The role of the *foraging* gene (*for*) on Social Interaction Networks of *Drosophila melanogaster*

By Nawar Alwash

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Cell & System Biology
University of Toronto

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Abstract

Social interactions are prevalent in the lives of many animals, including *Drosophila melanogaster*. *D. melanogaster* aggregate on fermenting fruit, creating complex social environments within which they display various social behaviours. Deciphering the genetic underpinnings of these social behaviours is a difficult task, and few studies have attempted to address this. Here, I investigate how the *foraging* gene (*for*) influences social networks in *D. melanogaster*. *for* is a well-established example of a pleiotropic gene that modifies behavioural phenotypes. In fruit flies, there are two naturally occurring alleles known as rover and sitter, that differ in their foraging behaviour. *for* regulates multiple phenotypes, the best known of these involves larval food-related behaviours. Recent studies suggest that *for's* influence extends to social behaviours across a variety of taxa. In my thesis, I report that *for* plays a role in influencing both behavioural elements of social networks and social network measures. Rover flies are characterized by having greater interaction rates, moving more during the trial, and having higher global efficiency values. While sitter flies spend more time interacting, are more likely to reciprocate an interaction, and create more homogeneous networks in which they display higher clustering coefficient values. My initial findings establish that this natural polymorphism of *for* influences the behavioural elements and social network measures. Using gene dosage manipulations, I show that differences in behavioural and social network phenotypes are mainly due to differences in the *for* locus. To address this further, I attempt to investigate the critical period of *for* expression on behavioural elements and social network

measures. I separately knockdown *for* in the adult stage, as well as from the embryonic until the larval wandering stage. I did not find an effect of these developmental manipulations on behavioural elements and social network measures. These results suggest that the critical period of *for* expression in relation to behavioural elements and social network phenotypes is likely within the pupal stage, during metamorphosis. I also use promoter-driven increased expression of *for* and show that the different *for* promoters may independently regulate different phenotypes within the analysis of social networks. I report that *for*'s influence on social behaviour exhibits plasticity to the environment. I show that even though network measures are resilient to social isolation, food, and sleep deprivation, the behavioural elements of a network are responsive to these stressors. Finally, I examine networks of mixed groups of the rover and sitter flies to shed light on how these strains may interact when they are in the same group. In summary, this thesis characterizes the effects of a specific gene on social networks. My findings emphasize the complexity of *for*'s influence on *Drosophila* social behaviour and support the theory of genetic effects on social behaviour.

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I am incredibly grateful for my parents, Dunya and Mustafa Alwash for all their unwavering love and support, and for always being an inspiration. To my brothers, Ameer and Zaid Alwash, for reading all my reports and listening to all my presentations. I would also like to thank Mays Alwash, Sana Alwash, Yahya AlHashimi, Nawar Alwesh, and all the rest. It is through your encouragement and support that I am able to cross the finish line!

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Forward

This forward aims to provide a brief introduction to outline my main thesis objective and the specific aims the different chapters attempt to address. Chapters 1 and 2 provide a comprehensive introduction to the social network analysis approach. The main purpose of my thesis is to characterize the role the *foraging* (*for*) gene plays in social interactions and the group structure of *Drosophila melanogaster.* Specifically, my work addresses how *for* affects how flies interact in a group structure and their social network phenotypes.

The consequent two chapters address the method employed in studying group structure across this thesis. Chapter 1 is a literature review that aims to introduce the social network analysis (SNA) approach and its use in social insects. This chapter was accepted for publication in 2019 for the journal *Current Opinion in Insect Science* with the title "Network analyses reveal structure in insect social groups". The purpose of this review was to demonstrate the value of using the SNA approach to investigate group structure and social networks in social insects. It uses examples of studies that incorporate the SNA approach to explore several questions concerning insect social networks to illustrate the advantages of using this method. Chapter 2 is a comprehensive literature review that introduces the SNA approach and focuses on its use to study the social networks of *D. melanogaster.* Chapter 2 addresses the increased interest from the scientific community in applying the SNA approach in studies of *D. melanogaster,* the diverse research questions they attempt to address, and differences in the methodology of the SNA approach across studies. The purpose of this review was to compare the methods and findings in these studies in an effort to draw parallels between them.

Chapters 3 and 4 encompass the data chapters of my thesis. In chapter 3, I investigate the effect *for* has on social network phenotypes, and the findings were published in 2021 in the *Journal of Neurogenetics* with the title, "The *Drosophila melanogaster foragin*g gene affects social networks". This study shows that the *for* gene influences how flies interact in a group and the social network phenotypes. Using genetic manipulations, I examine the effect of dosage, as well as the genetic background, and confirm that differences seen between the rover and sitter social networks can be attributed to the *for* locus. These results establish a foundation for *for's* influence on social behaviour, and specifically on social interaction networks. Chapter 4 addresses follow-up questions and investigates the spatiotemporal effect of *for* on the social network phenotypes. Next, I explore *for's* plasticity in response to stress, pertaining to social

network phenotypes. Chapter 4 also explores how the natural polymorphism of *for,* rovers and sitters, interact when mixed in a group. Finally, chapter 5 provides a general discussion of my thesis work and its significance in the field.

Chapter 1:

Network Analyses Reveal Structure in Insect Social Groups

Nawar Alwash, Joel D. Levine

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Author contributions are detailed in the acknowledgments section of this chapter.

1.1 Abstract

Animals, from flies to humans, interact with each other, forming complex relationships and structured social interaction networks. These networks describe patterns of interactions that occur within a group. Social network analysis (SNA) is the statistical analysis of nodes, which represent individuals within a network who are connected by social ties, often called edges, that represent interactions between individuals. Here, we review recent studies on social interaction networks in insects with an emphasis on flies. In flies and other insects, SNA has revealed the contribution of group structure to disease transmission, feeding strategy, fighting, mating, and oviposition. The literature shows that SNAs are useful to understand mechanisms underlying group behaviour as well as the evolution of social structure.

1.2 Introduction

Social interactions are inescapable. Most animal species exist in a social world that consists of individual members interacting with each other to form complex relationships. Such interactions form patterns that give rise to human social networks (Fowler et al., 2009). Yet, social networks are not restricted to human societies; many species including other primates, birds, fish, and insects also form social networks and the application of social network analysis (SNA) is thought to be useful for characterizing the structure of social groups (Croft et al., 2004; Fischhoff et al., 2009; Krause et al., 2009; Lusseau, 2003; Pereira et al., 2019). Four common social network measures are shown in Figure 1.

Recently, SNAs have attracted increasing interest and curiosity from the research community, partly due to advances in machine vision and computational algorithms that facilitate identification and tracking of individuals in groups. These analyses have also been applied to understanding how information is transmitted within groups (Kacsoh et al., 2018; Pasquaretta et al., 2016). One key example related to information flow is the study of disease transmission (Drewe, 2010; Otterstatter & Thomson, 2007; Rushmore et al., 2013). High centrality values were found to drive the spread of diseases in a population of wild chimpanzees (*Pan troglodytes*) (Rushmore et al., 2013) and meerkats (*Suricata suricatta*) (Drewe, 2010). Also, interaction networks are important for determining parasite transmission dynamics in bumble bee (*Bombus*

impatiens) colonies: Otterstatter and Thomson (2007) showed that interaction rates of individuals are important predictors of parasitic transmission and infections spread more quickly in dense social networks. Understanding the spread of disease using SNA sheds light on characteristics of interactions between conspecifics as well as the use of social information within a network. For example, SNAs were used to look at the relationship between grooming interactions and the centrality of individuals within a network of mandrills (*Mandrillus sphinx*) (Pereira et al., 2019) and wild chimpanzees (*P. troglodytes*) (Shimada & Sueur, 2014).

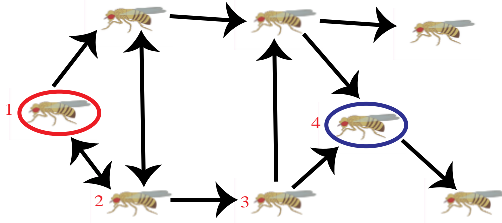
Bret et al. (2013) used SNAs to investigate the relationship between ranks, network stability, and cohesion in mandrills (*M. sphinx*). They found that the highest-ranking females played a central role in group structure and stability, and removal of these central individuals resulted in network fragmentation. Additionally, as social networks are studied in different species, interspecific comparisons may contribute towards understanding the evolution and maintenance of sociality (Fewell, 2003; Wey et al., 2008). SNA approaches provide a conceptual framework for researchers to study the organization of social behaviour at different levels such as individual organisms, interacting pairs, and group level operations. For example, consider that interactions such as aggression and courtship may co-occur within a group in a manner that highlights the complexity of networks. The co-occurrence of courtship networks and aggression networks suggests that group members make complex decisions about what to do and with whom. Indeed, Golemiec et al. (2016) have shown that the ability to correlate networks defined by different functional interactions can enrich understanding of group behaviour in kindergarten children and may challenge assumptions about social hierarchies.

Insect societies are well known for their social complexity and thus are great models for the study of social organization and evolution. SNAs of insects, such as *Apis mellifera* and *Drosophila melanogaster* highlight the social diversity of networks, providing insights into the complexity of these ‘simple’ organisms. Moreover, the ability to study social networks in flies is promising because *D. melanogaster,* in particular, is a genetically tractable organism (Bellen et al., 2010). Hypothetically, such mechanisms could include patterns of social interaction.

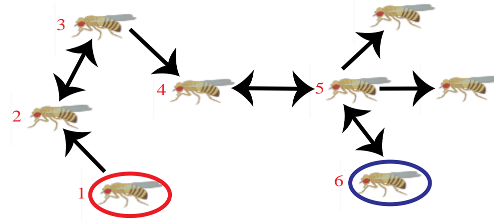
In this brief review, we summarize and discuss recent studies addressing group level social behaviour of insects. We emphasize studies that use SNAs to investigate networks of insects,

mostly, but not exclusively flies. The aim of this review is to illustrate insights provided by SNA in insects with the idea that such insights contribute to a more general understanding of animal social behaviour.

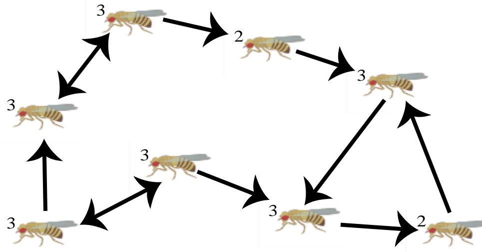
(a) High Global Efficiency



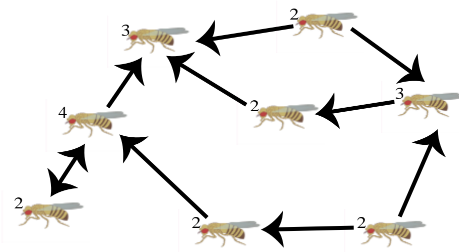
(b) Low Global Efficiency



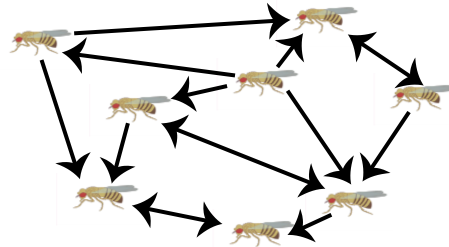
(c) High Assortativity



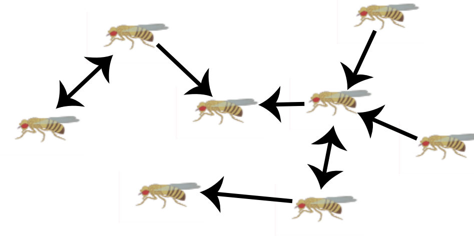
(d) Low Assortativity



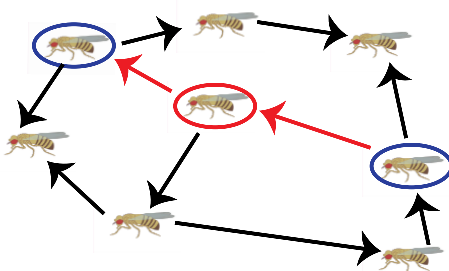
(e) High Clustering Coefficient



(f) Low Clustering Coefficient



(g) High Betweenness Centrality



(h) Low Betweenness Centrality

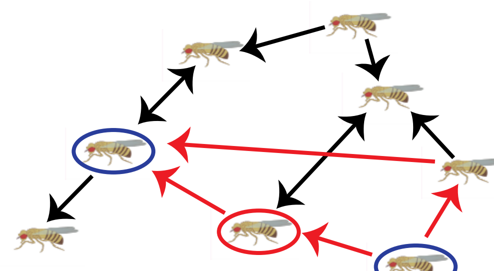


Figure 1.1 (Figure 1). Network measurements: (a, b) Global efficiency is a measure of redundant pathways and represents the efficiency of information flow in a network (Latora &

Marchiori, 2001a). Information flow begins at the fly circled in red and ends at the fly circled in blue. Numbers in red indicate steps for information flow from fly circled in red to the fly circled in blue. (a) High global efficiency network; for information to flow from red to blue it takes a total of four steps. (b) Low global efficiency network; for information to flow from red to blue it takes a total of six steps. More interactions are required for information relay in (b) than (a) indicating a less efficient flow of information. (c, d) Assortativity is the probability of an individual interacting with another individual of similar degree (Newman, 2010). Degree is defined as the number of interactions that transverse an individual (Newman, 2010). Numbers next to flies in networks indicate degree of flies. (c) High assortativity network; more homogenous network in which flies of similar degrees are more likely to interact. (d) Low assortativity network; more heterogeneous network in which flies of dissimilar degrees are more likely to interact. (e, f) clustering coefficient is a measure of network interconnectedness (Newman, 2010). (e) High clustering coefficient network; individuals that are more likely to interact with their neighbors. (f) Low clustering coefficient network; individuals are less likely to interact with their neighbors. (g, h) Betweenness centrality is a measure of the number of shortest paths that traverse an individual, indicating the relative importance of a given individual for information relay (Newman, 2010). Betweenness centrality measures of the flies circled in red are of interest for the information flow between the two flies circled in blue. Red arrows indicate possible paths of information flow between the individuals in blue. (g) High betweenness centrality network; the fly circled in red has a high betweenness centrality measure and if removed from the network, information can no longer flow between the two flies circled in blue. (h) Low betweenness centrality; the fly circled in red has a low betweenness centrality measure and if removed from the network, information can still flow via an alternative pathway.

1.3 Social nature of insects

As previously mentioned, many studies have addressed the social nature of insects. Like other animals, individuals within an insect population interact with one another to form complex relationships and social structures. Insect social interactions lead to the transfer of social information and the potential to reform behaviour (Battesti et al., 2012; Franklin & Franks, 2012; Kacsoh et al., 2018). This has been shown for mating and group foraging as well as social learning, disease transmission, and division of labor within insect populations (Alem et al., 2016; Battesti et al., 2012, 2014; Biganski et al., 2018; Danchin et al., 2018; Dawson et al., 2018; Dawson & Chittka, 2014; Durisko et al., 2014; Folly et al., 2017; Franklin & Franks, 2012; Grüter & Farina, 2009; Kacsoh et al., 2018; Leadbeater & Chittka, 2008; Loukola et al., 2017; Loyau et al., 2012; Mc Cabe & Farina, 2009; Mery et al., 2009; Molet et al., 2009).

More generally, interaction patterns between pairs of individuals lead to the formation of structured social networks that enhance the flow of information within a group. Social information allows individuals to modify their behaviour in response to conspecifics in the

environment (Heyes & Galef, 1996). Acquisition of novel social information from other individuals through observation or interaction is termed social learning (Heyes & Galef, 1996). Previous studies have shown that insect species exhibit social learning and use social information to make fitness-enhancing decisions (Battesti et al., 2012, 2014; Dawson & Chittka, 2012; Durisko et al., 2014; Grüter & Farina, 2009; Leadbeater & Chittka, 2008; Mc Cabe & Farina, 2009; Molet et al., 2009). In *D. melanogaster* oviposition site choice is influenced by social learning and interaction with conspecifics (Battesti et al., 2012, 2014). In addition, social learning provides members of a social network with useful information regarding foraging and food resources. Many species including *D. melanogaster* (Durisko et al., 2014), bumblebees *Bombus terrestris* (Dawson & Chittka, 2012; Leadbeater & Chittka, 2008; Molet et al., 2009), honeybees *A. mellifera* (Grüter & Farina, 2009), and stingless bees *Melipona quadrifasciata* (Mc Cabe & Farina, 2009) use social cues to gain information about profitable food resources and foraging skills.

Mate choice was also shown to be influenced by social learning. The use of social information in mate-choice decisions may enhance fitness (Danchin et al., 2018; Mery et al., 2009), for example, *D. melanogaster* females learn to avoid mating with semen-limited males by observing males' previous copulating activities (Loyau et al., 2012). Social learning can also aid in predator avoidance and response (Dawson & Chittka, 2014; Kacsoh et al., 2018). Bumblebees *B. terrestris*, for example, appear to use social information to identify safe flowers specifically in dangerous environments. This is shown by their preference to land on flowers occupied by conspecifics (Dawson & Chittka, 2014). Furthermore, task-solving behaviour is also observed in insects via social learning. Bumblebees can learn to solve tasks and obtain rewards and this task-solving behaviour can be socially learned through observation of trained individuals (Alem et al., 2016; Loukola et al., 2017). For instance, naïve bees can learn how to pull strings by observing trained demonstrators (Alem et al., 2016).

Moreover, the transmission of disease is influenced by interactions between individuals within a population. In fact, multiple studies have looked at social interactions between individuals and the rate of disease transmission in insect networks. In flies and bees, susceptible individuals may avoid interactions with infected individuals, or infected individuals may themselves reduce their

social interactions and isolate themselves in order to inhibit disease transmission (Biganski et al., 2018; Dawson et al., 2018; Folly et al., 2017).

Social isolation was found to have adverse effects on the fitness of insects, suggesting the importance of network dynamics. Social isolation leads to a reduced lifespan in flies and ants (Koto et al., 2015; Ruan & Wu, 2008) and also leads to an increase in aggression in *D. melanogaster* (Zhou et al., 2008). Insects that are socially isolated also exhibit impairment in the development of the mushroom body, a brain structure used to integrate multimodal input in carpenter ants, *Camponotus floridanus*, and the vinegar fly, *D. melanogaster* (Seid & Junge, 2016; Technau, 2007). Social isolation also leads to reduced foraging activity and reduced ability to find a mate in the cockroach, *Blattella germanica* (Lihoreau et al., 2009). Prior social isolation results in an increase in social space between individual *D. melanogaster* while increased social experience such as mating decreases social space (Simon et al., 2012).

Social interactions and social learning are not limited to conspecifics. For instance, Kacsoh et al. showed that 15 species across the genus *Drosophila* were able to convey a predatory threat to members of their own kind and additionally to members of several closely related *Drosophila* species (Kacsoh et al., 2018). Bumblebees also learned from the behaviour of sister species to locate valuable floral resources (Dawson & Chittka, 2012).

In this section, we have indicated the presence of interaction patterns and the potential contribution of the implicit social networks to feeding, mating and predator avoidance via social learning as well as to the spread of disease. We touched on the possibility that a specific network might provide information to other species facing similar threats. We also noted how the effects of isolation reinforce the contribution of a network on its members. In the next section, we discuss studies that have explicitly applied SNA to insects.

