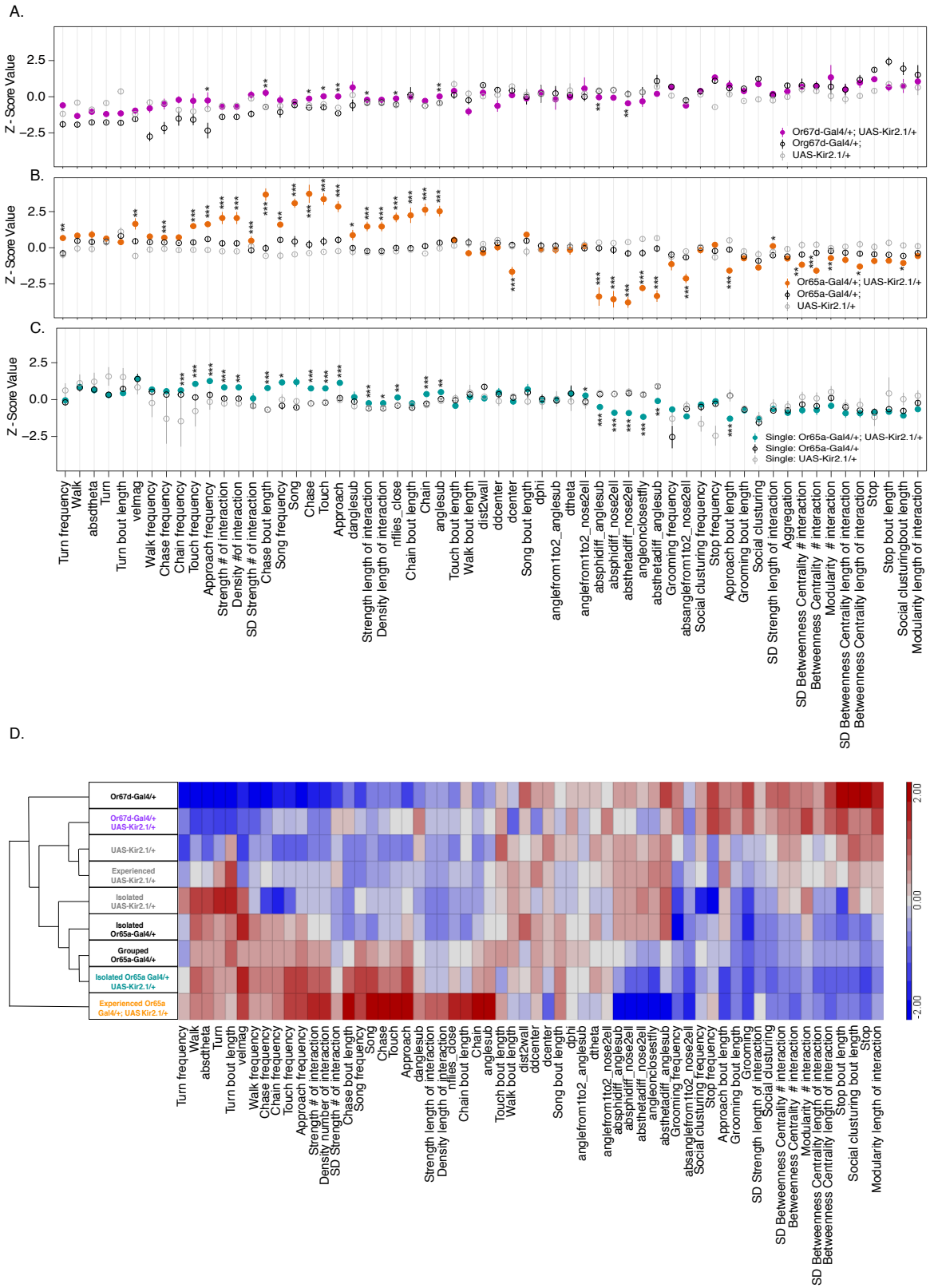

Figure 5. cVA sensing via Or65a neurons shapes social group interaction. A Behavioral group signatures (as normalized z scores) of socially experienced Or67d-Gal4/+; UAS-Kir2.1/+ flies compared to genetic controls, N= 7. Behavioral group signatures of Socially experienced Or65a-Gal4/+; UAS-Kir2.1/+ flies compared to genetic controls, N=13. C. Behavioral group signatures of previously isolated Or65a-Gal4/+; UAS-Kir2.1/+ flies compared to genetic controls, N=8. 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<0.05, **P<0.01, ***P<0.001. Error bars signify SEM. D. Hierarchical clustering of behavioral group signatures of all experimental conditions in A-C using Partek.