1.4 Social analyses of insect networks

The presence of networks in a widespread variety of species (Krause et al., 2009) suggests that there may be evolutionarily conserved principles of social interaction. Until recently, however, the social network approach has been mainly restricted to the description of groups of fish, birds, and other organisms. More recently, an increasing number of studies have begun to investigate

insect social networks. These studies often highlight phenomena that are relevant to natural behaviours in groups.

1.4.1 Disease transmission and division of labor

Dawson et al. (2018) investigated cancer progression and sociality by studying interactions between frequency and duration of social interactions were also influenced by the fly state and its social environment; a cancerous fly placed in a group of similarly cancerous flies had more frequent social interactions and longer interaction durations compared to those placed in a group of healthy flies. These results suggest that the composition of a group, that is, the social environment, strongly affects social interactions of cancerous flies. Another study by Otterstatter and Thomson looked at contact networks of *B. impatiens* and pathogen transmission. They found that the rate of interactions with infected nestmates was positively associated with a higher risk of infection (Otterstatter & Thomson, 2007). Social networks of *Camponotus fellah* provided novel insights into the division of labor. They found that these species form three interconnected social sub-groups that perform different tasks within the colony, and that these groups differ in their interaction patterns, both within and between groups (Mersch et al., 2013). Individuals in networks of cancerous and healthy *D. melanogaster* (Dawson et al., 2018). The social environment of cancerous flies affected their tumor progression; faster tumor progression was observed in isolated flies and those kept in a healthy group relative to flies kept in a cancerous group.

1.4.2 Feeding, fighting, copulation, and social structure

Researchers that have used the SNA approach to investigate insect behaviour have focused on those formed by *D. melanogaster*. *D. melanogaster* display an array of social behavioural interactions including courtship, mating, aggression, and foraging in groups. Schneider et al. (2012) used the SNA approach to show that *D. melanogaster* form non-random complex social interaction networks. Placing *D. melanogaster* in groups affects their patterns of behaviour, as well as directly influences individual members within the social network. Two different wild-type strains of *D. melanogaster* were tested (*Canton-S* and *Oregon-R*) that are known to carry genetic differences. These wild-type strains formed social networks with different properties.

Specifically, centrality measures differed between the two strains indicating that there are significant differences in the average number of individuals that are important for information relay. Because of the fact that these two wild-type strains are known to carry genetic differences, these results suggest that there is a genetic component that influences network phenotypes. In addition, sensory modalities were found to be important in the formation of social networks. Olfactory mutants formed networks that were different from controls, with a significantly lower rate of movement and interactions. In addition, olfactory mutants formed networks with lower global efficiency compared to the control and so information flow was less efficient in networks of olfactory mutants. These results suggest that chemosensory cues influence interactions within the group. Similarly, tactile cues were also shown to be important for interactions and the structuring of networks (Schneider et al., 2012; Schneider & Levine, 2014).

1.4.3 Oviposition

Pasquaretta et al. (2016) used SNA to investigate how decision making in *D. melanogaster* is influenced by its social environment. To address this issue, they demonstrated how oviposition site choice is influenced both by interactions between informed and uninformed mated females, and by the homogeneity of the individuals in their network. Homogeneous networks are those in which the majority of individuals make the same choice. Informed flies were conditioned to prefer one of two scents for their egg-laying medium. Naïve flies were then tested for preference. Results of this study showed that variance in centrality measures affected decision making. Decreased variance in the centrality parameters leads uninformed flies to adopt the oviposition choices of their informed counterparts. These results suggested that flies adjust their choices based on how well coordinated the behaviour of informed individuals in their network appears to be, that is, it was influenced by the homogeneity of the network (Pasquaretta et al., 2016).

1.4.4 Network analyses in non-dipteran insects

Additional studies have used SNAs in other insect species. The stability and reproducibility of social networks of fungus beetles, *Bolitotherus cornutus*, were investigated after a disturbance. The disturbance consisted of capturing and isolating individuals from the network for four days. Both clustering coefficient and betweenness centrality measures were more consistent in the

disturbed networks compared to their undisturbed controls. These results showed that disturbed networks produce consistent, reproducible network configurations that are consistent with a response to some external threat (V. Formica et al., 2017). Networks of cockroaches *Diploptera punctate* were examined. Females were significantly more gregarious and clustered than males in the network. This female social clustering was found to be beneficial in terms of limiting male harassment and improving fitness (Stanley et al., 2018). Finally, Stroeymeyt et al. (2018) used the SNA approach to investigate networks of the ant *Lasius niger* after exposure to a pathogen. Networks that were exposed to the pathogen displayed changes in network properties; an increase in clustering coefficient and a decrease in global efficiency measures. In addition, an increase in task assortativity was observed and this increased social segregation. Pathogen exposure leads to behavioural changes in both ants exposed to the pathogen and their nestmates in order to inhibit disease transmission, this was reflected in changes in properties of the networks formed (Stroeymeyt et al., 2018).

1.5 Conclusion

SNA provides researchers with a powerful tool for studying emergent patterns of behavioural interaction. SNAs provide a single conceptual framework for evaluating various interactive behaviours. Using SNA in the study of animal behaviour allows us to capture and quantify-specific attributes of social interactions and relationships. Although they are abstract, network measures provide a handle on group structure and information flow. Recent studies have shown that the presence of these structures corresponds to enhanced fitness. Furthermore, network analyses have gained popularity as a method for exploring principles of self-organization and information flow within groups. For example, how disease or a novel behaviour like the aforementioned string pulling (Alem et al., 2016) can be transmitted and spread within a group of interacting individuals.

Social network analyses in insects may facilitate hypotheses about the evolution and inheritance of social dynamics and social structure. It is now possible to test hypotheses about genetic and neural contributions to patterns of behaviour in groups. This brief review has touched on social networks, a complementary approach looks at how such patterns may emerge from a lexicon of individual behaviours and the rules that govern them (Berman, 2018). Stay tuned!

1.6 Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Chapter 2:

The Application of Social network analyses in *Drosophila*

Jacob Jezovit, Nawar Alwash, Joel D. Levine

This chapter is currently being prepared for publication.

Author contributions are detailed in the acknowledgments section of this chapter.

2.1 Abstract

Many animal species live in groups and interact with each other, creating a complex social environment. Group dynamics are shaped by the interplay between the behaviours of individuals within these groups and the group-level structure. Our understanding of collective behaviour and the social structure has advanced due to the application of the social network analysis (SNA) approach. SNA is a statistical tool that aids in revealing and understanding the patterns of interacting individuals. Recently, there has been increased interest from researchers in applying the SNA approach to model organisms, and in particular *Drosophila melanogaster*. Although the number of studies investigating the social networks of *Drosophila* are still limited, they address a wide array of questions and provide incredible insights into the complexity of sociality. However, many of these studies differ in the methodology of the SNA approach. Here, we aim to review these studies, comparing their scopes and the methods used, to draw parallels between them and the broader body of knowledge available. Indeed, we find similarities across studies investigating social isolation despite the different methods of the SNA approach being employed. Finally, this review aims to highlight this emerging field of *Drosophila* social networks. We also attempt to generate hypotheses and predictions that inspire future researchers and address burning questions in the field.

2.2 Introduction

Social interactions are pervasive in the lives of most animal species, and thus animals rarely act in isolation. Collective behaviour can be defined as the manifestation of group-level patterns produced by simple interactions between individuals (Sumpter, 2010). Animals provide a wealth of interesting collective behaviours, such as migrating geese flying in V-shaped formation; flocks of starlings turning in unison; schools of fish splitting and reforming while outmaneuvering a predator; collective foraging in honeybees; and the division of labour in ant colonies. The ubiquity of collective behaviours and emergent group structures across many animals suggests that individuals enhance their survival and reproductive output by organizing in groups. Our understanding of collective behaviour and social structure has advanced over the recent years, partially due to the use of social network analyses to study these behaviours.

Social network analysis (SNA) is an invaluable statistical tool that aids in understanding the patterns, underpinnings, and consequences of social structure and dynamics (Krause et al., 2009). Applications of SNAs originated in the 1930s to study sociological factors of human populations (Lewin, 1951; Moreno, 1934; Scott, 2000; Wasserman & Faust, 1994). Later, SNAs were applied to studying exclusively the social structure of nonhuman primates (Fedigan, 1972; Kudo & Dunbar, 2001; Pearl & Schulman, 1983; Sade, 1965; Sade et al., 1988; reviewed in Brent et al., 2011). Following pioneering studies by Lusseau (2003) and Croft (2004), SNA have been applied to additional animals such as birds (Boogert et al., 2014); fish (Croft et al., 2004); insects (Formica et al., 2017; Otterstatter & Thomson, 2007; Stanley et al., 2018; Stroeymeyt et al., 2018); and other mammals such as dolphins (Ilany & Akçay, 2016), elephants (Goldenberg et al., 2016), and giraffes to name a few (Shorrocks & Croft, 2009). In recent years SNA experiments have been more commonly conducted in the laboratory. This shift from applying SNAs in the field to the laboratory is a result of recent advancements in technology and analytical software that facilitate the automated identification and tracking of individuals in a network (Branson et al., 2009; Greenwald et al., 2015; Hong et al., 2015; Robie et al., 2017; Straw & Dickinson, 2009). SNAs provide researchers with a conceptual framework and the analytical tools to explore different aspects of social dynamics, structure, and function. It also allows researchers to capture and examine the interplay between different aspects of the social structure at the individual level, dyad level, group level, and population level, providing a deeper understanding of social complexity.

A *social network* can be defined as any number of nodes interconnected via social ties between them (Krause et al., 2009). *Nodes* are defined as social entities that represent an individual. *Edges* represent the connection between two nodes (social relationship or interaction), and these can be *weighted* or *unweighted*. Unweighted networks are binary and consider only the presence or absence of an interaction between individuals. Weighted networks, on the other hand, assign numerical values to all edges in the network which typically reflects the strength or frequency of interactions between nodes. In a *directed network* edges represent both the connection of nodes and the directionality of an incoming or out-going interaction, while in a *non-directed network*, edges only indicate that nodes are connected (see Figure 1). Finally, a social network represents connections between nodes over time. Conventionally, social networks may be *static*, meaning

all connections between nodes over some periods of time are represented in a single network that can be viewed as a history of social connections. More recently *iterative* networks have been studied, which refers to a process of generating multiple transient social networks over a set period to measure dynamic social properties of animal groups (Schneider et al., 2012; reviewed by (Blonder et al., 2012; Farine, 2017)).

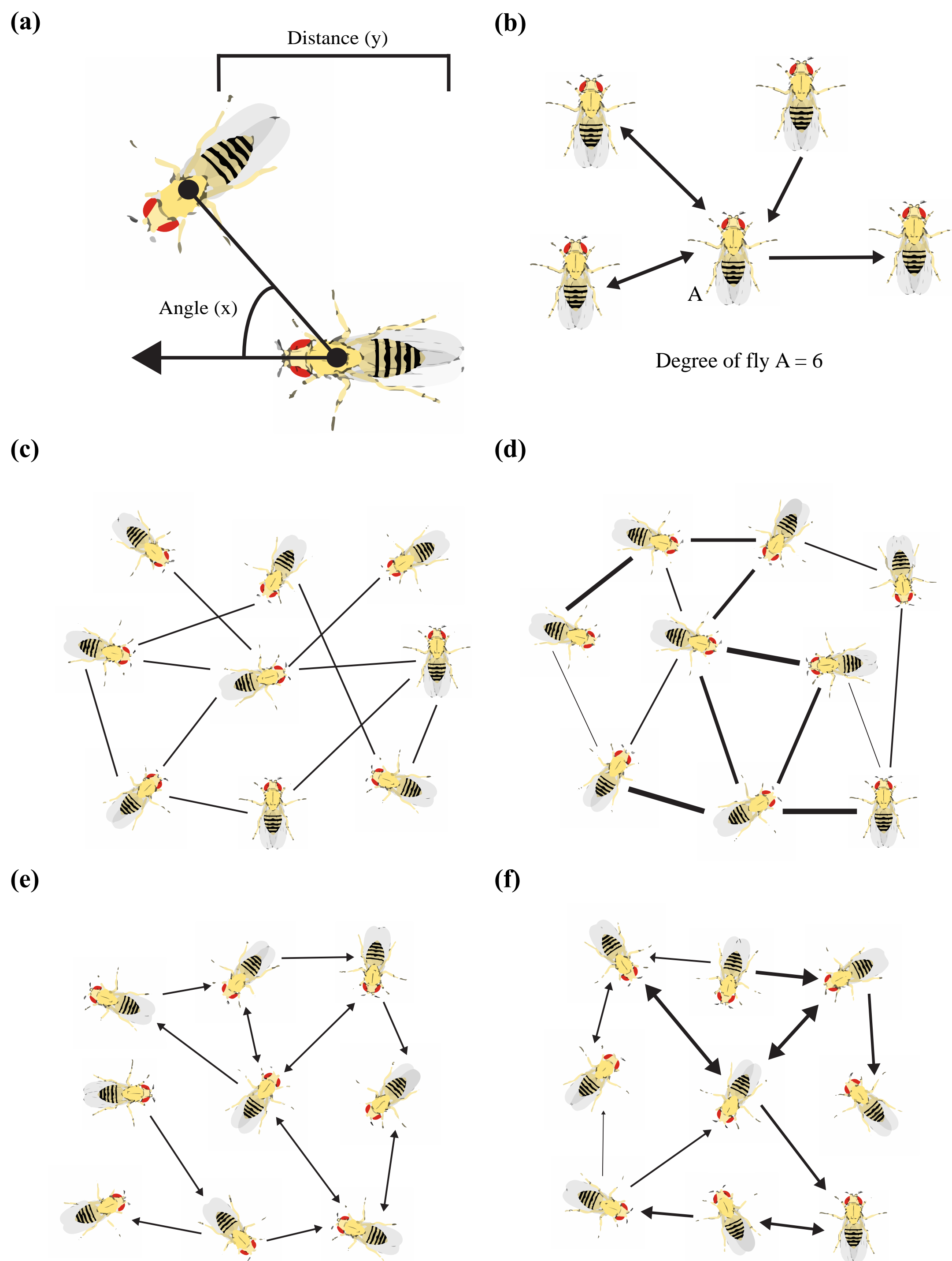

Figure 2.1 (Figure 1). Visualization of social space criteria and various forms of social networks. (a) Social space criteria for interactions between two flies is defined using (i) the angle

(x) connecting the center of the interactee fly relative to the interactor fly (shown with the arrow), (ii) the distance (y) between the two flies, and (iii) how long these conditions must be maintained for. (b) Degree is the number of incoming and outgoing interactions that transverses an individual. The fly labeled “A” has 3 incoming and 3 outgoing interactions giving it a degree of 6. (c) – (f) Visualization of network properties generated. Unweighted networks only consider the presence or absence of interactions between individuals (c & e), whereas interactions in a weighted network (d & f) reflect the strength or frequency of these interactions. In a directed network (e & f) the directionality of the interaction is recorded while this is absent in a non-directed network.

Both static and iterative social networks are scored through a variety of measures that are calculated from mathematical formulae and offer objective scores of individual and group sociality. Network measures may range from describing individual nodes to qualities of the entire network. *Degree* is the number of edges connected to a single node. In a directed network, *in-degree* represents the sum of incoming interactions and *out-degree* represents the sum of outgoing interactions from a single node. In weighted networks, the weighted degree of a node, also known as *strength*, is calculated as the sum of the edges’ weights connected to that node. In a directed and weighted network, the *in-strength* is the sum of the incoming edge weights and *out-strength* is the sum of the outgoing edge weights. The *density* of the network is defined as the proportion of actual connections between nodes over the potential number of connections between nodes in the network. This measurement dictates how densely individuals are connected throughout the network. There are a variety of *centrality* measures that aim to identify influential nodes in a network for information transmission. One example, *betweenness centrality*, is a measure of the number of shortest paths that traverse a node, which is informative of the overall role that node plays in social transmission or network cohesion. *Clustering coefficient* is a measure that reflects the cliquishness of nodes. *Assortativity* is a measure that reflects homophily as it measures the probability of an individual interacting with another individual of similar degree. Social network measures include global efficiency and modularity. *Global efficiency* is a measure of redundant pathways, and it represents the efficiency of transmission in a network. Although these measurements conventionally are calculated for single nodes in a network, one can average the score of all nodes to describe the overall structure of a social network. *Modularity* is a measure designed to quantify the degree to which a network could be divided into different groups or clusters. Networks with high modularity have dense connections within the modules but sparse connections between the modules. Modularity can be computed in

weighted, binary, directed or undirected networks. Additional social network measures referenced throughout this review, in addition to the terminology mentioned above, are defined in Table 1.

Table 2.1 (Table 1). A list common social network measurements defined by their both technical definition and their general applications.

Network Measure	Definition	Application	Reference
Degree	Number of edges connected to a single node. In-degree refers to the number of interactions a node receives and out-degree refers to the number of interactions a node outputs.	In all types of networks, degree informs how popular a single node is towards receiving and/or relaying connections.	Wasserman & Faust 1994
Strength	In networks with weighted edges, strength is the sum of all edge weights connected to a node. In-strength refers to the sum of all edge weights a node receives and out-strength refers to the sum of all edge weights a node outputs.	In weighted networks, strength informs overall how popular a single node is towards receiving and/or relaying connections relative to the weight of each connection.	Bentzur et al., 2020
Density	Proportion of actual connections in a network over the number of theoretically possible connections.	Measures to what extent the network connections are filled out between nodes.	Bentzur et al., 2020
Betweeness Centrality	Number of shortest paths that traverse a node.	Measures how central a node is in a network for relaying information and maintaining the network cohesion.	Newman, 2010
Weighted Closeness Centrality	Calculated as inverse between the shortest path between two nodes, from one node to all other nodes in the network and weighted for number of connections among nodes.	Measures how central a node is in a network for relaying information and maintaining the network cohesion.	Pasquaretta, Battesti, et al., 2016
Eigenvector Centrality	Directly related to the number of contacts a node has and to the relative weight of the nodes to which it is connected.	Measures how central a node is in a network for relaying information and maintaining the network cohesion.	Pasquaretta, Battesti, et al., 2016
Information Centrality Index	Calculated by combining all the paths present in a network and assigning a weight to them that is equal to the inverse of the path length.	It reflects the amount of information per individual contained in all possible paths that originate from and end with that individual.	Pasquaretta, Battesti, et al., 2016
Clustering Coefficient	A measure of how interconnected nodes are to one another.	Typically used to measure how cliquish nodes are in a network.	Newman, 2010

Modularity	A measure of how a network can be subdivided into clusters of sub-networks.	Typically used to measure how cliquish nodes are in a network.	Pons & Latapy, 2005
Assortativity	A measure of the homogeneity of the degree distribution of a network.	Distinguishes whether nodes in a network all have a similar degree.	Newman, 2010
Global Efficiency	A measure of redundant pathways in the overall network and how efficient information can spread.	Distinguishes whether the overall network has shorter paths between nodes or longer path.	Latora & Marchiori, 2001

The SNA approach has been used by scientists to shed light on the social structures of different animal species, their organization and stability. Studying the structure of social networks and their organization is important in understanding the biological basis of sociality. For instance, SNA allows researchers to consider how individuals drive the structure and properties of a network and how the entire network structure affects individuals nested within it. Various factors may influence an individual's position in a social network such as age (Baracchi & Cini, 2014; Liao et al., 2018), life history (Boogert et al., 2014; Brandl et al., 2019), and dominance rank (Mishra et al., 2020). For instance, Liao (2018) found an age dependent feature of rhesus macaque social networks where younger individuals tend to maintain higher numbers of social connections than older individuals. Also, Baracchi and Cini (2014) resolved a highly compartmentalized structure to honeybee colonies where younger bees are in closer contact with the queen. The effect of early life development on future network positions is apparent in birds. Boogert et al. (2014) experimentally demonstrated that zebra finches exposed to increased levels of an avian stress hormone resulted in individuals interacting less with kin, leading to a more centralized position in foraging networks compared to control finches. An additional experiment on wild zebra finches demonstrated that increased brood size produces similar effects, highlighting how the social environment plays a role in shaping the behaviour of animals later in life (Brandl et al., 2019). Similarly, animals in groups often assort into hierarchies and the dominance rank of an individual may be visible in a network structure. Mishra et al. (2020) investigated the relationship between hierarchy and centrality in a grooming network of long-tailed macaques and found that middle-ranking, and not high-ranking individuals, had the most central roles in a grooming network. In ant colonies, Mersch et al. (2013) identified through SNA how individuals form separate clusters based on division of labour roles. Worker ants were divided into nurses that exclusively interact with the queen and her brood, while foragers and cleaners assemble separate clusters due to their different roles within the colony (Mersch et al.,

2013). On the contrary the position an individual holds in a network may influence their fitness. It has been demonstrated in multiple animals that individuals that are more central in a social network and who are less cliquish tend to have a higher copulation success (Farine & Sheldon, 2015; Formica et al., 2012; Oh & Badyaev, 2010).

In recent years, there has been increased interest in applying SNAs to the genetic model organism, *Drosophila melanogaster*. Although the number of these studies is limited, the research questions addressed are surprisingly diverse. The studies also provide insight into the social diversity and complexity of these 'simple' organisms. However, many of these studies differ in the core methodology of SNA at the experimental, statistical, and conceptual levels. Our aim in this review is to compare the scope, objectives and methods of these studies, and attempt to draw parallels between them and the broader literature of animal social networks. Finally, in this review we intend to highlight the benefit of *Drosophila* insects towards studying complex social phenomena. We also attempt to generate hypotheses and predictions that may inspire future experiments in this growing field of animal behaviour.

2.3 *Drosophila* Social Networks

2.3.1 Social space

SNA relies on a concrete definition of social behaviour to fill connections between nodes. Across animal species this varies – for example, primate social networks are often represented by grooming interactions while ant social networks are represented by physical contact between the antennae of multiple ants in proximity (Blonder & Dornhaus, 2011). For the fruit fly, courtship and aggression have long been considered their exclusive social behaviours. Decades of investigation into the genetic, neurological, and physiological basis of social behaviour in *Drosophila melanogaster* offers the consensus that various combinations of visual, acoustic, tactile, and chemosensory cues are involved in the communication between conspecifics (Agrawal et al., 2014; Bontonou & Wicker-Thomas, 2014; von Schilcher, 1976). Yet, to our knowledge, no courtship nor aggression networks have been successfully applied to *Drosophila*. What forms of social communication occur in a homogenous, single-sex group? As we will discuss below, social networks in *Drosophila* involve physical encounters between conspecifics,

like social network analysis in ants and bees (Blonder & Dornhaus, 2011; Gernat et al., 2018). In this section, we discuss the *social space* of flies, defined as spatiotemporal positions between multiple flies in proximity, and justify it as a reliable method of scoring social communication in any social context.

The first observation of social space in *Drosophila* is credited to Sexton and Stalker who noticed that groups of female *Drosophila paramelanica* touch one another with their forelegs to maintain uniform spacing at high group density (Sexton & Stalker, 1961). This observation was rediscovered by Schneider et al. (2012) over 50 years later in *Drosophila melanogaster*. Repeated video recordings of flies in a homogenous group revealed repeated 'touching' behaviour, which involves an 'interactor' touching, with its foreleg, either the body, wing, or leg of an 'interactee'. Before touching, the interactor would typically approach the side of the interactee's body, unlike in courtship when males tap the rear of a female's abdomen. This behaviour can be classified using three interaction criteria parameters: i) *distance* of the shortest line segment connecting the centre of mass between the interacting flies; ii) *angle* of the line segment connecting the centres of mass of both flies and the line segment protruding from the head of the interactor; iii) the *time* fulfilled during these touch encounters (Figure 1). Through observation, Schneider et al. defined a social encounter between multiple flies as distance ≤ 2 body lengths, angle ≤ 90 degrees, and time ≥ 1.5 seconds. Since this was repeatedly observed in a social context devoid of courtship behaviour, these social space criteria arguably represent the most basic unit of social communication in flies. As flies house gustatory taste receptors within bristles on their legs (Vosshall, 2007), it is possible flies touch conspecifics to taste cuticular hydrocarbons and other chemical secretions as a form of social communication. Additional studies have applied similar interaction criteria for scoring social interactions, with some modification that involved relaxing the angle criterion (Bentzur et al., 2020) and restricting the distance criterion to as low as one body length (Dawson et al., 2018; Liu et al., 2018).

A critical limitation to the criteria defined by Schneider et al. (2012) is the fact that it was derived by subjective observation of video footage. This was largely solved by the development of an algorithm that analyzes spatial positioning between all flies across merged video recordings and maps the typical *social space* of flies in an unsupervised fashion (Schneider & Levine, 2014). More specifically the algorithm automatically computes distance, angle, and time criteria

that are over-represented across videos of real flies interacting in a group versus randomized spatial positions of null flies. For the remainder of this review, we will refer to criteria generated from this algorithm as “automated criteria” and all other criteria derived from human observation as “defined criteria”.

The automated criteria algorithm was first applied to male and female *Canton-S* and *Oregon-R* strains. The automated social space criteria that was computed differed from the previous defined criteria (Schneider et al., 2012). The distance parameters ranged between 1.75 and 2 body lengths, the angle parameters ranged between 115 and 160 degrees and the time parameters ranged between 0.4 and 0.6 seconds (Schneider & Levine, 2014). Using different methods of image analysis over time, Simon et al. (2012) and Jiang et al. (2020) demonstrated that the average nearest neighbour distance between flies studied in a group converges between 1.5 and 2 body lengths. Other researchers studying group dynamics in *Drosophila* have also applied a distance criterion between 1 and 2 body lengths based on their own observation (Bentzur, 2020; Pasquaretta 2016). Overall, multiple studies suggest 2 body lengths may be a reliable consensus for the physical interactions that occur between individual flies in a group setting.

When using social space criteria to score the social behaviour of flies in a group, it is important to consider how to minimize false-positive interactions. For instance, the automated criteria estimated by Schneider & Levine (2014) displayed an increase in the distance and angle parameters and a decrease in the time parameters compared to the previous defined criteria (distance ≤ 2 body lengths, angle ≤ 90 degrees, and time ≥ 1.5 seconds; Schneider et al., 2012). A wider distance and angle parameter and a shorter time parameter naturally would increase the number of interactions and indeed Schneider & Levine (2014) reported an increase of a couple of thousand social interactions with the revised criteria. Additionally, false-positive social interactions may occur when two flies, interacting over long periods of time, momentarily slip outside the bounds of interaction criteria. Stricter social space criteria have been applied by other researchers, likely with the intention of minimizing the number of interactions. One straightforward approach is reducing the distance parameter so that social interactions are only counted when flies are in close proximity. For example, Liu et al. (2018) recorded interactions exclusively when one fly’s head approached and touched another fly’s rear. Another strategy is the implementation of a ‘gap length’ parameter, which is a set time interval required to elapse

before additional interactions between the same pair of flies are counted (Bentzur et al., 2020; Liu et al., 2018). Bentzur et al. (2020) reported that implementing a gap length of 4 s substantially reduced the number of consecutive interactions occurring between the same pairs of flies. Overall, when defining social space criteria, there is a trade-off between filtering false-positive and accepting the loss of true positive interactions and balancing this depends on the experimental approach whether it is generating social networks or monitoring aggressive or sexual interactions in flies. Flies engaging in aggressive or sexual acts may posture their bodies differently than the touch events described previously and adjusting social space criteria to reflect this may become useful towards future pursuits in automated behavioural classification of *Drosophila.*

2.3.2 Social networks

Within recent years, there has been an increased interest in applying SNA to study the sociality of *Drosophila* insects from computational, behavioural, neurobiological, and evolutionary perspectives (Alwash et al., 2021; Bentzur et al., 2020; Jezovit et al., 2020; Jiang et al., 2020; Liu et al., 2018; Pasquaretta, Battesti, et al., 2016; Pasquaretta, Klenschi, et al., 2016; Rooke et al., 2020; Schneider et al., 2012; Schneider & Levine, 2014). All these studies consist of analyzing video footage tracked by machine vision software and applying social space criteria to generate social networks based on physical encounters between multiple flies. Across these studies, similar experimental questions have been addressed (see Table 3), but this is also obfuscated by differences in the methodology of social network analysis (see Table 2). In this section, we review some recent social network analyses applied to *Drosophila* insects, we comment on the similarities, and we point out differences. We also attempt to connect key findings from these experiments to the broader animal social network literature.

Table 2.2 (Table 2). A comparison of all published-to-date *Drosophila* social network studies with their network analysis methods summarized.

Publication	**Social Space Criteria**	**Summary of network analysis**	**Group size**	**Length of video recordings**	**Tracking Software**	**Post-tracking correction**
(Schneider et al., 2012)	Time: 1.5 seconds Distance: 2 body lengths Angle: 90°	Unweighted, directed, iterative	12 flies	30 minutes	Ctrax	Yes (Fixerrors)
(Pasquaretta, Battesti, et al., 2016)	Time: 0.5 seconds Distance: 1 body length	Weighted, directed, iterative*	12 flies	4 hours	Ctrax	Yes (Fixerrors)
(Pasquaretta, Klenschi, et al., 2016)	Time: 0.5 seconds Distance: 1.1 body length	Weighted, directed, static	12 flies	4 hours	Ctrax	No
(Liu et al., 2018)	Touch only: Head to tail contact for 0.5 seconds Gap length between interactions: 0.5 seconds	Weighted, undirected, static	16 flies	1 hour	Flytracker	No
(Bentzur et al., 2020)	Time: 2 seconds Distance: 2 body lengths Angle: <0°	Unweighted, directed, iterative	10 flies	15 minutes	Ctrax	Yes (FixTRAX)
(Jezovit et al., 2020)	Automated method (Schneider 2014)	Unweighted, directed, iterative	12flies	30 minutes	Ctrax	Yes (Fixerrors)
(Rooke et al., 2020)	Automated method (Schneider 2014)	Unweighted, directed, iterative	6 flies, 12 flies, 24 flies	30 minutes	Ctrax	Yes (Fixerrors)
(Alwash et al., 2021)	Automated method (Schneider 2014)	Weighted, directed, static	12 flies	30 minutes	Ctrax	Yes (Fixerrors)

Table 2.3 (Table 3). A summary of the research objectives and hypotheses tested in all published-to-date *Drosophila* social network studies.

Research objective	**Publications**
Quantification of *Drosophila* social networks and group formation	Schneider et al., 2012; Pasquaretta, Battesti et al., 2016; Liu et al., 2018; Bentzur et al., 2020; Jezovit et al., 2020; Rooke et al., 2020; Jiang et al., 2020; Alwash et al., 2021
The experimental effects of social isolation on social networks and group formation	Schneider et al., 2012; Liu et al., 2018; Bentzur et al., 2020
The experimental effects of sensory deprivation on social networks and group formation	Schneider et al., 2012; Bentzur et al., 2020; Jiang et al., 2020; Rooke et al., 2020
Analysis of social space	Schneider & Levine, 2014; Jiang et al., 2020
Diffusion analysis - modeling spread of information flow between flies	Pasquaretta, Battesti et al., 2016; Pasquaretta, Klenschi et al., 2016
The experimental effects of density and group size on social networks	Rooke et al., 2020
Phylogenetic comparative methods - modeling the evolution of social networks and group formation	Jezovit et al., 2020
Genetic underpinnings to social networks and group formation	Alwash et al., 2021
Investigation of social networks from mixed groups	Pasquaretta, Battesti et al., 2016

2.3.2.1 Video acquisition, tracking and network generation

First, the precision of social network data depends on reliable, error-free video tracking. The number of errors generated by video tracking is dependent on the level of contrast between the flies and the background, the length of the videos, and the number of flies (Robie et al., 2017). The most common tracking platform across the *Drosophila* social network literature is Ctrax, an open-source machine vision tracker (Branson et al., 2009). An inconvenient limitation of Ctrax is the requirement to tediously review the tracking data for errors that involve, for example, inconsistent identification of the same individual fly, or changes in the size and orientation of the tracks. Each of these errors would require manual review and correction. In a recent experiment that repeatedly filmed 10 flies in an arena for 15 minutes, an automated error fixing script was applied to edit the tracking errors of Ctrax (Bentzur et al., 2020). An alternative tracker, called

Flytracker, has been developed that claims to produce error-free tracking and was applied in an experiment that repeatedly filmed 16 flies for one hour (Liu et al., 2018). While these alternatives may increase the speed of data collection, there is always the danger of harboring tracking errors that could lead to a loss of precision and integrity of the SNA. This can be minimized if the tracking data is thoroughly reviewed and corrected by the user as done by other studies (Alwash et al., 2021; Jezovit et al., 2020; Rooke et al., 2020; Schneider et al., 2012).

All *Drosophila* social network studies published to date have utilized both the static and iterative network approach. Multiple studies have applied an iterative approach first published by Schneider et al. (2012). This method generated directed, unweighted, iterative networks in replicated groups of 12 flies from multiple video sources (n=43 videos). The iterative networks were all generated at a controlled network density from a sliding boxcar filter (Kossinets & Watts, 2006). To summarize, one network iteration is generated exclusively from unique interactions. When an additional unique interaction is observed, the oldest unique interaction is removed from the network and the newest interaction is added and this forms the second network iteration., This pattern continues and can produce hundreds or thousands of social network interactions in a single video, all offering snapshots of changing network structure over time. To score and compare the network measures of different types of fly groups, the properties of every node within a network iteration are averaged. Then each mean iteration is standardized to thousands of networks generated by null flies with similar in-degree and out-degree characteristics. The result is an averaged z-score of all network iterations per video, which aims to score the properties of the entire organized group of flies (Schneider et al., 2012). Aside from the studies utilizing these exact methods (Alwash et al. 2021, Jezovit et al. 2020, Rooke et al., 2020), we are unaware of other *Drosophila* SNA experiments that normalize networks in this fashion.

2.3.2.2 Social Isolation

While the methods of generating and analyzing *Drosophila* social networks differ, one feature most of the studies have in common is how social experience affects social structure (Bentzur et al., 2020; Liu et al., 2018; Schneider et al., 2012). Schneider et al. (2012) examined the effect of three-day social isolation on repeated network properties of a group of 12 flies. This was done by

measuring network properties from a fully isolated group, compared to controls. However, no significant differences were found in the average network measures between these treatments (Schneider et al., 2012). A limitation to this study was the use of the same defined criteria (2 body lengths, 90 degrees, 1.5 seconds) for the isolated flies and socialized flies. This does not consider potential differences in the social space of isolated versus socialized flies. Therefore, to better understand how social experience influences the behaviour of flies we re-analyzed the Schneider et al. (2012) data using automated criteria (Figure 2). Indeed, we find that socially isolated flies tend to engage in longer social interactions than their controls (housed-together treatment). On the other hand, socialized flies that were paired with unfamiliar flies (mixed-together treatment) during experiments tend to have a shorter interaction time compared to the housed-together treatment. We then generated social networks using the iterative approach and found that social isolation significantly affects the network structure. For example, global efficiency is significantly higher in isolated flies indicating that isolated flies have more redundant connections in their networks. Isolated flies also display a significantly lower betweenness centrality, which indicates that there are fewer central individuals serving as a hub in the network. Across all measures, we observe greater variability in networks of isolated flies compared to the controls, particularly in assortativity and clustering coefficient. Lack of social experience in these groups of isolated flies may be contributing to these less predictable network measures. Other behavioural measures, such as the average interaction rate and percentage of interactions reciprocated, were also significantly lower in groups of isolated flies. Therefore, social isolation affects both individual flies and the social structure of the group (Figure 2).

Another experiment socially isolated flies differently. Rather than isolate virgin flies for three days, Liu et al. (2018) isolated 9-day old flies for six days. These authors applied the more conventional static network generation methods. Replicates of directed and weighted social networks were generated from multiple video sources and then averaged using the Graduated Assignment algorithm (Gold & Rangarajan, 1996). This algorithm averages replicates of static networks from multiple video sources (n = 10 videos), corresponds individual nodes with similar characteristics from separate networks and produces a composite social network. Analysis of these networks revealed that groups of 16 flies that had been isolated for six days tend to have a higher clustering coefficient than groups of socialized flies. Additionally, a time course of one-

day to six-day long isolation treatments showed that the average clustering coefficient is significantly greater than that of socialized flies at all time points. This suggests that a single day of isolation is sufficient to alter the social structure of flies. Although not significant, we find a similar trend for clustering coefficient in the re-analyzed Schneider et al. (2012) data (Figure 2). Liu et al. also found assortativity displayed no significant difference between isolated and socialized flies, which also agrees with our re-analyzed data (Figure 2).

A more recent experiment by Bentzur et al. (2020) also found social isolation affects fly networks. Like Schneider et al., these authors collected flies as virgins and isolated or socialized them for three days before recording their behaviour. In this study, the authors generated a single static network, per video, and networks were analyzed two ways: 1) weighting nodes by the number of interactions to emphasize short and acute social patterns; 2) weighting nodes by the length of interactions to emphasize long-lasting social interaction patterns. The authors found that isolated flies displayed a lower average betweenness centrality than socialized flies in networks weighted both by the number of interactions and length of interactions, which agrees with the re-analyzed data (Figure 2). However, they also found that, on average, isolated flies socially cluster less and have a lower modularity score in networks weighted both by the number of interactions and length of interactions. This disagrees with the findings that flies in isolation tend to produce networks with higher clustering coefficient ((Liu et al., 2018), see also Figure 2). In addition to measuring social networks of isolated versus socially experienced flies, Bentzur et al. (2020) measured an impressive repertoire of ethogram-like fly behaviours using the machine learning algorithm JAABA (Kabra et al., 2013). The authors found that isolated flies were more active and displayed a higher incidence of physically touching other flies, chasing other flies, and approaching other flies. Higher locomotor activity and increased social interactions of isolated flies is consistent with results from Liu et al. (2018). However, higher locomotor activity may lead to more frequent social interactions, resulting in a higher network density, and a higher average degree/strength. Indeed, this was reported in isolated flies by Liu et al. (2018) and Bentzur et al. (2020) and highlights a limitation of static networks. Without controlling the network density, groups of flies that interact frequently may produce different network measures. Therefore, the network measures would not be comparing differences in group organization, but rather differences in individual interactions within groups. Interestingly, in networks weighted by

length of interactions, Bentzur et al. (2020) found no significant difference in the network density between isolated and experienced flies. Perhaps measuring networks weighted by length of interactions, and not number of interactions, reduces the confounds that arise from differences in like locomotor activity and frequency of social encounters. The iterative approach by Schneider et al. (2012) certainly overcomes this confound by generating networks of controlled density over time. Like the Schneider study, Liu and Bentzur also applied the same defined criteria to both isolated and socialized flies (Bentzur et al., 2020; Liu et al., 2018; Schneider et al., 2012). As we have demonstrated, complete social isolation during adulthood affects the social space of flies. Unlike the other studies, we find socially isolated flies are just as active as socialized flies and engage in fewer social interactions on average (Figure 2). This highlights another benefit of using automated criteria for generating social networks because the behaviour of flies between different treatments and between separate experiments may change. The automated criteria can correct for such changes and produce social networks that best reflects how groups of flies interacted within an experiment.