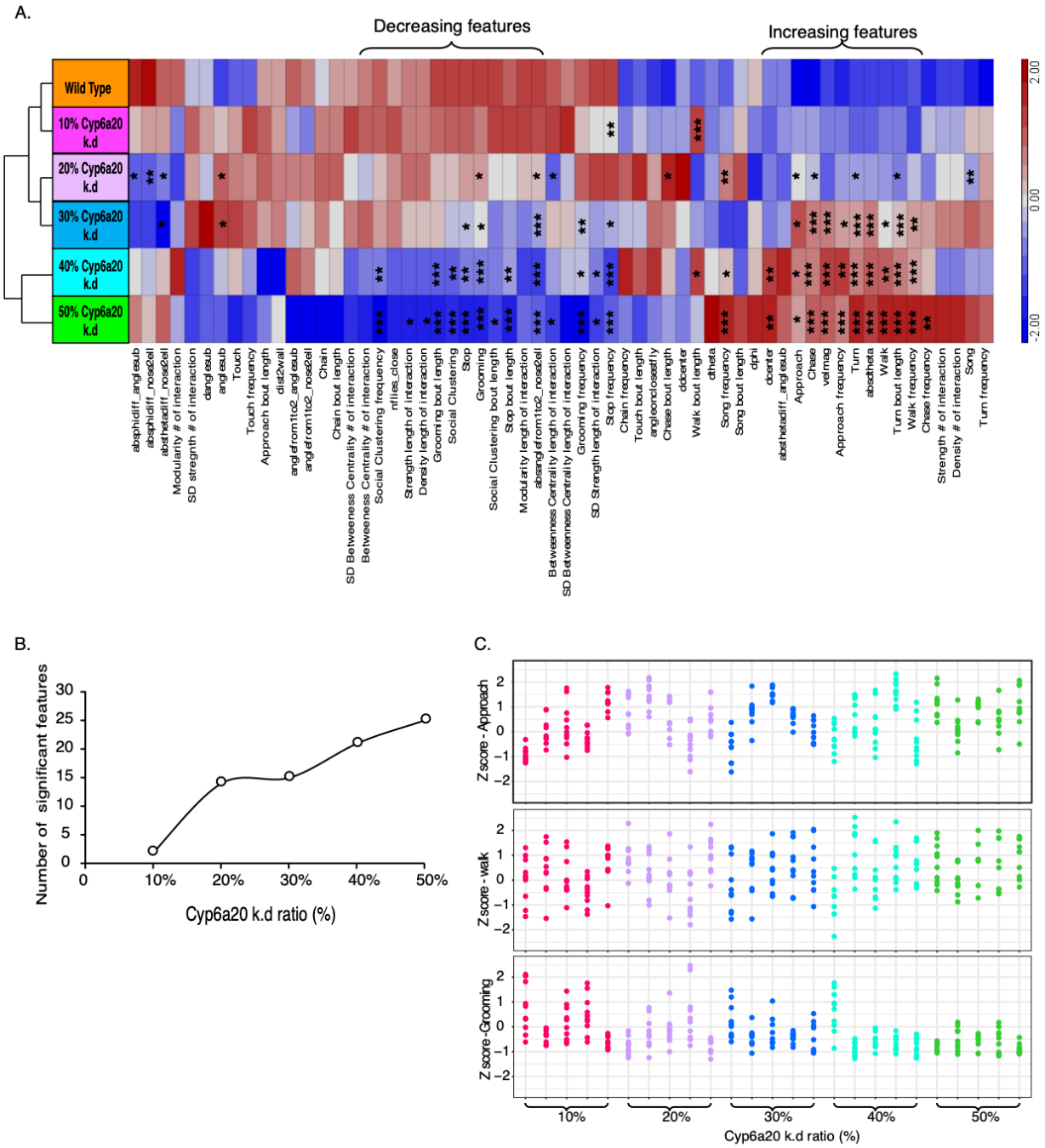

Figure 6. Sub populations of aggressive flies in a group affect different features of group behavior. A. Hierarchical clustering of behavioral signatures of groups composed of different ratios of socially isolated Cyp6a20-Gal4/+; UAS-Cyp6a20-RNAi/+ and socially experienced WT flies (0-50%). Data of each experimental group was normalized to a WT control group which 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. Hierarchical clustering of conditions and behavioral features were performed using Partek. 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 with 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. B. Number of significantly different behavioral features compared to controls as a function of the ratio of isolated Cyp6a20-Gal4/+; UAS-Cyp6a20-RNAi/+ to experienced WT flies in a group (10-50%). C. Per-fly distribution of three behavioral features (interaction, walk, grooming) in groups containing increasing ratios (0-50%) of isolated Cyp6a20-Gal4/+; UAS-Cyp6a20-RNAi/+ to socially experienced WT male 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.

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

Definition	Description
Walk	Fly moves.
Stop	Fly is still.
Turn	Changes in fly's direction.
Touch	Fly actively touches another fly.
Approach	Fly approaches another fly and perform interaction (active or passive).
Aggregation	Fly sits in a group of 3 or more flies.
Grooming	Fly grooms.
Chase	Fly chases another fly.
Chain	Chase with 3 or more flies.
Song	Fly moves one wing next to another fly.
Behavior bout length	Length of the longest sequence of frame in which the behavior occurred for each fly.
Behavior frequency	Length of the movie minus the length of the longest sequence of frames in which the behavior didn't occurred for each fly.
Density interactions' length	Accumulated interactions' length relative to the maximum interactions' length possible.
Modularity interactions' length	Representation of how much the network is divided into modules according to interactions' length.
Strength interactions' length	Length of interactions of a certain fly.
SD Strength interactions' length	Standard divination of the strengths according to interactions' length of flies from the same movie.
Betweenness Centrality interactions' length	A measure of centrality of a certain fly based on shortest paths according to interactions' length.
SD Betweenness Centrality interactions' length	Standard divination of the betweenness centralities according to interactions' length of flies from the same movie.
Density interactions' number	Interactions' number relative to the maximum interactions' number possible.
Modularity interactions' number	Representation of how much the network is divided into modules according to interactions' number.
Strength interactions' number	Number of interactions of a certain fly.
SD Strength interactions' number	Standard divination of the strengths according to interactions' number of flies from the same movie.
Betweenness Centrality interactions' number	A measure of centrality of a certain fly based on shortest paths according to interactions' number.
SD Betweenness Centrality interactions' number	Standard divination of the betweenness centralities according to interactions' number of flies from the same movie.