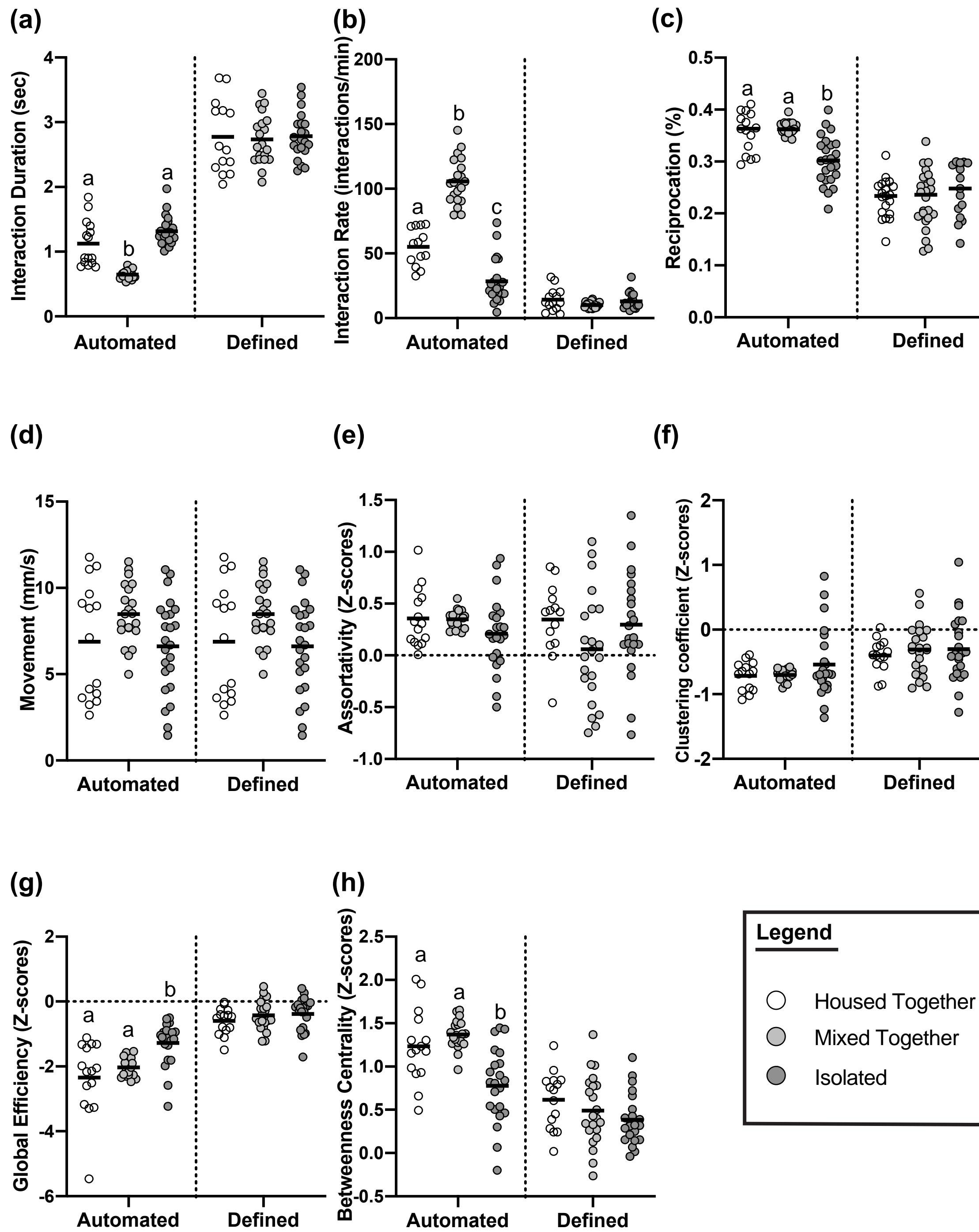

Figure 2.2 (Figure 2). Data from Schneider et al. (2012) re-analyzed with automated criteria compared to the original published data with defined criteria reveals social experience affects

both social interaction and social network measures. Flies were divided into three treatments: i) Housed together meaning all 12 flies in one video trial were raised together; ii) Mixed together meaning all 12 flies in one video trial were unfamiliar with each other from being raised with other flies; iii) Isolated meaning all 12 flies in one video trial were completely socially isolated since eclosion. (a) Flies of the mixed group have significantly lower average interaction duration when analyzed using the automated criteria but did not differ across groups when using the defined criteria (Automated: ($p \leq 0.0001$) (Housed together n = 15, Mixed group n = 20, Isolated n = 22) - Defined: ($p = 0.2549$) (Housed together n = 14, Mixed group n = 20, Isolated n = 21)). (b) Flies of the isolated treatment have significantly lower rates of interaction when analyzed using the automated criteria but did not differ across groups when using the defined criteria (Automated: ($p \leq 0.0001$) (Housed together n = 13, Mixed group n = 21, Isolated n = 21) - Defined: ($p = 0.2995$) (Housed together n = 14, Mixed group n = 16, Isolated n = 19)). (c) Proportion of interaction that were reciprocated of mixed group were significantly higher than isolated group and did not differ from control when analyzed using the automated criteria, whereas using the defined criteria proportion of reciprocation of mixed group was significantly lower than control but resembled level of isolated treatment (Automated: ($p \leq 0.0001$) (Housed together n = 15, Mixed group n = 22, Isolated n = 24) - Defined: ($p = 0.5640$) (Housed together n = 15, Mixed group n = 21, Isolated n = 24)). (d) Movement did not differ between the three treatments and when using the automated versus defined criteria (Automated: ($p = 0.0909$) (Housed together n = 15, Mixed group n = 21, Isolated n = 24) - Defined: ($p = 0.0909$) (Housed together n = 15, Mixed group n = 21, Isolated n = 24)). (e) No significant differences between the three treatments were observed for assortativity when analyzed using both the automated and defined criteria (Automated: ($p = 0.1027$) (Housed together n = 15, Mixed group n = 19, Isolated n = 22) - Defined: ($p = 0.1010$) (Housed together n = 15, Mixed group n = 22, Isolated n = 22)). (f) No significant differences between the three treatments were observed for clustering coefficient when analyzed using both the automated and defined criteria (Automated: ($p = 0.9540$) (Housed together n = 14, Mixed group n = 17, Isolated n = 23) - Defined: ($p = 0.8404$) (Housed together n = 14, Mixed group n = 22, Isolated n = 23)). (g) Global efficiency values of networks formed by isolated treatments were significantly higher those formed by the controls when using automated criteria, but no significant differences were found between the three groups when using the defined criteria (Automated: ($p \leq 0.0001$) (Housed together n = 15, Mixed group n = 19, Isolated n = 21) - Defined: ($p = 0.2956$) (Housed together n = 15, Mixed group n = 21, Isolated n = 21)). (h) Betweenness centrality of networks of isolated treatment were significantly lower when compared to controls (Automated: ($p \leq 0.0001$) (Housed together n = 15, Mixed group n = 21, Isolated n = 22) - Defined: ($p = 0.1369$) (Housed together n = 15, Mixed group n = 22, Isolated n = 21)). *a-h* were analyzed with one-way ANOVA with ranks to determine if statistical differences exist between the groups Outliers were removed from all the datasets. Bars indicate mean. Letters indicate statistical significance. *e-g* Measurements were standardized using z-scores as described by Schneider et al (2012).

2.3.2.3 Effect of density and group size on social networks

Each of the studies that compared social networks of isolated and socialized flies all examined groups of different sizes. A recent experiment by Rooke et al. (2020) demonstrated that group size and arena density impact features of social networks by comparing groups of 6, 12, and 24

flies across three different arena sizes. First, the authors found the average locomotor activity of flies was similar across different group sizes and arena density; suggesting flies regulate their movement to compensate for increased arena density. In terms of the social networks, the authors generated iterative, unweighted, and directed networks at controlled network density as published by Schneider et al. (2012). Rooke et al. (2020) found that flies in groups of 6 and 12 form networks with a significantly lower clustering coefficient than flies in groups of 24 and this was consistent across three arena densities. Additionally, flies in groups of 12 and 24 form networks with a significantly higher betweenness centrality than flies in groups of 6. This suggests that larger groups of flies, on average, form more interconnected groups with greater cohesion. More importantly, although the number of social interactions increases as the arena density and group size increase, properties of the social networks remain consistent across different densities of the same group size. Since the social networks were all generated at a controlled network density, differences in the network measures can be attributed fully to differences in group size. No matter how confined or dispersed a group of flies may be, the properties of the group shift only when the size of the group increases. Perhaps flies track the number of individuals that are present and organize themselves in the group according to the detected group size.

2.3.2.4 Sensory and genetic aspects of group formation

With *Drosophila melanogaster* being one of the most widely used organisms for behavioural genetic experiments, the wide availability of mutant strains and genetic tools to manipulate gene expression have been applied to social network experiments. To date, social networks have been generated for flies with disrupted visual, olfactory, gustatory, and acoustic modalities. Schneider et al. reported that the gustatory mutant *poxn*$^{\Delta XBs6}$ displayed an extreme reduction in the ability to form social networks (Schneider et al., 2012). More specifically, 40% of the videos filmed of these mutants did not harbour enough social interactions to form a single iterative network (Schneider et al., 2012). More recently, Jiang et al. also reported that *poxn* mutants, in addition to a range of other gustatory mutants, displayed an impaired ability to form physical social clusters (Jiang et al., 2020). Together, this suggests gustatory receptors are crucial for maintaining the sociality of flies.

Schneider et al. also demonstrated that hearing-impaired *inactive* mutants (*iav*^{1}) produced social networks that were not significantly different from wild-type flies (Schneider et al., 2012). Surgical removal of aristae to ablate auditory perception in wild-type flies also had no effect on social clustering of wild-type flies (Jiang et al., 2020). However, Jiang et al. (2020) reported that *iav*^{1} mutants socially cluster differently than wild-type flies where *iav*^{1} mutants tend to be more dispersed. This is also reflected in social space data for *iav*^{1}mutants where the social distance parameter was estimated to be larger than wild-type flies (Schneider & Levine, 2014). Although auditory mutants may socially interact and cluster less than wild-type flies, there is currently no evidence that manipulating auditory cues within a group of single sex flies affects its social network measures (Schneider et al., 2012).

To disrupt vision, experiments have been conducted on wild-type flies in the dark. Schneider et al. reported that groups of flies filmed in the dark display a lower clustering coefficient and higher betweenness centrality, but these effects were not considered significant when accounting for multiple test correction (Schneider et al., 2012). Bentzur et al. (2020) found that groups of socially isolated flies behave more similarly in the light and dark compared to socially experienced flies. The authors reported that in networks of socially experienced flies, visual disruption leads to a significantly lower average betweenness centrality; opposite of what was reported by previous studies (Schneider et al., 2012). Despite disagreement in the social network data when subjecting flies to darkness, multiple studies report similarity in how flies aggregate and physically cluster. Using automated behavioural classification, Bentzur et al. (2020) reported that groups of flies in the dark physically cluster less often and for shorter periods of time on average. Data by Jiang et al. (2020) also found that wild-type flies in the dark, along with *noprA33* visual mutants, cluster together less than wild-type flies. These two recent studies reinforce observations by Schneider et al. (2012) that darkness decreases the average interaction duration among groups of flies.

Arguably olfaction is the dominant sensory mechanism *Drosophila* depends on to locate foraging sites and conspecifics. As a result, ablating olfaction is complex because *Drosophila* insects possess multiple olfactory receptors that are encoded by multiple genes. The olfactory mutant, *orco*, is known to have a severe loss of smell because it is deficient for a co-receptor that complexes with a variety of odorant receptors (Vosshall & Hansson, 2011). Social networks of

orco mutants have been shown to have a significantly lower global efficiency than wild-type flies, with *orco* heterozygotes displaying an intermediate score (Schneider et al., 2012). This may indicate that the copy number of the *orco* gene leads to social interactions that, on average, results in a greater distance between individuals in the network. In the same study, the *orco* mutants also displayed a higher clustering coefficient and a higher assortativity compared to controls, although the differences were not statistically significant after multiple test correction (Schneider et al., 2012). The *orco* fly mutant has also been observed to form less clusters with conspecifics compared to wild-type flies (Jiang et al., 2020). Overexpressing an *orco* transgene in the olfactory system of these mutants led to the flies forming clusters like wild-type flies (Jiang et al., 2020). This reinforces a similar result in *orco* ant mutants that had a reduction in the ability to follow pheromone trails and cluster with other ants (Trible et al., 2017). This cross-species reduction in clustering suggests that olfaction is crucial for insect sociality and it is no surprise that olfactory mutants produce social networks different from wild-type flies.

In addition to studying the social behaviour of fly mutants, the *Drosophila* model system offers genetic tools to manipulate the expression of genes in a tissue-specific manner through the GAL4-UAS system (Elliott & Brand, 2008). This system has been applied to recent social network studies to examine the downstream behavioural effects of ablating specific olfactory sensing neurons (Bentzur et al., 2020; Rooke et al., 2020). One experiment examined the social networks of flies where the olfactory receptor neurons Or65a and Or67d were inhibited by driving the expression of *kir2.1* in those cells. These olfactory receptors are known to be sensitive to cVA, a male-specific pheromone that serves as an attractant to flies and mediates aggressive and copulatory behaviours in male flies (Bontonou & Wicker-Thomas, 2014). Interestingly, flies with inhibited Or67d neurons did not produce social networks drastically different from wild-type flies, but inhibition of Or65a neurons leads to a significantly decreased average betweenness centrality (Bentzur et al., 2020). Another experiment focused on inhibiting the olfactory gene *lush*, which is expressed in trichoid sensillae of flies and aids in the binding of ligands to olfactory receptors (Rooke et al., 2020). By driving the expression of *kir2.1* in all *lush*-expressing cells, Rooke et al. found that *lush*-inhibited flies produce social networks different from controls in larger group sizes. More specifically, groups of 6 *lush*-inhibited flies social networks with an average clustering coefficient and betweenness centrality that resembles the

wild-type controls. However, in groups of 12 and 24 the *lush*-inhibited flies generate social networks with a significantly higher betweenness centrality and clustering coefficient than wild-type controls (Rooke et al., 2020). Together, these results indicate that different olfactory genes, expressed in different tissues, may play different roles in regulating group-wide social connections in flies.

From a behavioural genetic perspective, Alwash et al. (2021) investigated the effect of the *foraging* (*for*) gene on social behaviour. This gene expresses natural polymorphism in flies called rover and sitter. The authors show that networks of rovers and sitters form social networks with different phenotypes. On average, networks of sitter flies tend to have longer interaction durations and were more likely to reciprocate interactions, whereas rover flies are more active and display higher interaction rates. For network measures, sitter networks have higher assortativity and clustering coefficient, but there is a less efficient flow of information. Alwash et al. (2021) also use transgenic lines that manipulate *for* gene dosage and show a gene dosage effect on all network phenotypes. The authors confirm that many of the social network differences observed between rovers and sitters are influenced by the *for* locus. These findings are one of the first to characterize the influence of a specific gene on social network dynamics in *Drosophila*, shedding light on the genetic underpinnings of sociality.

Multiple independent experiments that measured the social behaviour of *Drosophila* mutants and transgenic flies with inhibited neurons revealed that social clustering in flies is multisensory. In unisex groups of *D. melanogaster*, auditory sensory systems do not appear to play a role in social organization. Visual, gustatory, and olfactory manipulations cause flies to behave differently than wild-type flies in several ways (Bentzur et al., 2020; Jiang et al., 2020; Rooke et al., 2020; Schneider et al., 2012) However, all studies to date have only examined the social structure of unisex groups and it is possible that mixed groups of male and female flies may generate social structures that depend on a wider range of sensory systems since, for example, auditory cues are critical for courtship in flies (von Schilcher, 1976). Future studies should consider manipulating the composition of the social groups when inhibiting genes of interest to widen our knowledge of *Drosophila* social structures, like how Rooke et al. (2020) studied flies with *lush* inhibition at a variety of group sizes. It remains difficult to define how differences in precise network measures of mutants translates to differences in social organization, especially since various social network

experiments utilize different methods of generating and analyzing networks. However, experiments that focused on social attraction and aggregation of flies used similar mutants and transgenic tools and found overlapping results to social network studies (Jiang et al., 2020; Sun et al., 2020). Various mutations and neuronal inhibitions reduce the tendency of flies to socially cluster and form wild-type social networks. This suggests that social networks capture some aspect of group-level social organization that is genetically and neurologically controlled. Further experimentation in social attraction and aggregation of flies at the neuronal and genetic level can assist in unraveling how abstract social network measures translate to a real-world group structure.

2.3.2.5 Social transmission

To date, one group analyzed *Drosophila* social networks to study diffusion of information flow, like many social network studies on ant colonies (Blonder & Dornhaus, 2011; Mersch et al., 2013; Stroeymeyt et al., 2018). For context, social communication within *Drosophila* groups can inform naïve flies about the presence of oviposition sites (Battesti et al., 2014) and the presence of predatory insects (Kacsoh et al., 2018). Pasquaretta, Battesti et al. (2016) applied a SNA to further examine how information spreads within a group of informed and naïve flies. This was done by video recording a 4-hour training phase designed to inform focal flies of an oviposition site. Then social networks were generated within groups consisting of 8 informed flies and 4 uninformed flies (Pasquaretta, Battesti, et al., 2016). Iterative, directed, and weighted social networks were generated every 15 minutes from 4 hours of video footage. Afterwards, every trained and untrained female fly were subjected to an oviposition site choice assay to determine if the mean and variance of social network measures predict the tendency of uninformed flies to follow or avoid the choices made by informed flies. Uninformed flies followed the correct choice when informed flies had less variable network distances from other individuals, as measured by weighted closeness centrality. Uninformed flies also followed when informed flies all had a similar number of social contacts, as measured by eigenvector centrality. Uninformed flies also followed when informed flies exchanged information to a similar extent, as measured by information centrality index. On the contrary, uninformed flies were more likely to avoid copying the choices made by informed flies when uninformed flies had a high betweenness centrality in the social network. Taken together, this suggests that when informed flies

participate in most social interactions within the group, information is more likely to transmit from the informed to the uninformed flies.

Pasquaretta, Klenschi et al. (2016) also modeled the decision making of the informed flies following their oviposition site choice assay. Informed flies were found to avoid the preferred medium when they formed clusters, as measured by having a high mean clustering coefficient. This highlights how cluster formation is not always beneficial to members of a group, since formation of clusters may impede or slow information transmission. This has also been found in network diffusion experiments on ants, where information transmission has been modeled to be slower in groups of ants than theoretical simulations of virtual ants (Blonder & Dornhaus, 2011). While information may travel rapidly through a network theoretically, groups of interacting animals likely slow the flow of information through competitively occupying territories in environments with limited resources.

Disease can also be transmitted via social interactions within a group. Social network analysis in bumble bee colonies, for example, shed light on the relationship between interaction rate and parasitic transmission (Naug, 2008; Otterstatter & Thomson, 2007). Although no studies to date have used social network analysis to explore how social interaction and network properties affect disease transmission in *Drosophila,* Dawson et al. (2018) used similar methods to investigate how the social environment affects cancer progression. In a homogenous group, cancerous flies were found to have higher interaction rate and duration than in heterogeneous groups consisting of cancerous and healthy flies. The rate and duration of interaction of a healthy fly were all similar in different social contexts. Additionally, Dawson et al. (2018) showed that tumor progression is slower when cancerous flies are kept in a homogenous group and tumor progression is faster when cancerous flies are in isolation or within a group of healthy individuals. The use of the social network analysis approach can allow us to investigate the relationship between disease progression and social interactions even further by looking at social network measures.

2.3.2.6 Evolution of social organization

Recently, a social network comparative study was conducted on 20 drosophilid species. Generating iterative, directed, unweighted networks from groups of 12 male flies and groups of 12 female flies across all species, Jezovit et al. (2020) found phylogenetic patterns for the species differences observed in assortativity, clustering coefficient, betweenness centrality, and global efficiency. This mirrors the results of a social network comparative study on primates that also reported no evidence of phylogenetic signal in species differences (Pasquaretta et al., 2014). Averaged climate data was extracted from the geographic range of each drosophilid species and tested for correlations with the relative species differences in social networks. Jezovit et al. (2020) found that variation in the climate data predicts species differences in the social network measures better than the differences found in behavioural characteristics such as average locomotor activity, average interaction duration and average tendency to reciprocate interactions. Considering that each species studied descends from an inbred stock domesticated to the laboratory environment, it is surprising that factors of each species' environment predict differences in their social network measures. Additionally, Jezovit et al. (2020) collected two independent datasets of social networks for 5 species, separated by two years at the time of collection. Consistent trends in the relative species' differences were found for average assortativity, clustering coefficient, betweenness centrality, and global efficiency. Thus, species-specific social networks appear to represent robust phenotypes that capture differences in social organization behaviours. Together, this suggests that social organization in drosophilids, measured by social networks, may be an adaptive trait that evolved in response to differences in the environmental characteristics of each species' habitat.

This comparative study also made interesting observations beyond social network measures. Generating automated criteria for each species revealed that the average social distance of each species ranges between 1 and 3 body lengths and this variation maps onto the drosophilid phylogeny. In fact, the authors observed the average leg length of each species relative to their body size positively correlates with social distance. That result suggests the conserved social distance score can be explained at least partially by the morphology of each species. It also demonstrates how the evolution of social behaviour can be indirectly influenced by the evolution

of morphology. This study showcases the potential of drosophilid insects towards studying the evolutionary basis of animal social behaviour.

2.3.2.7 Putting Together the Various Drosophila SNA Methods

Social network measures are affected by the number of nodes, the degree distribution of all nodes and network density. The first factor can easily be controlled in the laboratory by ensuring all behavioural trials are filmed with a consistent group size. The second factor can be controlled by normalizing social networks to randomized networks. Schneider et al. accomplished this by computing all network measures as z-scores, where every social network generated was normalized to thousands of randomly generated. Additionally, Schneider et al. (2012) controlled the density of networks by capping the number of social interactions per network and analyzing iterations of subsequent density-controlled networks. Other recent SNA's applied to *Drosophila* applied more conventional methods of network generation where all social interactions in an entire experimental trial are filled out into a larger static social network (Bentzur et al., 2020; Liu et al., 2018). The conventional approaches are best used if the research interest is focused on individual nodes in a network and how they influence the entire network structure. If that is the research goal, then edges in a network can be weighted to identify influential individuals in processes such as information transmission (Pasquaretta, Battesti, et al., 2016). Otherwise, if the goal is to simply average all nodes in a network to study overall group structure, the dynamic iterative approach more precisely measures repeatable patterns of group organization because it controls for the degree distribution and density.

Social networks and their interpretation rely on what a social interaction is defined as. Many *Drosophila* SNA studies relied on subjective social space criteria based on human observations (Bentzur et al., 2020; Liu et al., 2018; Pasquaretta, Battesti, et al., 2016; Pasquaretta, Klenschi, et al., 2016; Schneider et al., 2012). The main shortcoming to using subjective criteria is its inflexibility. This issue was solved through an algorithm that automatically estimates the social space of flies (Schneider & Levine, 2014). Combined with the iterative approach, social networks can be generated with flexibility in the social space criteria to best represent the

repeated patterns of group organization in flies. So far, this methodology was applied to study: 1) the effects of group size and density on social networks in wild-type flies and olfactory deficient flies (Rooke et al., 2020); 2) the social networks of multiple drosophilid species in an evolutionary context (Jezovit et al., 2020); 3) to investigate the genetic underpinnings of social networks (Alwash et al., 2021). All these studies were able to control for social space variation that could occur between experiments, fly strains and social contexts, and generate social networks from the most appropriate criteria. A final consideration regarding *Drosophila* social interaction criteria is reducing the number of false positive social interactions. Two flies may enter a social space, momentarily slip out and re-enter. The re-entry may be considered as a second interaction when it is truly an extended single interaction. One solution to this issue is applying a gap length parameter, which has been demonstrated to reduce the number of recorded interactions (Bentzur et al., 2020). Ultimately future studies that apply SNA to *Drosophila* insects should consider all social space approaches reviewed here and find a balance between using criteria that reduces false social interactions without severe information loss.

2.4 Final section: Biological role of social networks

Social network analyses (SNA) provide researchers with a powerful tool that contributes to our understanding of mechanisms underlying collective behaviours. The aim of SNA across animal species has been increasingly directed towards connecting collective behaviour to their consequences on ecological and evolutionary purposes. For instance, there is evidence that wild animals occupy consistent positions in social networks when introduced to new environments (Canteloup et al., 2020; Krause et al., 2017), and across changing seasons (Blaszczyk, 2018; Rose & Croft, 2020; Stanley et al., 2018). Also, social network structures of animals analyzed in captivity are consistent with those studied in the wild (Brandl et al., 2019; Ripperger et al., 2019), suggesting that there is order to animal social groups that can be predictably recreated. In *Drosophila melanogaster*, the network position of individual flies (degree) fluctuates, but the overall network structure remains consistent over time (Schneider et al., 2012). A similar finding was reported in ants where an individual's degree offered no predictive power over their degree later in the experiment (Blonder & Dornhaus, 2011). Despite the evidence of individual animals occupying fixed positions in a network over time (Blaszczyk, 2018; Brent et al., 2013; Canteloup et al., 2020; Krause et al., 2017; Stanley et al., 2018), individuals also shift roles to maintain the

stability of the group and this flexibility maintains the group after individuals are lost due to predation and other stresses (Firth et al., 2017; Formica et al., 2017; Goldenberg et al., 2016; Naug, 2008). There is also evidence that various drosophilid species maintain consistent group structures across separate experiments, suggesting social networks are phenotypic (Jezovit et al., 2020). This was corroborated by another recent experiment that found the *foraging* gene and its polymorphisms affect the social networks of *Drosophila* (Alwash et al., 2021). Heritability in social network measures have also been reported in humans and rhesus macaques (Brent et al., 2013; Fowler et al., 2009), reinforcing the idea that robust social network measures may represent phenotypes of collective group structures.

Throughout this review, we have outlined experiments that all suggest *Drosophila* insects form organized and reproducible social networks when individuals aggregate (Schneider et al., 2012). There remains much to study regarding both animal behaviour and network theory and *Drosophila* insects offer an opportunity to enrich these fields. Despite *Drosophila* having long been considered asocial, a variety of organized collective behaviours have been uncovered in recent years. For example, flies in groups collectively escape from environmental threats (Ramdya et al., 2015) and enhance survival of offspring through communal oviposition (Lihoreau, Poissonnier, et al., 2016). Information exchanged during social interactions have been shown to influence fitness-enhancing decisions (Pasquaretta, Battesti, et al., 2016). Oviposition site choice is influenced by social interaction with conspecifics. Battesti et al. (2012) demonstrated that when "teacher flies" are trained to deposit eggs on one of two food options, naïve "student" flies follow the same choice as the teachers after socially interacting. In addition, female flies arrest oviposition upon detection of predatory threats and can transmit this response to flies unaware of the threat (Kacsoh et al., 2015). Furthermore, flies in smaller group sizes exhibit a higher tendency to freeze their movement upon detection of a predator (Ferreira & Moita, 2020), emphasizing the fitness benefits individuals gain from group formation. Applying SNA to these behavioural studies offers the opportunity to explore the rate of information transmission more precisely, and how other factors such as group size, density and individual status contributes to the group-level output. So far one study applied SNA methods to study information transmission more precisely in *Drosophila* and the authors found that oviposition site choice is influenced by both interactions between teacher and student flies, as well as, by the

homogeneity of the network. Unlike the other *Drosophila* social networks reviewed, this is the only study to date that examined the social networks of mixed groups. The greater animal social network literature will benefit from experiments dedicated to measuring *Drosophila* social networks from mixed groups. For instance, Jezovit et al. (2020) found that male-only social networks differ from female-only social networks in some species. Would mixing the sexes provide an intermediate social network phenotype, or could some interaction effect be observed? Additionally, layers of different network types could be analyzed when males and females are mixed in the same group. Social space criteria can be refined to measure courtship and mating interactions, or aggressive interactions. How would the properties of courtship and aggression networks compare to the properties of the general social networks? A variety of studies on courtship networks exist in the broader animal social network literature (Fisher et al., 2016; Formica et al., 2012; Oh & Badyaev, 2010; Ryder et al., 2008) and it would be worthwhile to determine if the *Drosophila* courtship networks overlap with these other studies. Finally, *Drosophila* has a long history of serving as a model organism to the genetic basis of social behaviour. Applying social network methods to well-studied mutants may aid in uncovering genetic mechanisms of sociality. For instance, a recent study found a potential role the *foraging* gene plays in the collective behaviour of flies that can be measured using social networks (Alwash et al., 2021). Further experimental efforts using *Drosophila* and the vast genetic reagents available within this system would shed more light onto genetic and neurological mechanisms governing collective behaviour. These findings may one day contribute towards identifying ancient mechanisms of sociality similar to how other pervasive mechanisms, like the circadian clock, have been uncovered in *Drosophila.*

2.5 Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Chapter 3:

The *Drosophila melanogaster foraging* gene affects social networks

Nawar Alwash, Aaron M. Allen, Marla B. Sokolowski, Joel D. Levine

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3.1 Abstract

Drosophila melanogaster display social behaviours including courtship, mating, aggression, and group foraging. Recent studies employed social network analyses (SNAs) to show that *D. melanogaster* strains differ in their group behaviour, suggesting that genes influence social network phenotypes. Aside from genes associated with sensory function, few studies address the genetic underpinnings of these networks. The *foraging* gene *(for)* is a well-established example of a pleiotropic gene that regulates multiple behavioural phenotypes and their plasticity. In *D. melanogaster,* there are two naturally occurring alleles of *for* called rover and sitter that differ in their larval and adult food-search behaviour as well as other behavioural phenotypes. Here, we hypothesize that *for* affects behavioural elements required to form social networks and the social networks themselves. These effects are evident when we manipulate gene dosage. We found that flies of the rover and sitter strains exhibit differences in duration, frequency, and reciprocity of pairwise interactions, and they form social networks with differences in assortativity and global efficiency. Consistent with other adult phenotypes influenced by *for*, rover/sitter heterozygotes show intermediate patterns of dominance in many of these characteristics. Multiple generations of backcrossing a rover allele into a sitter strain showed that many but not all of these rover-sitter differences may be attributed to allelic variation at *for.* Our findings reveal the significant role that *for* plays in affecting social network properties and their behavioural elements in *Drosophila melanogaster*.

3.2 Introduction

Forty years ago, as a graduate student, Sokolowski observed that *Drosophila melanogaster* larvae display different foraging strategies (Sokolowski, 1980). When positioned on food, some larvae, called sitters, move less than others, called rovers; sitters also stay near a food patch whereas rovers wander off. The foraging path-length difference on food was quantified in a behavioural assay (Anreiter et al., 2016; Sokolowski, 1980; Sokolowski et al., 1997). In the absence of food, the locomotion of rovers and sitters did not differ, suggesting that the feeding environment acts to stimulate the expression of rover-sitter behavioural differences in larval foraging path-length.

Quantitative genetic analysis of the rover-sitter difference in larval foraging path-length supported a primarily Mendelian model of an autosomal gene with the rover phenotype (long path-length phenotype) exhibiting complete genetic dominance to the sitter (short path-length phenotype) (de Belle & Sokolowski, 1987). The mapping of an autosomal gene responsible for rover-sitter differences showed that it mapped to the left arm of chromosome-2 (de Belle & Sokolowski, 1989). To more precisely map the larval path-length phenotype de Belle et al. (1989) developed the lethal tagging technique. Lethal tagging involved mutagenesis of the rover strain, crossing it to the sitter strain, and screening for sitter behaving larvae that carry a recessive lethal allele that tagged the gene responsible for rover-sitter differences in foraging path-length. Five alleles of the gene named *foraging (for)* were identified, all of which failed to complement for foraging path-length behaviour (de Belle et al., 1989). Three of these five lines also failed to complement the pupal lethality and identified the lethal tag of the gene. The other two lines carried viable sitter alleles. Recombination and deletion mapping localized the rover-sitter foraging path-length difference to cytological region 24A3-5 (see also de Belle et al., 1989, 1993). To further localize the gene, de Belle et al. (1993) generated a complementation map of the region by inducing additional mutants in the rover strain and mapping the lethality and behavioural alteration (from rover to sitter) to within the 5' end of the *dg2* gene which encodes cGMP-dependent protein kinase (de Belle et al., 1989, 1993; de Belle & Sokolowski, 1987, 1989; Kalderon & Rubin, 1989). Molecular analyses further demonstrated that *for* is synonymous with *dg2*. *for* null alleles carrying a full deletion of the 35 kb genomic region of *for* are pupal lethal (Allen et al., 2017; Anreiter et al., 2021). The *for* null was rescued with a full genomic fragment of *for/dg2* (Allen et al., 2017), and increasing *for/dg2* with *for*-cDNA in a sitter resulted in rover larval path-lengths and higher PKG enzyme activities characteristic of rover confirming that *for* is *dg2* (Allen et al., 2017; Osborne et al., 1997). Further research confirmed that *for* encodes a cGMP-dependent protein kinase (PKG), and plays an important role in food-related behaviours more generally (Allen et al., 2017; Anreiter and Sokolowski, 2019; Osborne et al., 1997). The *for* based rover-sitter differences in larval path length are for the most part robust to changes in genetic background whether on lab or field derived genetic backgrounds (Sokolowski et al., 1997).

Over the past three decades it has been shown that *for* is a pleiotropic gene that influences several behavioural and metabolic phenotypes in both the larval and adult stages of *D. melanogaster* (reviewed in Anreiter & Sokolowski, 2019). Examples of *for* related phenotypes are adult sucrose responsiveness and habituation to sucrose (Belay et al., 2007; Scheiner et al., 2004), larval food intake (Allen et al., 2017; Anreiter et al., 2017; Kaun et al., 2008), adult exploratory behaviour (Burns et al., 2012), habituation (Eddison et al., 2012; Engel et al., 2000), larval nociception (Dason et al., 2020), larval thermotolerance (Dawson-Scully et al., 2007, 2010), larval and adult learning and memory (Foucaud et al., 2013; Kaun et al., 2007; Kuntz et al., 2012; Mery et al., 2007; Wang et al., 2008), sleep and responses to sleep deprivation (Donlea et al., 2012), dispersal (Edelsparre et al., 2014, 2020), triglyceride levels (Allen et al., 2017; Anreiter & Sokolowski, 2018; Kaun et al., 2008), Malpighian tubule function (MacPherson, Broderick, et al., 2004; MacPherson, Lohmann, et al., 2004) and additional characteristics involved in the functional and developmental plasticity of neural circuits (Dason et al., 2019, 2020; Peng et al., 2016; Renger et al., 1999). In summary, *for* is a pleiotropic gene that acts as a modifier of physiology and behaviour (Anreiter & Sokolowski, 2019).

There is also evidence that rover-sitter differences in *for* affect social behaviours in *D. melanogaster*. Rover males exhibit higher levels of aggression compared to sitters and sitter mutants generated on a rover genetic background, suggesting a role of *for* in aggression (Wang & Sokolowski, 2017). Sitter flies learn better when trained and tested in groups compared to when they are alone whereas rovers learn equally well when they are trained and tested alone or in groups (Kohn et al., 2013). Sitter flies are more sensitive to their social context (Foucaud et al., 2013). Sitter adults are more likely to aggregate and tend to choose a more crowded refuge from heat than rovers; this aggregation behaviour depends on the ratio of rovers to sitters in a group (Philippe et al., 2016). *for* affects the choice of an oviposition site, with rover females laying more eggs on low-nutrient patches than sitter females (McConnell & Fitzpatrick, 2017). Together, these studies suggest that *for* affects social behaviour in *D. melanogaster*.

for orthologues are also associated with social dynamics in a variety of taxa (reviewed in (Anreiter & Sokolowski, 2019)). The gene expression levels of *for* orthologues correlate with the division of labor and social organization in eusocial insects ((Ben-Shahar et al., 2002, 2003; Bockoven et al., 2017; Ingram et al., 2005, 2011; Lucas and Sokolowski, 2009; Lucas et al.,

2015; Manfredini et al., 2014; Page et al., 2018; Tobback et al., 2008). In honey bees, *for* expression levels depend on the task that the bee performs in the hive; nurses who work in the hive have lower levels of expression of the honey bee *for* orthologue than do foragers who work outside the hive (Ben-Shahar et al., 2002, 2003). In several other eusocial insect species the converse is found (e.g. Kodaira et al., 2009; Page et al., 2018). Social dance is used by honeybees to communicate the location of a high-quality food (Seeley, 1994). Honey bee *for* expression levels are negatively correlated with the number of social dance circuits performed after a forager encounters high but not low-quality sucrose (George et al., 2020). The caterpillar *Drepana arcuate* transitions from a social to a solitary lifestyle during the third instar when *for* along with several other genes thought to be involved in social behaviour are upregulated (Yadav et al., 2020). Sitter flies develop smaller ovaries that contained fewer eggs than rovers when rover and sitter flies are exposed to honeybee queen mandibular pheromone (Camiletti et al., 2014).

Broadly speaking, *for* and its rover and sitter genetic variants exhibit many of the elements important for the evolution of social behaviour (Sokolowski, 2010). These include pleiotropic effects on a number of genetic modules (for example, foraging/feeding, learning and memory, stress, social behaviour) as well as the responsiveness of the gene to the social environment leading to plasticity in group-level social behaviours (Sokolowski, 2010). These considerations led us to evaluate whether *Drosophila* social networks vary as a function of *for* gene dosage and whether rovers and sitters contribute to plasticity at the group level. We addressed these questions using social network analysis on groups of *D. melanogaster* genetic variants to assess contributions of the *for* locus to group dynamics.

Here, we ask whether rovers and sitters display different patterns of behaviour in groups by analyzing properties of the social networks they form. Social network analysis is a statistical tool that reveals patterns in groups of interacting individuals. When such patterns are evident, they denote a group level phenotype that may be affected by the genotype(s) of its group members (Bentzur et al., 2020; Jezovit et al., 2020; Liu et al., 2018; Pasquaretta, Battesti, et al., 2016; Rooke et al., 2020; Schneider et al., 2012). These social network measures yield replicable patterns in *D. melanogaster* that depend on inheritance (Alwash & Levine, 2019; Schneider et al., 2012). Due to *for*'s effect on several *D. melanogaster* social behaviours and its influence on

social organization in various eusocial insects, it is reasonable to inquire whether *for* influences *D. melanogaster* social networks.