Table S1: Definitions of behavioral features used in this work. Kinetic (red) features were obtained from Kabra et al. Classified behavioral features (blue) were generated using JAABA. Network (green) features were calculated in R using igraph.

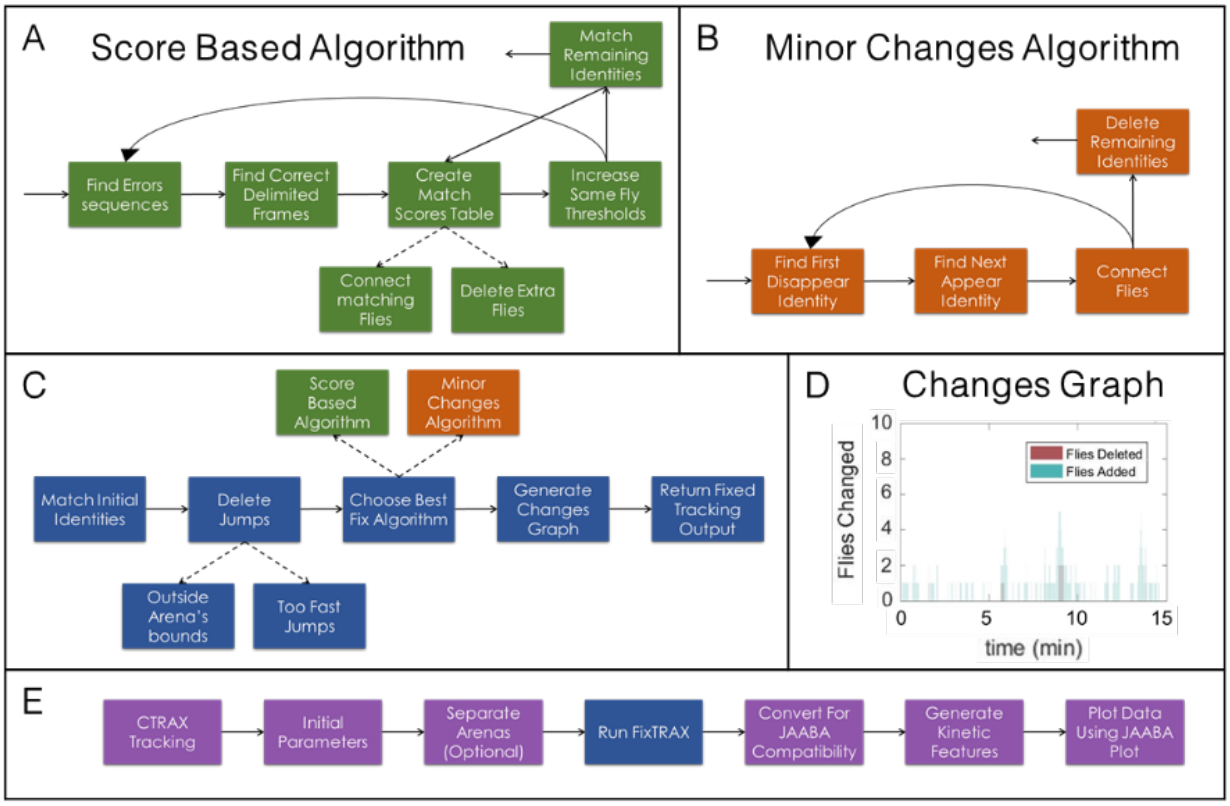

Figure S1. FixTRAX and analysis pipeline. A. Schematic illustration of the Score Base fix algorithm. B. Schematic illustration of the Minor Changes fix algorithm. C. Schematic illustration of FixTRAX framework. D. Example of a changes graph output. Blue lines represent the number of identities that were added per-frame. Red lines represent the number of identities that were deleted in per-frame. E. Schematic illustration of the analysis pipeline.

Individual features:

a. Strength: Represents individual social activity, as the total duration/number (depending whether analysis is based on length or number of interactions) of all interactions each fly participated in (Fig. 1F; size of each vertex represents its strength).

b. Betweenness Centrality: Reflecting the importance of that individual to the network structure, measured by the proportion of shortest paths between any two individuals that pass via the focal fly, (Fig. 1F. High for red vertices and low for blue vertices).

Group features:

a. Density: Reflecting network saturation (Fig. 1F, green sub-network has high density whereas yellow sub-network has low Density).

b. Modularity: Reflecting the propensity of the network to be divided into clear modules, or sub-groups (in Fig. 1F the network has two distinct modules (green and yellow)).

c. Standard Deviation of Strength and Betweenness Centrality for each network (SD strength and SD betweenness Centrality respectively).

Box 1. Explanation of social network features.

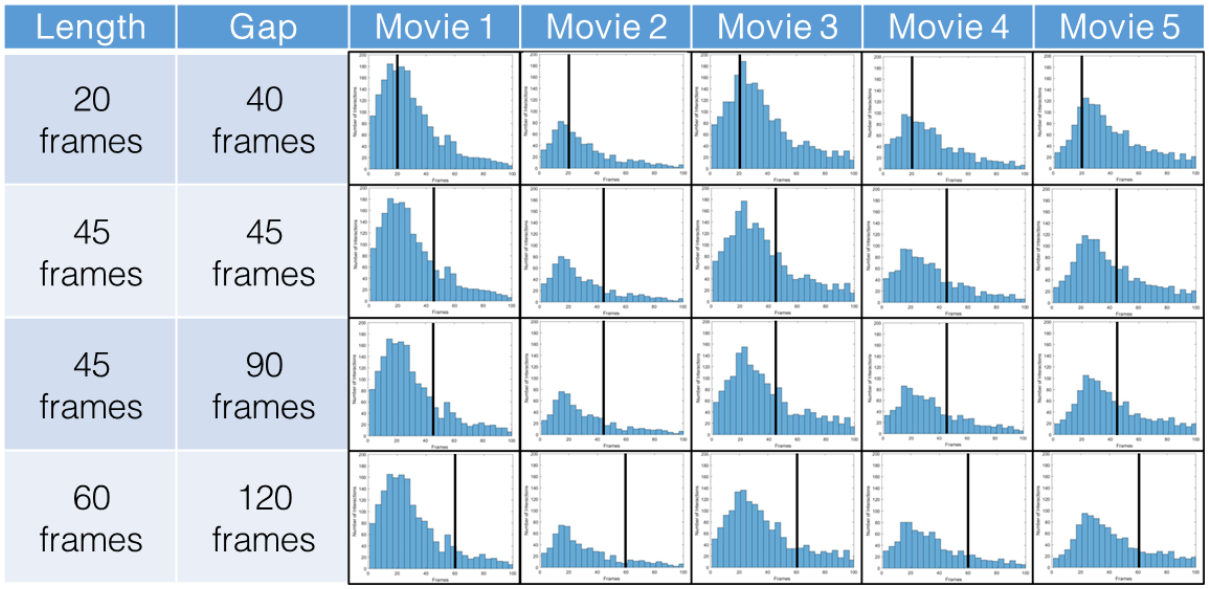

Figure S2. Defining interaction duration and gap thresholds affects the number of very short interactions. Number of encounters that meet the minimal distance and angle requirements for interaction as a function of encounter duration in five movies, with different combinations of duration and gap parameters (20-60 frames/0.4-2 sec and 40-120 frames/1.2-4 sec respectively). Each row represents a combination of duration and gap values. Each column represents one movie. Black lines represent the minimal threshold for an interaction according to the specific duration parameter.