In this paper, we show that rover and sitter flies differ in the behavioural elements that act as precursors of social networks and that they form quantitatively different network structures. We use a rover/sitter heterozygote to assess whether rovers or sitters are phenotypically dominant in their social network measures. We also investigate whether *for* exhibits a dosage effect on *D. melanogaster* networks. Finally, we examine social networks using R1, a line with a rover allele backcrossed into a sitter genetic background. Together, these experiments establish that *for* influences the structure of social groups in *D. melanogaster* and provide a foundation for subsequent investigations into how *for* contributes to social behaviour.

3.3 Methods

3.3.1 Fly strains, crossing, and rearing

All flies were maintained in standard medium containing agar, glucose, sucrose, yeast, cornmeal, wheat germ, soy flour, molasses, propionic acid, and Tegosept in a 12-hr light/dark cycle (LD 12:12) at 25°C.

The *D. melanogaster* rover *(*for^R*)* and sitter (*for^S*) strains have been previously described (Allen et al., 2017; Anreiter et al., 2017). The rover and sitter strains have been in the lab for over 30 years. These strains have been isogenized multiple times over the years, most recently between 2010 and 2012. At the time, crosses were done so that these share common first and third chromosomes. *for* resides on chromosome-2 at cytological position 24A3-5.

Crosses of rover/sitter heterozygotes were generated by crossing rover males to sitter virgin females to test for patterns of dominance in the network measures. To assess whether *for* specifically affects behavioural elements of social networks and social network measurements we performed *for* gene-dosage experiments. Null mutants of *for (*for^0*)* are pupal lethal and on a for^S genetic background (Allen et al., 2017). We tested heterozygous animals of for^0 crossed to for^S to generate a line with only one copy of *for.* We also tested homozygous animals of a rescue line *(*for^0*; {*for^{BAC}*})* which carries two copies of the wild-type transgenic BAC construct, a

35 kb genomic copy of *for* introduced onto the third chromosomes, also on a sitter genetic background (Allen et al., 2017). As the 35kb sequence used to generate the *{for^{BAC}}* line originated from the *y^1;cn^1,bw^1,sp^1* strain (Venken et al., 2009), which was also used for the reference genome project, it may represent a distinct allelic variant of *for*. Homozygous animals of an over-expression line (*for^S*; *{for^{BAC}}*) carry both copies of the endogenous *for^S* allele on the second chromosomes and two copies of the wild-type *{for^{BAC}}* construct on the third chromosomes in a sitter genetic background. These lines were generated and described in Allen et al. (2017). In summary, lines described above carry 1 (*for^0* / *for^S* heterozygote), 2 (*for^0* with 2 copies of the *{for^{BAC}}*), and 4 (homozygous *for^S* alleles with 2 copies of the *{for^{BAC}}*) copies of the *for* allele. For ease of understanding, we refer to these lines as *1for,* 2*for,* and 4*for* respectively.

We sought to disentangle the social behaviour and network effects that were attributed to the *for* gene from effects of genetic background. To this end, we used a line called R1 which has the rover *for* allele backcrossed into a sitter genetic background. This was accomplished by conducting two, nine generation backcrosses. In the first, null *for* mutants were backcrossed to the sitter strain for nine generations. In the second, the resulting line was used as a sitter background donor for an allele swap that put a rover allele into a sitter genetic background using another 9 generations of backcrossing. Since the *for* null mutant background donor had no *for* sequence, recombination of the rover allele could not occur at the *for* locus during these crosses. Thus, we used the *for* null mutation to introgress the rover *for* locus into a sitter genetic background to generate the R1 strain.

3.3.2 Acquiring SINs

Three days prior to the experiment, we collected 12-16, 3-day-old virgin female *D. melanogaster* under light anesthesia (CO_2) and placed them in vials (25mm diameter, 95 mm height). These vials were kept at constant conditions in a 12L:12D, 25°C incubator for three days. Fifteen minutes before each experiment, 12 flies were transferred without anesthesia into a plexiglass arena (60 mm diameter, 2mm height) and covered with a glass lid. Thirty minutes of video were recorded using fview, an application used to record footage from digital cameras (Straw & Dickinson, 2009). Videos were recorded 9.5 hrs. after lights on. We collected ~20 videos for

each treatment group. For example, for each genotype about 20 different collections of 12 flies each defined the sample size (n) for that genotype (rover, sitter, etc). The videos were then analyzed using an applied machine vision system, Ctrax, to obtain fly identity, orientation, position, and trajectories in an arena throughout the 30-minute trial (Branson et al., 2009). MATLAB was then used to import the videos and a program within MATLAB was used as a classifier to identify interactions that occur between specific flies based on the three criteria set (proximity of flies, their angular orientation during the interaction, and the duration of the interaction). An automated method was used to identify values for interaction criteria. This program was used to identify each experimental group's social spacing patterns by getting estimates on the distance, angle, and time parameters for a social interaction to occur (Schneider & Levine, 2014).

3.3.3 Behavioural Elements of Social Networks

We calculated four measures associated with interactions as behavioural elements of the networks. The interactions we analyze are directed and unweighted. An interaction between one fly and another is not assumed to be reciprocated. MATLAB code analyzed:

(1) interaction duration - the mean time, in seconds elapsed between socially interacting flies.
(2) interaction rate - the mean number of social interactions expressed as interactions per minute.
(3) Reciprocation – the proportion of social interactions that are reciprocated by the receiving fly.
(4) Movement - the average distance a fly walks per second for each genotype.

3.3.4 Network measurements

Social network measurements are calculated based on interactions between two flies. Four social network measurements were analyzed using MATLAB:

(1) Global efficiency – a measure of redundant pathways (Latora & Marchiori, 2001a). Networks with high global efficiency have smaller average

distance between nodes compared to networks with a lower global efficiency.

(2) Clustering coefficient – a measure of how interconnected neighbors are to one another (Newman, 2010).

(3) Assortativity - the probability of an individual interacting with another individual of similar degree. Degree is defined as the sum of incoming and outgoing interactions for an individual (Newman, 2010).

(4) Betweenness centrality – the number of shortest paths that traverse a node. Betweenness centrality indicates the relative importance of an individual for relaying information (Newman, 2010).

3.3.5 Statistical analyses

All networks were normalized using *z*-scores to allow for the comparison between networks of different *Drosophila* strains. Networks and *z*-scores were generated as described in Schneider et al. (2012).

A two-tailed t-test was used to determine if the behavioural elements of social networks and the networks themselves differed between rovers and sitters. For the dosage experiments, ANOVA was used to determine whether network properties were significantly different. The Tukey-Kramer method was used as the post-hoc test. Outliers were removed from the data (outliers were calculated as follows: A trial's mean was considered an outlier if it was less than the 25th quartile-(1.5*IQR) or greater than the 75th quartile + (1.5*IQR)). The null hypothesis is that there is no difference between groups and is tested with $\alpha = 0.05$.

3.4 Results

3.4.1 Rover and Sitter flies form networks with different phenotypes

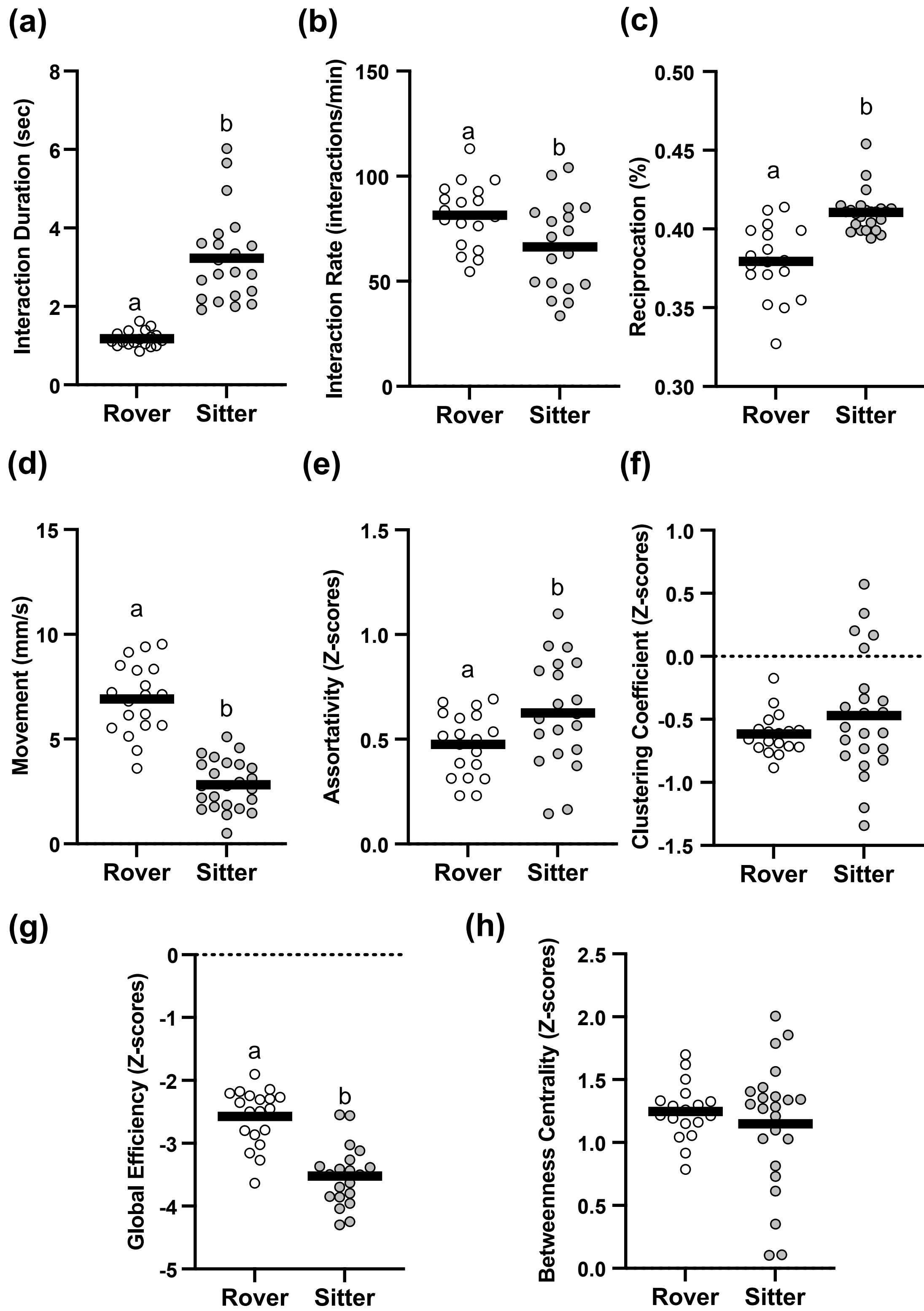
In a pilot study we looked at both males and females separately. Although trends were the same, the female data showed stronger effects, so we continued to study social networks in virgin females (see supplementary figure S1 and table S1 for comparison). To test whether *for* might

affect behavioural elements of *D. melanogaster* we acquired and analyzed networks of rover and sitter virgin female flies. Table 1 shows interaction criteria of the rover and sitter strains.

Behavioural elements of social networks and the networks themselves differ between rover and sitter flies (Fig. 1). Rover and sitter flies significantly differed in all measures (Fig. 1a-g) except for betweenness centrality (Fig. 1h). Sitter flies had significantly higher interaction duration, spending almost two and a half times longer interacting than rovers on average (Fig. 1a). Sitters were significantly more likely to reciprocate a directed interaction compared to rovers (Fig. 1c). Rover flies moved significantly more on average compared to sitter flies during the 30-minute trial and had significantly higher rates of interaction (Fig. 1d, 1b). Sitter networks showed significantly higher assortativity (Fig. 1e) compared to rover networks. No significant differences were found between rover and sitter for clustering coefficient or betweenness centrality (Fig. 1f, 1h). Finally, rover networks exhibited significantly higher global efficiency, an indication of faster information relay (Fig. 1g).

Table 3.1 (Table 1). Interaction criteria for rover and sitter flies, from the first experiment. Interaction criteria for each strain were automatically computed for distance, angle and time criteria across the replicates in order to capture interactions objectively across videos.

Strain	**Distance (body lengths)**	**Angle (°)**	**Time (sec)**
Rover	2.00	185	0.55
Sitter	1.50	110	0.80



(a)
Interaction Duration (sec)
8
6
4
2
0
a
b
Rover
Sitter
(b)
Interaction Rate (interactions/min)
150
100
50
0
a
b
Rover
Sitter
(c)
Reciprocation (%)
0.50
0.45
0.40
0.35
0.30
a
b
Rover
Sitter
(d)
Movement (mm/s)
15
10
5
0
a
b
Rover
Sitter
(e)
Assortativity (Z-scores)
1.5
1.0
0.5
0.0
a
b
Rover
Sitter
(f)
Clustering Coefficient (Z-scores)
1.0
0.5
0.0
-0.5
-1.0
-1.5
Rover
Sitter
(g)
Global Efficiency (Z-scores)
0
-1
-2
-3
-4
-5
a
b
Rover
Sitter
(h)
Betweenness Centrality (Z-scores)
2.5
2.0
1.5
1.0
0.5
0.0
Rover
Sitter

Figure 3.1 (Figure 1). Rover and sitter strains differ in behavioural elements of social networks and network properties**.** (a) Average duration of an interaction was different between rover and sitter groups, with rovers having significantly shorter interactions (t = 7.35) (df = 37) ($p \leq 0.0001$) (rover n = 18, sitter n = 21 groups). (b) Interaction rate was significantly higher in rover relative to sitter (t = 2.18) (df = 34) ($p \leq 0.05$) (rover n = 18, sitter n = 18). (c) Percentage of interactions that were reciprocated by the receiver was higher in sitter (t = 5.56) (df = 40) ($p \leq 0.0001$) (rover n = 18, sitter n = 24). (d) Rovers moved significantly more during the trial compared to sitters (t = 9.38) (df = 41) ($p \leq 0.0001$) (rover n = 19, sitter n = 24). (e) Assortativity values of the networks were significantly higher in sitters (t = 2.21) (df = 37) ($p \leq 0.05$) (rover n = 19, sitter n = 20). (f) Clustering coefficient values did not differ between rover and sitter networks (t = 1.24) (df = 39) ($p = 0.18$) (rover n = 18, sitter n = 23). (g) Global efficiency values were significantly higher in networks of rovers relative to sitter networks (t = 6.38) (df = 37) ($p \leq 0.0001$) (rover n = 19, sitter n = 20). (h) Betweenness centrality measures did not differ between the rover and sitter variants (t = 0.78) (df = 39) ($p = 0.41$) (rover n = 18, sitter n = 23). *a-h* were analyzed with two-tailed t-tests (*Methods)*. Outliers were removed from all the datasets. Bars indicate mean. Letters indicate statistical significance. *e-h* Measurements were standardized using *z*-scores.

3.4.2 Rover/sitter heterozygotes are intermediate to rover and sitter

To determine the dominance patterns of these different network phenotypes, we tested rover/sitter heterozygotes in parallel with their homozygous rover and sitter controls. Table 2 shows interaction criteria of the heterozygotes and homozygote controls. Once again, rovers show significant differences in interaction duration with rovers interacting for the shortest mean amount of time (1.49 s) and sitters the longest (4.47 s).

Heterozygotes had a significantly intermediate interaction duration compared to rovers and sitters (Fig. 2a). Heterozygotes significantly reciprocate interactions (Fig. 2c) at levels between rovers and sitters and move at levels between rovers and sitters (Fig. 2d). For social network measures, networks of heterozygotes tended to display intermediate levels however for clustering coefficient (Fig. 2f) and global efficiency (Fig. 2g) heterozygotes did not significantly differ from rovers and for global efficiency heterozygotes significantly differed from rovers and not from sitters. Mean values of heterozygotes for assortativity (Fig. 2e) and betweenness centrality (Fig. 2h) was intermediate between rovers and sitters, however differences between the three groups were not significant. Heterozygotes tended to exhibit interaction rate values that were significantly different from rovers (Fig. 2b). Overall, heterozygotes were intermediate on many but not all of the behavioural elements and social network measures.

Table 3.2 (Table 2). Interaction criteria of the rover/sitter heterozygotes and their rover and sitter controls. Interaction criteria for each strain were automatically computed for distance, angle and time criteria across the replicates in order to capture interactions objectively across videos.

Strain	**Distance (body lengths)**	**Angle (°)**	**Time (sec)**
Rover	1.50	125	0.60
Sitter	1.50	120	0.75
Rover/Sitter Heterozygote	1.50	115	0.55

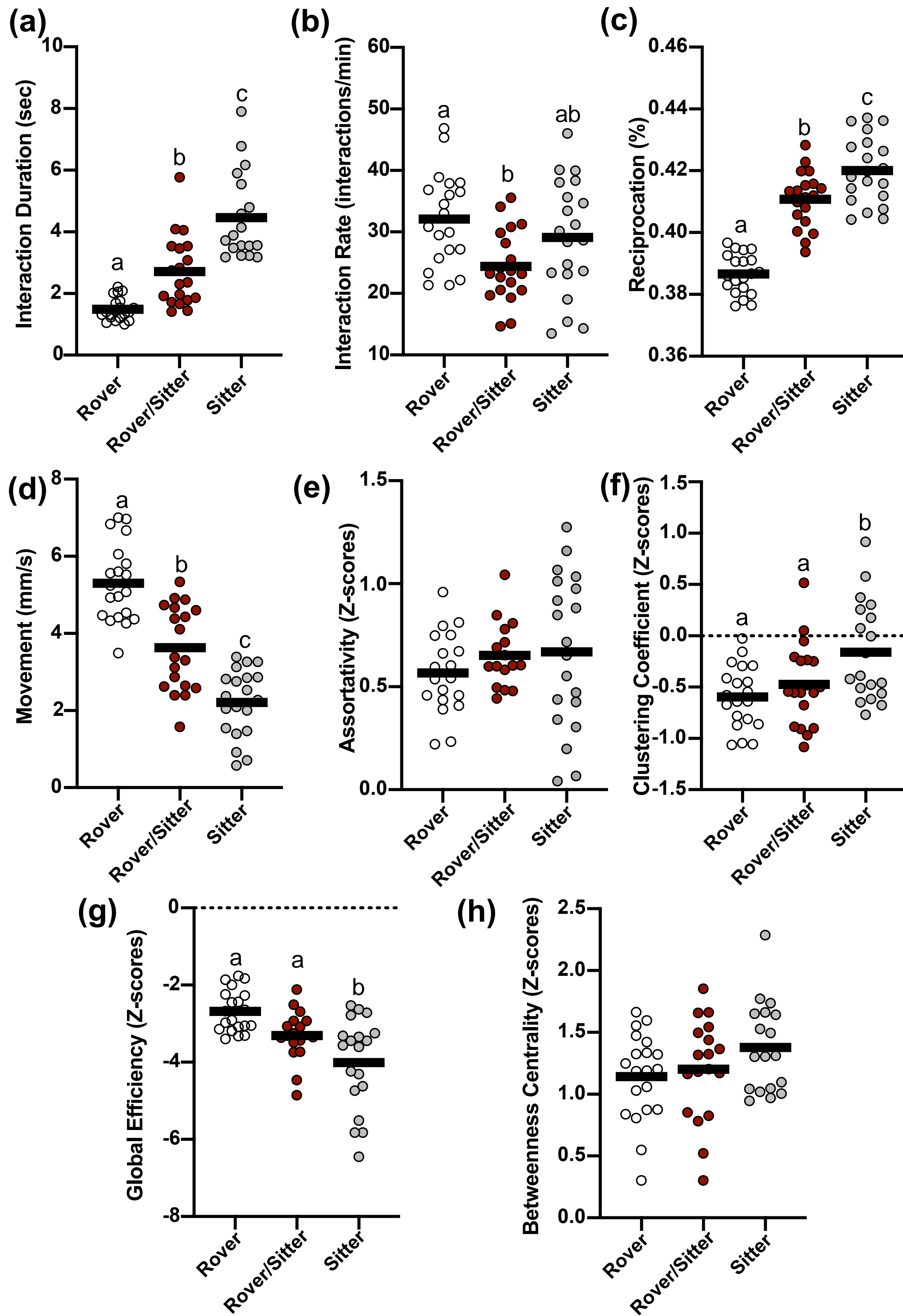

(a)
Interaction Duration (sec)
a
b
c
Rover
Rover/Sitter
Sitter
(b)
Interaction Rate (interactions/min)
a
b
ab
(c)
Reciprocation (%)
a
b
c
(d)
Movement (mm/s)
a
b
c
(e)
Assortativity (Z-scores)
(f)
Clustering Coefficient (Z-scores)
a
a
b
(g)
Global Efficiency (Z-scores)
a
a
b
(h)
Betweenness Centrality (Z-scores)

Figure 3.2 (Figure 2). Social behavioural elements and network properties in rover/sitter heterozygotes compared to rover and sitter. (a) rover/sitter heterozygotes had intermediate interaction duration values compared to rover and sitter, sitters had the highest average duration of interactions, followed by the rover/sitter heterozygote and finally the rover ($F_{(2,54)} = 37.79$) ($p \leq 0.0001$) (rover n = 20, rover/sitter n = 19, sitter n = 18). (b) Interaction rate was significantly higher in rover compared to rover/sitter heterozygotes ($F_{(2,56)} = 4.87$) ($p \leq 0.05$) (rover n = 20, rover/sitter n = 18, sitter n = 20). (c) Percentage of interactions that were reciprocated by the receiver were highest in sitter, lowest in rover and intermediate in the heterozygotes ($F_{(2,56)} = 73.45$) ($p \leq 0.0001$) (rover n = 20, rover/sitter n = 19, sitter n = 20). (d) Movement of flies during the trials was the highest in rover, followed by the heterozygotes, and the lowest in sitter ($F_{(2,56)} = 47.90$) ($p \leq 0.0001$) (rover n = 20, rover/sitter n = 19, sitter n = 20). (e) Assortativity values did not differ between the three groups compared ($F_{(2,52)} = 0.80$) ($p = 0.46$) (rover n = 19, rover/sitter n = 16, sitter n = 20). (f) Clustering coefficient values were highest in networks of sitter, while rover and rover/sitter formed networks with similar clustering coefficient values ($F_{(2,55)} = 6.09$) ($p \leq 0.01$) (rover n = 20, rover/sitter n = 19, sitter n = 19). (g) Sitter had the lowest global efficiency values, while rover and rover/sitter formed networks with similar global efficiency values ($F_{(2,53)} = 11.93$) ($p \leq 0.0001$) (rover n = 20, rover/sitter n = 17, sitter n = 19). (h) Betweenness centrality values did not differ between the three groups compared ($F_{(2,53)} = 0.1565$) ($p = 0.86$) (rover n = 20, rover/sitter n = 18, sitter n = 18). *a-h* were analyzed with one-way ANOVA to determine if statistical differences exist between the groups. The Tukey-Kramer method was used as the post-hoc test (*Methods)*. Outliers were removed from all the datasets. Bars indicate mean. Letters indicate statistical significance. *e-h* Measurements were standardized using *z*-scores.

3.4.3 for dosage on social behavioural elements and social network measures

Overall, network phenotypes showed an effect of *for* gene-dosage. Average duration of an interaction decreased as *for* copy number increases (Fig. 3a), with *1for* and sitter flies having the longest interaction durations. Whereas interaction rate increased as *for* copy number increased (Fig. 3b). *for* gene dosage effects were observed for reciprocation (Fig. 3c), assortativity (Fig. 3e) and clustering coefficient (Fig. 3f). *4for* flies were significantly less likely to reciprocate interactions, than *1for* and sitter flies (Fig. 3c). Conversely, as *for* copy number increased, average movement significantly increased with *4for* flies moving significantly more on average (Fig. 3d). From the perspective of our social network measures, *1for* and sitter flies were significantly more assortative (Fig. 3e) and formed more interconnected networks (Fig. 3f) compared to flies with higher *for* copy numbers. And finally, as *for* copy number increased, global efficiency (Fig. 3g) significantly increased. Betweenness centrality showed a decreasing dosage trend, however no significant differences were found between the five groups (Fig. 3h).

Table 3 shows the criteria for an interaction for each of the lines used in our dosage experiment along with the rover and sitter controls.

Table 3.3 (Table 3). Interaction criteria of lines of *for* used in the gene-dosage experiments and their rover and sitter controls. Interaction criteria for each strain were automatically computed for distance, angle and time criteria across the replicates in order to capture interactions objectively across videos.

Strain	**Distance (body lengths)**	**Angle (°)**	**Time (sec)**
1for	1.75	130	1.15
2for	2.25	110	1.20
4for	2.25	75	0.95
Rover	2.00	125	0.95
Sitter	1.50	125	1.25

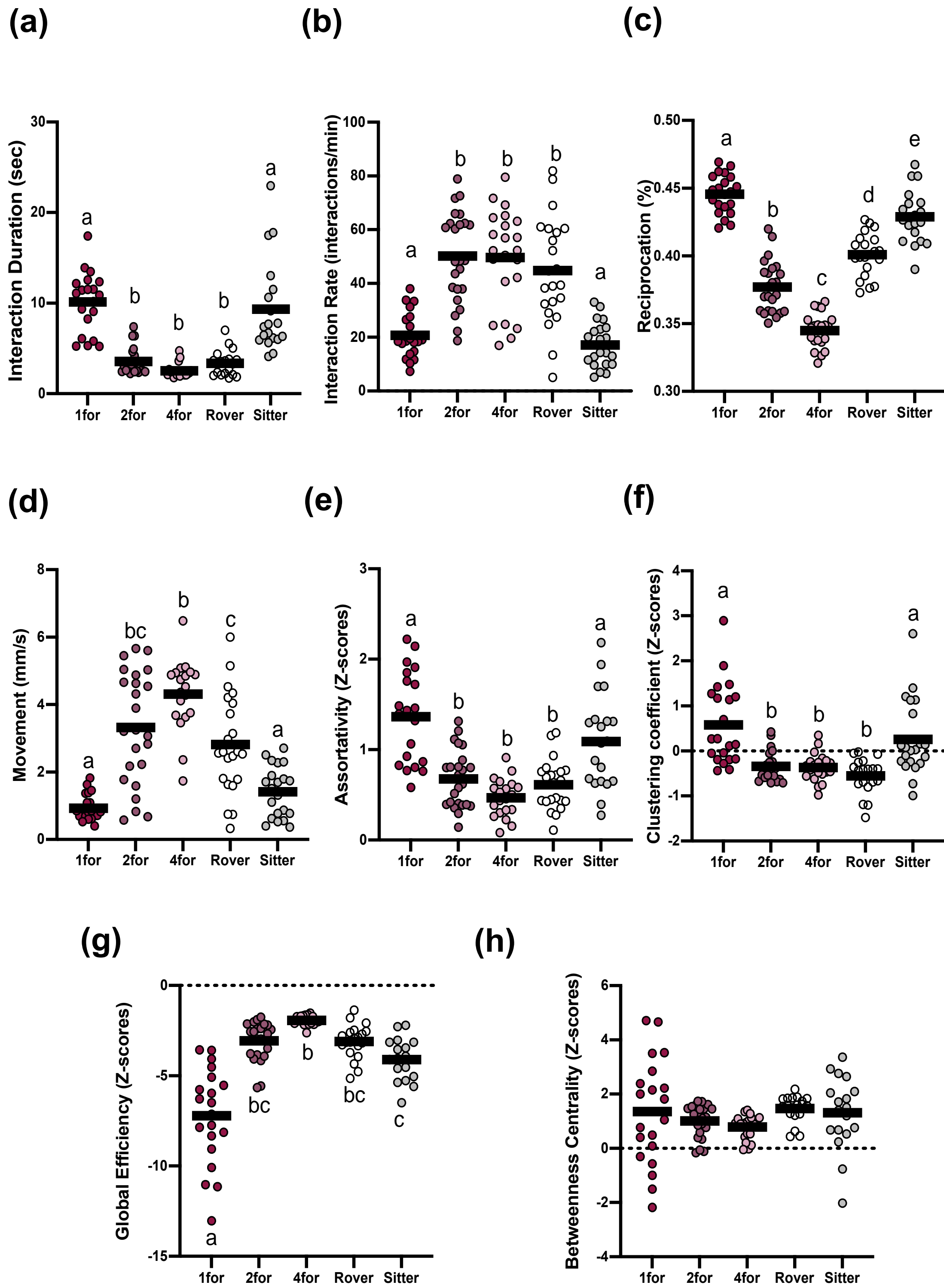

(a)
(b)
(c)
Interaction Duration (sec)
Interaction Rate (interactions/min)
Reciprocation (%)
(d)
(e)
(f)
Movement (mm/s)
Assortativity (Z-scores)
Clustering coefficient (Z-scores)
(g)
(h)
Global Efficiency (Z-scores)
Betweenness Centrality (Z-scores)
1for
2for
4for
Rover
Sitter

Figure 3.3 (Figure 3). *for* copy number affects social behavioural elements and social networks of *Drosophila melanogaster.* (a) Average duration of an interaction decreased as *for* copy number increased, and sitter had significantly higher average interaction duration compared to rover ($F_{(4,93)} = 30.85$) ($p \leq 0.0001$) (*1for* n = 20, *2for* n = 21, *4for* n = 19, rover n = 19, sitter n = 19). (b) Interaction rate was lower in networks of 1*for* and sitter flies ($F_{(4,104)} = 24.19$) ($p \leq 0.0001$) (*1for* n = 20, *2for* n = 24, *4for* n = 22, rover n = 22, sitter n = 21). (c) Percentage of interactions that were reciprocated by the receiver decreased as *for* copy number increased and were highest in networks of sitter flies ($F_{(4,102)} = 125.10$) ($p \leq 0.0001$) (*1for* n = 21, *2for* n = 24, *4for* n = 21, rover n = 21, sitter n = 20). (d) Movement of flies during the trial increased as *for* copy number increased, rover moved more on average during trials compared to sitter ($F_{(4,102)} = 27.79$) ($p \leq 0.0001$) (*1for* n = 21, *2for* = 24, *4for* n =19, rover n = 22, sitter n = 21). (e) As *for* copy number increased, assortativity values of the networks decreased, assortativity was significantly higher in sitter ($F_{(4,100)} = 18.79$) ($p \leq 0.0001$) (*1for* n = 20, *2for* n = 24, *4for* n =20, rover n = 21, sitter n = 20). (f) Clustering coefficient value of networks decreased as *for* copy number increased and sitter networks had higher clustering coefficient compared to rover ($F_{(4,98)} = 12.46$) ($p \leq 0.0001$) (*1for* n = 21, *2for* n = 22, *4for* n =20, rover n = 19, sitter = 21). (g) Global efficiency values increased as *for* copy number increased ($F_{(4,92)} = 36.51$) ($p \leq 0.0001$) (*1for* n = 20, *2for* n = 22, *4for* n = 18, rover n = 20, sitter = 17). (h) Betweenness centrality values did not differ across the five groups ($F_{(4,97)} = 36.51$) ($p = 0.27$) (*1for* n = 20, *2for* n = 23, *4for* n = 22, rover n = 19, sitter = 18). *a-h* were analyzed with one-way ANOVA. The Tukey-Kramer method was used as the post-hoc test (*Methods)*. Outliers were removed from all the datasets. Bars indicate mean. Letters indicate statistical significance. *e-h* Measurements were standardized using *z*-scores.

3.4.4 Rover-sitter allelic variants, social behavioural elements and social network measures

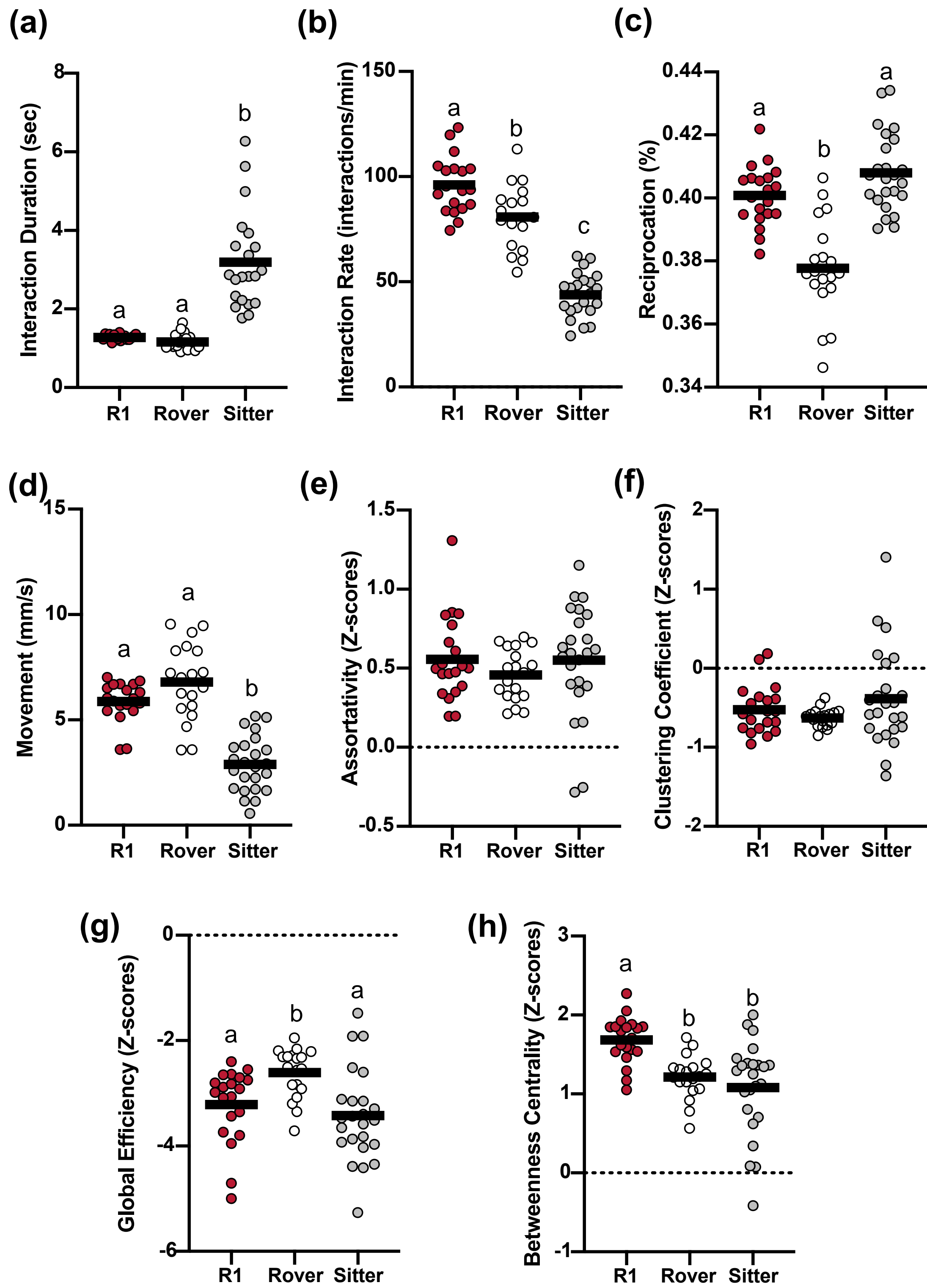
The R1 strain has a rover allele backcrossed (9 generations) into a sitter-like genetic background. We used this strain to investigate whether the introduction of a rover allele into a sitter genetic background changed the behavioural elements of social networks and social network phenotype when compared to rover and sitter flies.

Table 4 shows the interaction criteria of the lines tested. As was the case for all of the other experiments described above, sitter's time parameter in the estimate of social spacing was higher than rover and R1 is intermediate but closer to rover (Table 4). From the perspective of social behavioural elements, the interaction durations of R1 and rovers did not significantly differ from each other and were significantly lower than sitter. Sitters spent about three times longer interacting (Fig. 4a). In this comparison, R1 flies display higher interaction rate values compared to rover and sitter flies, but more closely resembled rovers (Fig. 4b). In contrast, the percentage of reciprocated interactions did not significantly differ for R1 flies and sitter flies, while rover

flies reciprocated significantly fewer interactions on average (Fig. 4c). Movement did not differ between R1 and rover and was significantly higher than in sitters (Fig. 4d). Together, these data suggest that the substitution of the rover allele influences interaction duration, interaction rate and movement while the sitter genetic background likely influences reciprocal interactions. From the perspective of social network measures, no significant differences were found for assortativity (Fig. 4e) or clustering coefficient (Fig. 4f) between the three lines, however the rover-sitter differences showed trends in the same direction of our previous experiments. Finally, rover-sitter differences in global efficiency were replicated from our previous experiments, however, mean global efficiency of R1 networks was similar to that of sitters (Fig. 4g). Betweenness centrality values of R1 networks were significantly higher but did not differ between rovers and sitters (Fig, 4h). The use of the R1 strained helped us determine that the rover-sitter differences in interaction duration interaction rate and movement traits were robust to differences in genetic background (Fig. 4). In contrast, rover-sitter differences in reciprocation and global efficiency resulted from differences in genetic background. These results indicate that rover-sitter differences are evident in the behavioural elements of social networks and the networks themselves.

Table 3.4 (Table 4). Interaction criteria of the R1 strain and the rover and sitter controls. Interaction criteria for each strain were automatically computed for distance, angle and time criteria across the replicates in order to capture interactions objectively across videos.