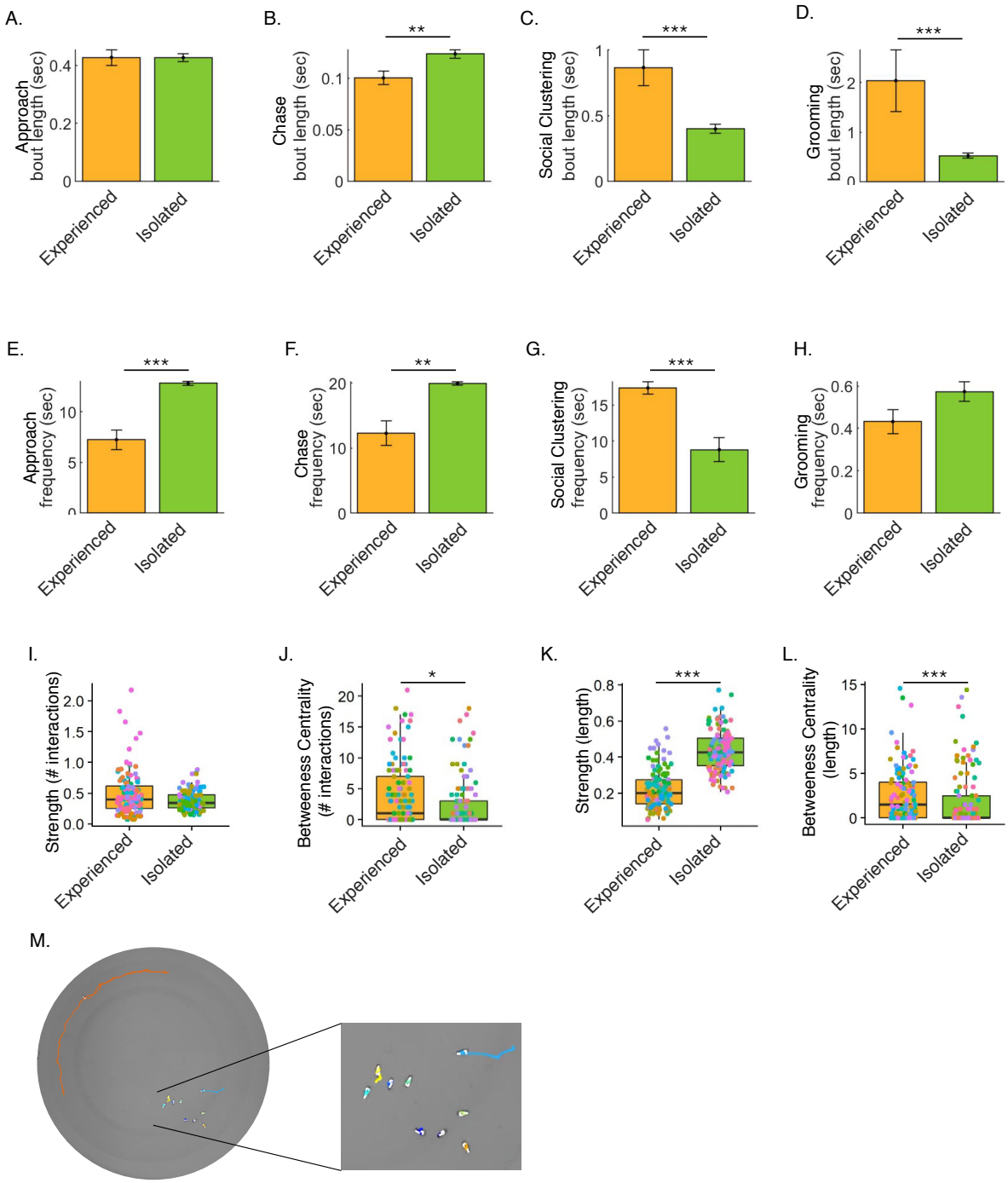

Figure S3. Prior social experience affects bout-length and frequency of specific behaviors and changes network structure. A-H: Average bout-length (A-D) and frequency (E-H) of specific behaviors (Interaction, Chase, Social clustering and Grooming, respectively) of socially experienced (orange) vs. isolated (green) WT male flies. I-L: Per-fly network features (Strength and Betweenness Centrality) in which network weights were calculated according to duration of interactions (I-J) or number of interactions (K-L) between socially experienced (orange) vs isolated (green) WT male flies. t-test for normally distributed features or Wilcoxon test for non-normally distributed features. N=18, * P<0.05, ** P<0.01, *** P<0.001. Error bars signify SEM. M: Picture of a social clustering event, performed by socially experienced WT male flies within a FlyBowl arena, colored lined represent tracking trajectories over the next 60 frames/2 sec.

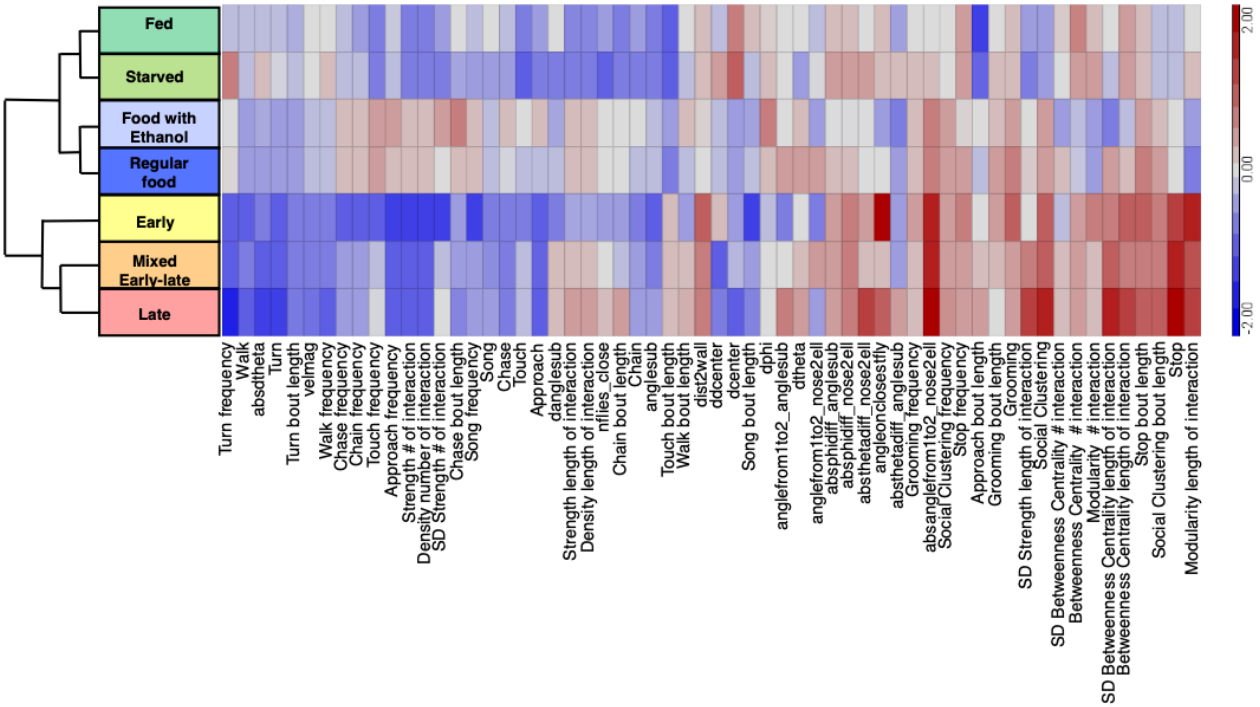

Figure S4. Different internal motivational states in socially experienced flies do not affect group signatures. Socially experienced flies which were starved for 24 hours prior to behavioral test (fed/starved), exposed to ethanol for 3 days prior to behavioral test (food with ethanol/regular food) or tested at different times during the day (early/mixed/late) do not display any differences in group behavior, compared with controls. Hierarchical clustering of conditions reveals a similarity between each experimental group and its control group (left, hierarchy tree). t-test for normally distributed parameter or Wilcoxon test for non-normally distributed parameters in starvation and ethanol experiments. One-way ANOVA with Bonferroni post hoc test for normally distributed parameters or Kruskal Wallis followed by Wilcoxon signed-rank test for non-normally distributed parameters in different times experiment. N=13, 14 and 6 for ethanol, starvation and time difference tests respectively, n.s. Hierarchical clustering of conditions was performed with Partek.

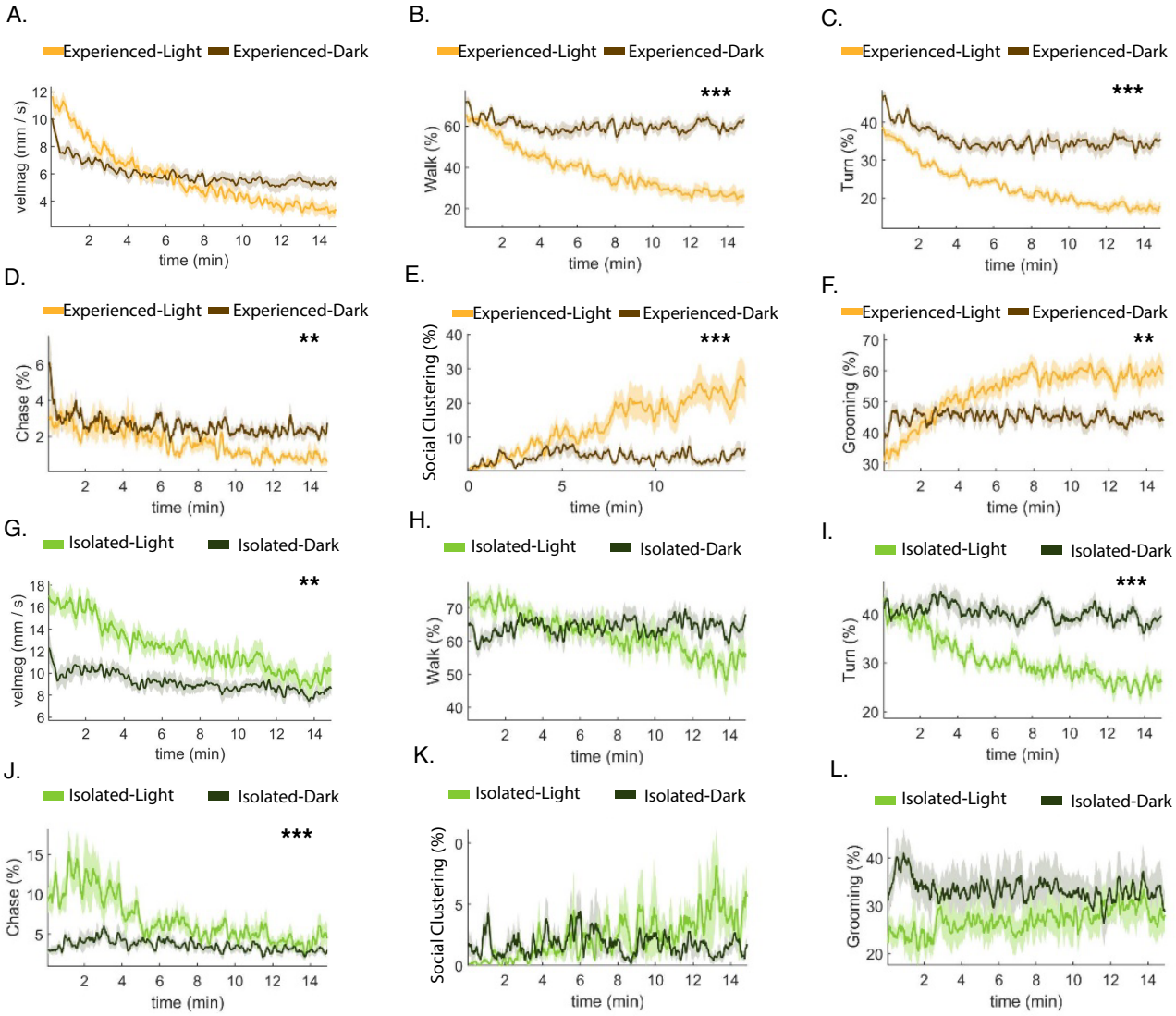

Figure S5. Visual cues are required for habituation during test. A-F: Average per-frame of velocity (velmag), walk, turn, chase, social clustering and grooming of socially experienced WT male flies tested in normal lighting (experienced light - orange) or in the dark (experienced dark - brown). G-L: Average per-frame of velocity (velmag), walk, turn, chase, social clustering and grooming of socially isolated WT male flies tested in normal lighting (Experienced light - orange) or in the dark (Experienced dark - brown). Statistical analysis was performed on the average of each behavior for the entire duration of the test (15 min). t-test for normally distributed features or Wilcoxon test for non-normally distributed features. FDR correction for multiple testing was performed for all analyses, N=18 for experienced and N=11 for isolated experiments, ** P<0.01, *** P<0.001. Error bars signify SEM.

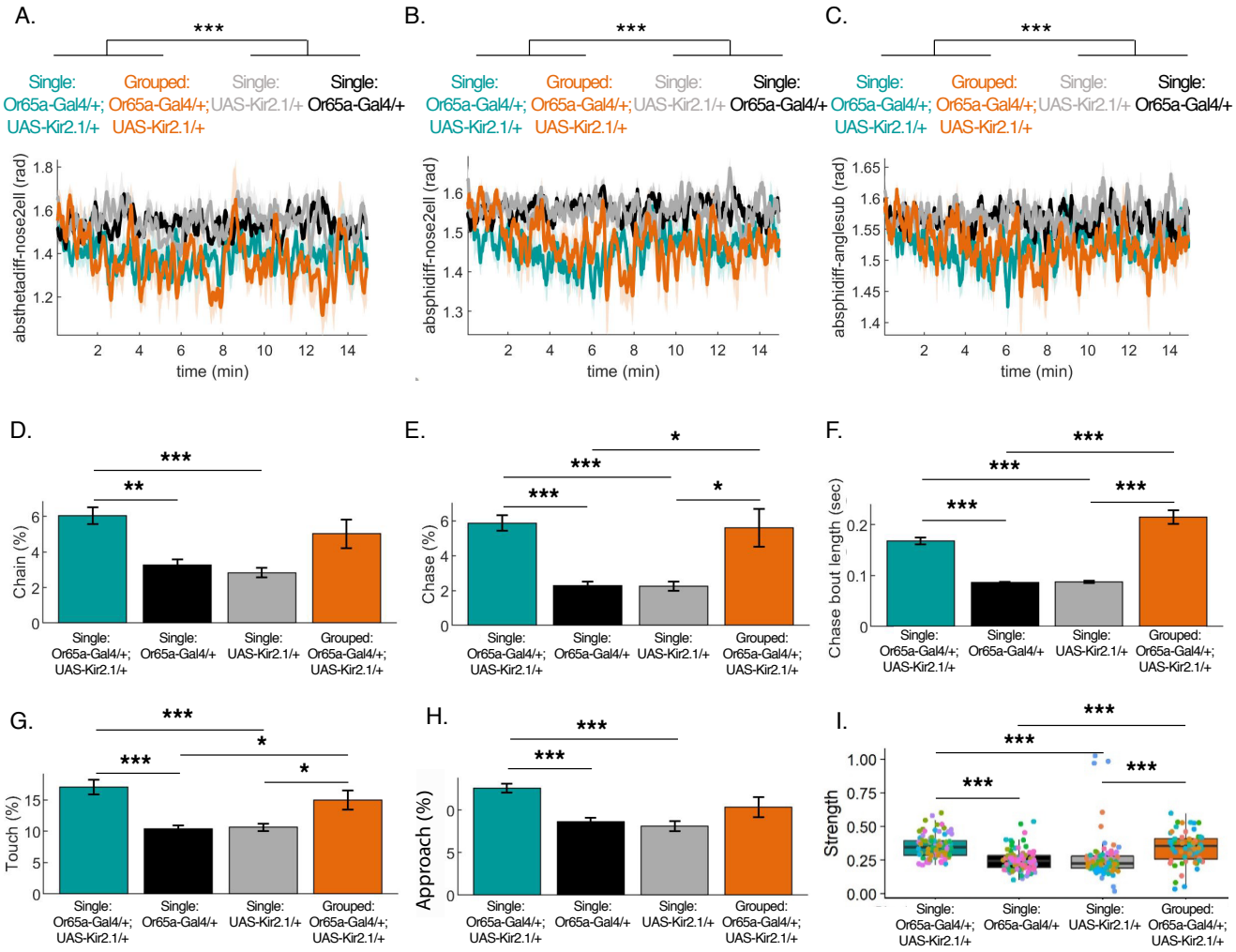

Figure S6. Socially experienced and isolated Or65a-Gal4/+; UAS-Kir2.1/+ male flies display similarities in group behavior compared to isolated genetic controls. A-C: Per-frame averages of three kinetic features (A: absthetadiff_nose2ell, B: absphidiff_nose2ell, C: absphidiff_anglesub) in socially experienced (orange) and isolated (blue) Or65a-Gal4/+; UAS-Kir2.1/+ flies compared to socially isolated genetic controls (black and gray). D-H: Average percentage of time socially experienced (orange) and isolated (blue) Or65a-Gal4/+; UAS-Kir2.1/+ male flies performed chain, chase, chase bout length, touch and interaction behaviors compared with socially isolated genetic controls (black and gray). I: Per-fly network strength of socially experienced (orange) and isolated (blue) Or65a-Gal4/+; UAS-Kir2.1/+ male flies compared to socially isolated genetic controls (black and gray). One-way ANOVA with Bonferroni post hoc test for normally distributed parameters or Kruskal Wallis followed by Wilcoxon signed-rank test for non-normally distributed parameters. N=6, * P<0.05, ** P<0.01, *** P<0.001. Error bars signify SEM.

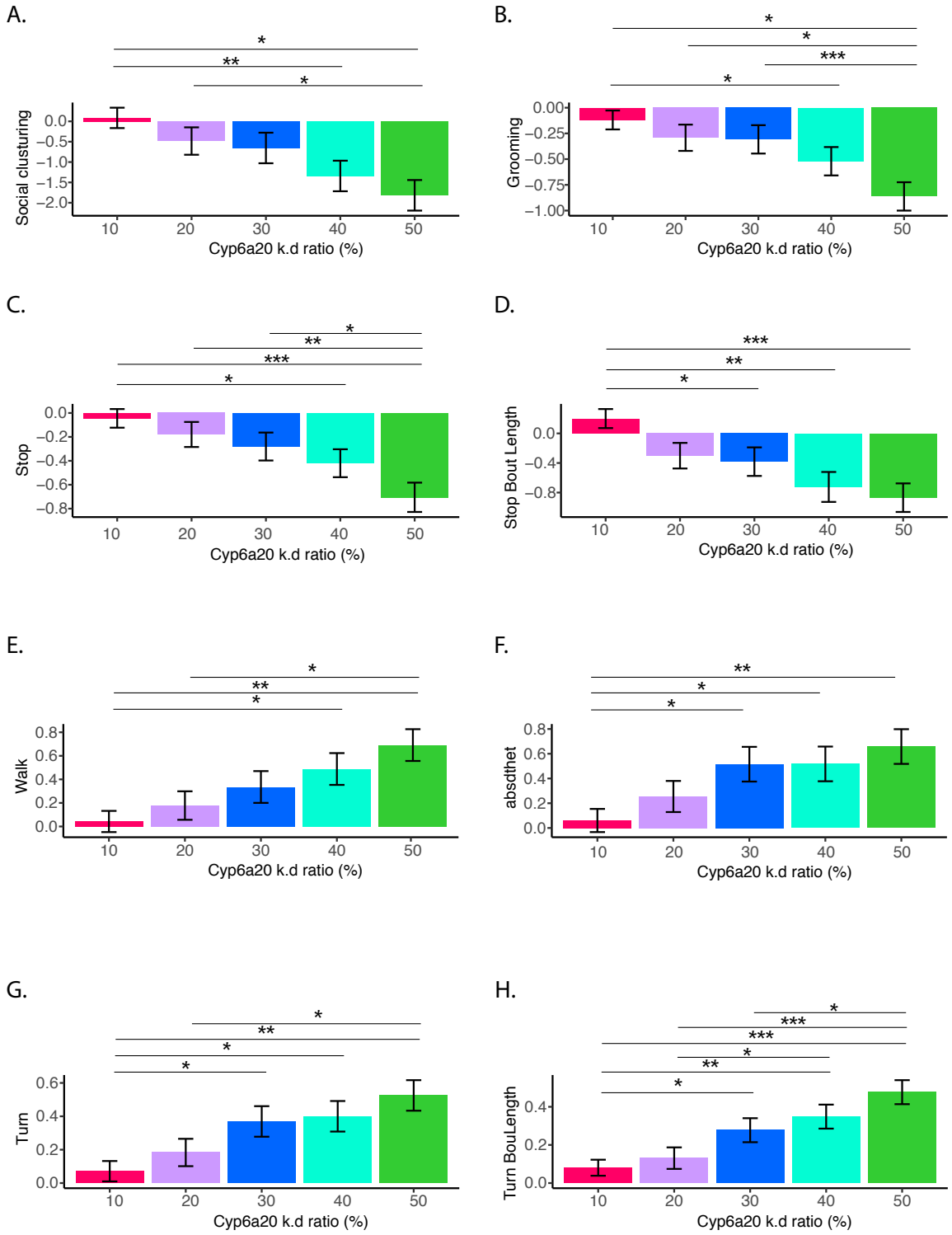

Figure S7. Sub populations in a group affect specific features within behavioral group signatures. A-H: log2 transformed averages of gradually decreasing behavioral features (A: Social clustering, B: Grooming, C: Stop, D: Stop bout length) and gradually increasing features (E: Walk, F: absdtheta, G: Turn, H: Turn bout length) in groups composed of 10%-50% isolated Cyp6a20-Gal-4/+; UAS-Cyp6a20-RNAi to socially experienced WT flies. 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 with by linear contrasts and FDR correction was applied to all comparisons. N=14, 8, and 6 for groups of 10%, 20-30% and 40-50%, respectively, * P<0.05, ** P<0.01, *** P<0.001. Error bars signify SEM.