Strain	**Distance (body lengths)**	**Angle (°)**	**Time (sec)**
Rover	1.50	115	0.55
Sitter	1.50	120	0.85
R1	1.50	120	0.65



(a)
Interaction Duration (sec)
8
6
4
2
0
a
a
b
R1
Rover
Sitter
(b)
Interaction Rate (interactions/min)
150
100
50
0
a
b
c
R1
Rover
Sitter
(c)
Reciprocation (%)
0.44
0.42
0.40
0.38
0.36
0.34
a
b
a
R1
Rover
Sitter
(d)
Movement (mm/s)
15
10
5
0
a
a
b
R1
Rover
Sitter
(e)
Assortativity (Z-scores)
1.5
1.0
0.5
0.0
-0.5
R1
Rover
Sitter
(f)
Clustering Coefficient (Z-scores)
2
1
0
-1
-2
R1
Rover
Sitter
(g)
Global Efficiency (Z-scores)
0
-2
-4
-6
a
b
a
R1
Rover
Sitter
(h)
Betweenness Centrality (Z-scores)
3
2
1
0
-1
a
b
b
R1
Rover
Sitter

Figure 3.4 (Figure 4). Rover-sitter allelic variants, social behavioural elements and social networks. (a) Average duration of an interaction differed with R1 and rover having significantly shorter interactions than sitter ($F_{(2,53)} = 43.77$) ($p \leq 0.0001$) (R1 n = 17, rover n = 18, sitter n = 21). (b) Rover had higher interaction rates than R1 and sitter ($F_{(2,55)} = 85.15$) ($p \leq 0.0001$) (R1 n = 19, rover n = 17, sitter n = 22). (c) R1 and sitter flies had higher reciprocity than rovers ($F_{(2,60)} = 32.33$) ($p \leq 0.0001$) (R1 n = 20, rover n = 19, sitter n = 24). (d) R1 and rovers moved significantly more than sitters ($F_{(2,59)} = 47.01$) ($p \leq 0.0001$) (R1 n = 19, rover n = 19, sitter n = 24). (e) No significant differences in assortativity were found ($F_{(2,60)} = 0.78$) ($p = 0.46$) (R1 n = 20, rover n = 19, sitter n = 24). (f) No significant differences in clustering coefficient values ($F_{(2,59)} = 1.68$) ($p = 0.20$) (R1, n = 20, rover n = 18, sitter n = 24). (g) Global Efficiency values were significantly higher in rovers relative to sitter and R1 ($F_{(2,60)} = 7.09$) ($p \leq 0.01$) (R1 n = 20, rover n = 19, sitter n = 24). (h) Betweenness centrality values of R1 line was significantly higher but did not differ between rovers and sitter ($F_{(2,60)} = 11.34$) ($p \leq 0.0001$) (R1 n = 20, rover n = 19, sitter n = 24). *A-h* were analyzed with one-way ANOVA to determine if statistical differences exist between the groups. The Tukey-Kramer method was used as a post-hoc test (Methods). Outliers were removed from all the datasets. Bars indicate mean. Letters indicate statistical significance. *e-h* Measurements were standardized using *z*-scores.

3.5 Discussion

for influence social behaviour in a variety of species and has been postulated to be part of a genetic toolkit involved in the evolution of eusocial insects (reviewed in (Rittschof & Robinson, 2016). Here, we reasoned that if *for* does play a role in group-level behavioural dynamics, a difference should be evident between rover and sitter genetic variants of *for* and it is (see Fig. 1 and S1). A rover-sitter difference in social network measurements allowed us to focus on *for* with regard to patterns of dominance (Fig. 2), to assess the contributions of the *for* locus using gene dosage (Fig. 3), and to manipulate genetic background (Fig. 4). Overall, our findings support the hypothesis and establish an empirical foundation for future studies on the contribution of the *for* gene to behaviour in groups.

How did we determine interaction criteria in our social network analysis? Schneider et al., (2012) showed that *D. melanogaster* form non-random social interaction networks using fixed criteria to define interactions. A critical limitation of the fixed criteria was that they were derived by subjective observations of video recordings and freely behaving flies. The use of fixed interaction criteria assumes that different strains interact using the same parameters. To address this assumption, an automated method of analyzing social spacing and defining interaction criteria in an unsupervised fashion was developed (Schneider & Levine, 2014). Both strain and

species differences affected interaction criteria, thus, using this automated criteria method seem to add precision to evaluating social networks (Jezovit et al., 2020; Schneider & Levine, 2014). The method used in the present paper for analyzing social networks uses automated criteria to identify interactions between flies (Schneider & Levine, 2014). Average criteria are determined for each group in each trial of each experiment. The use of experiment-specific criteria permits greater accuracy than the application of a single interaction rule for establishing the patterns of interaction that define a network. In our assay, three criteria define an interaction (interaction criteria): distance, angle, and time. These measurements for each experiment are given in Tables 1-4 and S1. Differences in the interaction criteria were observed between the groups tested within each experiment. The only consistent differences in the interaction criteria that we observed across experiments were in the temporal interaction criteria parameter. This criterion for duration of an interaction – given some distance and angle between two individuals – was always longer for sitters than for rovers. The difference in duration in Table 3 indicates not only that sitters have longer interaction times, but also that interaction time in rovers decreases as the number of copies of the rover allele is increased from 1 to 2 to 4.

The rover and sitter strains used in this study were established based on the larval foraging assay first established by Sokolowski in her classic paper (Sokolowski, 1980). The R1 strain was generated by backcrossing the rover allele into a sitter genetic background allowing for the comparison of R1, rover, and sitter as a first pass to investigate whether the rover allele influences our social behaviour and network measures. In these comparisons, statistical differences and some trends were evident in the duration of interactions between groups of rover compared to sitter flies (not limited to the interaction criteria), in the rate of formation of these interactions, and the percent of interactions that were reciprocated. Overall, sitters were higher than rovers in both measures of interaction duration and percentages of interactions that were reciprocated. Rovers moved significantly more quickly when in groups than did sitters and also had higher rates of interactions. In this experiment, speed correlates with other strain differences. However, we and others have previously shown that differences in the movement levels do not explain the differences in social network measures in *D. melanogaster* (Bentzur et al., 2020; Jezovit et al., 2020; Schneider et al., 2012). Further, when such correlations arise in species comparisons movement does not fully account for differences in social network measurements

(Jezovit et al., 2020). Here, we do not interpret the correlation between movement, elements of social networks, and the networks themselves in a causal manner.

Adult rover/sitter heterozygotes are known to exhibit intermediate behavioural phenotypes to rover and sitter [e.g., adult foraging behaviour (Pereira & Sokolowski, 1993); sucrose consumption in a foraging arena (Anreiter et al., 2017)]. A pattern of intermediate dominance was also found for most of the behavioural elements and social network measures that exhibited rover-sitter differences (see figure 2). Interestingly, there appeared to be a larger spread in the sitter compared to rover and rover-sitter heterozygotes for some of the measures suggesting that sitter group measures may be more plastic in response to the environment. Differences in behavioural plasticity have been reported for behavioural and physiological phenotypes influences by *for* (reviewed in (Anreiter & Sokolowski, 2019)).

We found differences between rovers and sitters in assortativity and global efficiency with a non-significant difference in clustering; the sitters demonstrated a higher value on average that did not attain statistical significance. Additionally, no differences were observed between rovers and sitters for betweenness centrality, showing that *for* has no influence on betweenness centrality measures. Figure 1 is consistent with the idea that *for* plays a role in determining behavioural elements and social network measures. New experimental measures of the rover-sitter strains in our social network assay were performed for the data in each of the four figures and the supplementary figure. The rover-sitter differences were mainly observed across these experiments, although not always reaching significance. For the behavioural elements of social networks, the average duration of interaction and the proportion of interactions reciprocated were significantly higher for sitter flies (Fig. 1-4a, S1a, 1-4c, S1c), whereas rover flies moved significantly more during the 30-min trial (Fig. 1-4d, S1d). Rates of interaction were consistently higher for rover flies across experiments, although not always reaching significance (significant: Fig. 1b, 3b, 4b, S1b, not significant: Fig. 2b). As for network measures, a trend was observed for assortativity and clustering coefficient with sitter networks having higher assortativity and clustering coefficient values across experiments, but the rover-sitter differences were not always statistically significant (significant: Fig. 1e, 3e, 2f, 3f, not significant: Fig. 2e, 4e, S1e, 1f, 4f, S1f). Global efficiency values were significantly higher in networks of rovers, indicating a more efficient flow of information in all but one experiment (significant: Fig. 1g, 2g, 4g, S1g, not

significant: Fig. 3g). Finally, there were no differences between rover and sitter networks in betweenness centrality measures across the five experiments (Fig. 1-4h, S1h). Overall, these results show the stability and reproducibility of the rover-sitter differences in our measures.

We further reasoned that manipulating the number of copies of *for* would reveal dosage effects, and this would provide evidence that the differences we observe depend on the *for* locus. We varied the number of copies of *for* (1, 2, or 4 and continued to compare them to the rover and sitter strains). Interestingly, we found that for all but one of the measures, on average, one copy of *for* (sitter hemizygotes) gives the same value as sitter (Fig. 3a-f). The exception is global efficiency, where one copy of *for* (sitter hemizygote 1for) produces a much lower score than all other groups (Fig. 3g). For all but one of these measures (reciprocity), 2 copies of *for* [flies homozygous for the {for^{BAC}} transgene (2*for*)] resembles rover, and 1 copy of sitter resembles sitter (Fig. 3b). The results suggest that 1 copy of *for* produces the least expression, followed by 2 copies of the for^{BAC} and rover, and that 2 or more copies of the locus produce a ceiling effect. The exception is for movement, which increases from 2 copies of the gene to 4 (Fig. 3d). Future studies will begin to untangle the contributions of dosage and *for* genetic variants on the social network phenotypes. Taken together, the results of varying gene dosage suggest that the *for* locus is exerting an effect on our social behaviour measures including, interaction, interaction duration, reciprocation, and movement. Interestingly, three of our network measures including, assortativity, clustering, and global efficiency, the social network parameters, were affected by *for* gene dosage.

We tested the effect of genetic background on our measures by using a newly developed strain called R1 (see methods). The R1 strain contains a rover allele of the *for* locus that has been backcrossed into a sitter background nine times. Thus, *for* is rover, but after 9 generations of backcrosses, most of genetic background is presumed to come from sitter. R1 looks like rover and not like sitter in all but two of our measures (see figure 4). Figure 4a and d hold up with statistical significance; Figure 4c, the percent of interactions that are reciprocated, all values are similar on average, but the rover is statistically lower than R1 and sitter; and clustering. Figure 4e and f, R1 and rover look the same and look different from sitter, but this is a non-significant trend. Overall, R1 provides results that indicate a clear effect for the rover variant of *for* on the non-network metrics, with a non-significant effect on two of the other three. Although not as

robust as we would have liked, the R1 strain appears to function as a tool for mapping effects of the rover allele to the *for* locus, and in the context of our other findings, here we view the results on the network measures as consistent, even if confidence exceeds 0.05.

Taken together, the results reported here indicate that *for* participates in social behavioural elements and social network measures in *D. melanogaster*. *for* is a complex locus with four promotors and a minimum of 21 transcripts and nine protein isoforms (Allen et al., 2017). Each of the *for* promoters acts in a modular fashion and differs in the subsets of *for* transcripts that they target to specific tissues and cells in both the larval (Allen et al., 2018) and adult fly (Allen & Sokolowski, 2021). Overall, *for* is expressed in subsets of tissues in the larval and adult nervous system, muscle, adipose tissue and gut, and the male and female reproductive systems (Allen et al., 2018; Allen & Sokolowski, 2021; Anreiter et al., 2017). *for* expression is also cell-specific, for example *for* is expressed in multiple glia subtypes in the adult brain (Allen & Sokolowski, 2021). *for*'s pleiotropic functions at the synapse have been well studied using the larval neuromuscular junction (nmj) model (Dason et al., 2019; Dason & Sokolowski, 2021; Renger et al., 1999). At the nmj, glial function influences nerve terminal growth, and presynaptic neuronal function influences endocytosis; expression of *for* in glia and neurons both affect neurotransmission (Dason et al., 2019; Dason & Sokolowski, 2021). *for* acts in synaptic development of the larva and also acutely suggesting that *for* acts on a number of time scales (Dason et al., 2019).

The effects of *for* on social networks reported here could be the consequence of expression in early life (embryonic and/or larval stages), the pupal stage, or in adulthood, or any combination of these stages. Various individual tissues or combinations of tissues might be involved in *for's* regulation of social networks. All of this remains to be identified. In any case, we have established a foundation that justifies further testing of the hypothesis that variation and plasticity in group-level behaviours involves *for* (Sokolowski, 2010).

Future studies will test the idea that *for* operates in a pathway that manages social and collective responses to the environment and, in this way, mediates the plasticity of social dynamics, similar to the way it mediates plasticity in foraging behaviour.

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3.7 Declaration of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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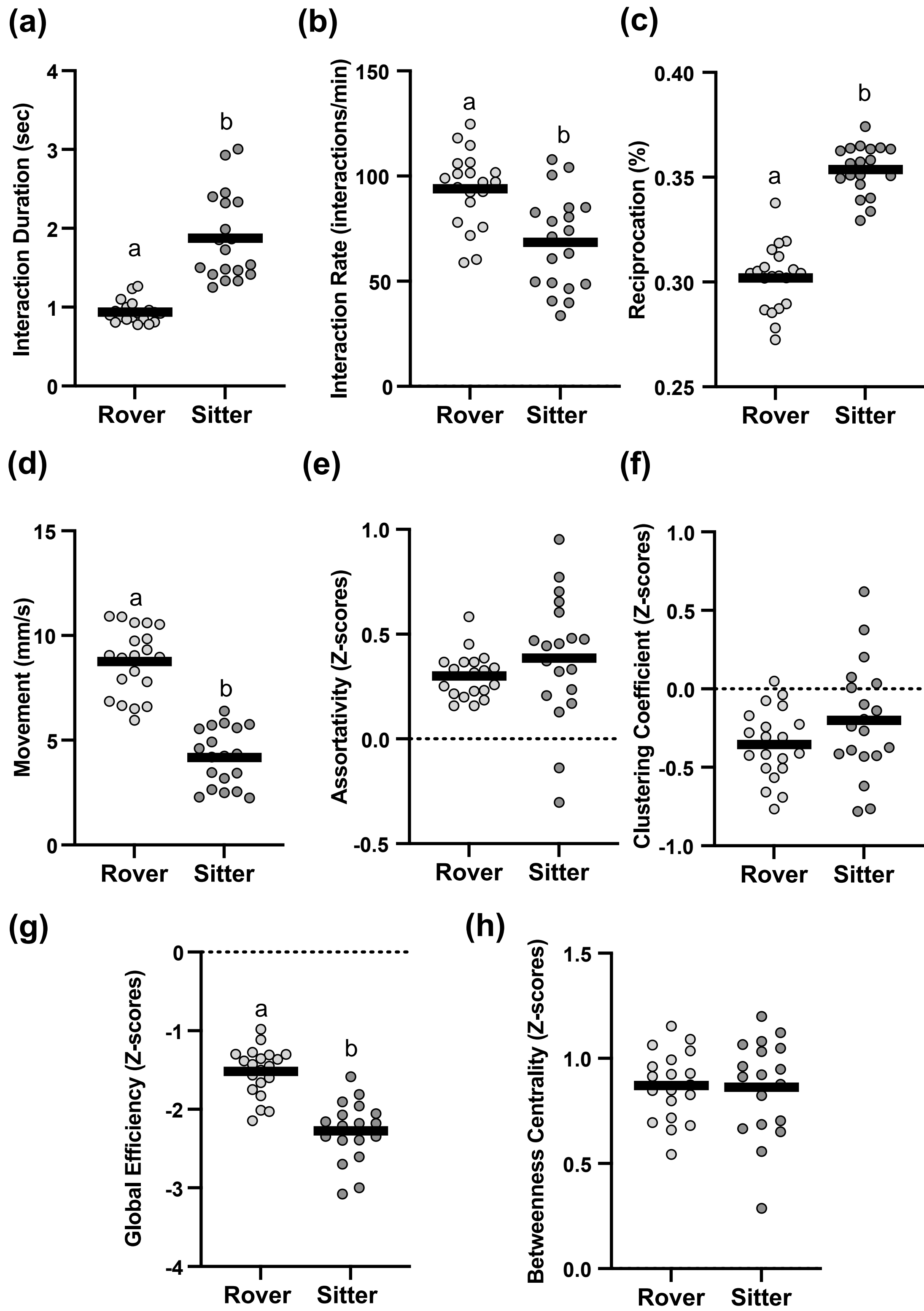
3.9 Supplemental Material:

Table S3.1 (Table S1). Interaction criteria of the rover and sitter male flies. Interaction criteria for each strain were automatically computed for distance, angle and time criteria across the replicates in order to capture interactions objectively across videos.

Strain	Distance (body lengths)	Angle (°)	Time (sec)
Rover	2.00	75	0.45
Sitter	2.25	85	0.60



(a)
Interaction Duration (sec)
4
3
2
1
0
a
b
Rover
Sitter
(b)
Interaction Rate (interactions/min)
150
100
50
0
a
b
Rover
Sitter
(c)
Reciprocation (%)
0.40
0.35
0.30
0.25
a
b
Rover
Sitter
(d)
Movement (mm/s)
15
10
5
0
a
b
Rover
Sitter
(e)
Assortativity (Z-scores)
1.0
0.5
0.0
-0.5
Rover
Sitter
(f)
Clustering Coefficient (Z-scores)
1.0
0.5
0.0
-0.5
-1.0
Rover
Sitter
(g)
Global Efficiency (Z-scores)
0
-1
-2
-3
-4
a
b
Rover
Sitter
(h)
Betweenness Centrality (Z-scores)
1.5
1.0
0.5
0.0
Rover
Sitter

Figure S3.1 (Figure S1). Male flies of the rover and sitter strain differ in their behavioural elements and social network properties and followed the same trend as females. (a) Sitter flies had significantly higher interaction duration that rover flies (t = 7.20) (df = 36) ($p \leq 0.0001$) (rover n = 19, sitter n = 19). (b) Interaction rates of rovers were significantly higher than those of sitters (t = 3.90) (df = 37) ($p \leq 0.001$) (rover n = 20, sitter n = 19). (c) Sitter flies were more likely to reciprocate an interaction and had significantly higher percentage reciprocation when compared to rover flies (t = 11.67) (df = 37) ($p \leq 0.0001$) (rover n = 20, sitter n = 19). (d) Rover flies moved significantly more during the trials (t = 9.48) (df = 37) ($p \leq 0.0001$) (rover n = 20, sitter n = 19). (e) Assortativity values did not differ between networks of male rovers and sitter (t = 1.15) (df = 36) ($p = 0.26$) (rover n = 19, sitter n = 19). (f) Clustering coefficient values did not differ between rover and sitter networks (t = 1.58) (df = 37) ($p = 0.12$) (rover n = 20, sitter n = 19). (g) Global Efficiency values were significantly higher in networks of rovers relative to sitter networks (t = 6.72) (df = 36) ($p \leq 0.0001$) (rover n = 20, sitter n = 18). (h) Betweenness centrality values did not differ between rovers and sitters (t = 0.12) (df = 35) ($p = 0.9063$) (rover n = 19, sitter n = 18). *a-h* were analyzed with two-tailed t-tests (*Methods)*. Outliers were removed from all the datasets. Bars indicate mean. Letters indicate statistical significance. *e-h* Measurements were standardized using *z*-scores.

Chapter 4:

Exploring the effect of *Drosophila melanogaster* *foraging* gene on social networks

Nawar Alwash, Marla B. Sokolowski, Joel D. Levine

This chapter is currently being prepared for publication.

Author contributions are detailed in the acknowledgments section of this chapter.

4.1 Abstract

The *foraging* gene, *for,* is a well-established example of a pleiotropic gene. It has been extensively studied in *Drosophila melanogaster* and other species for its effects on development, physiology and behaviour. *for* was also shown to influence several social behaviours across species, including social learning, aggression, and cross-species communication in *Drosophila*. A recent study employed the social network analysis (SNA) approach, showing that *for* influences social networks. The two naturally occurring variants of *for*, rovers and sitters, differed in their behavioural elements and their social network properties. Here, we aim to characterize *for*'s effect on social networks further. First, we investigate the critical period of *for* expression in relation to social networks. We find that knocking down *for* during the adult stages and till the late wandering stage does not affect behavioural elements or social network measures. Next, we investigate for promoters' contributions to the social network. Our results suggest that *for*'s different promoters may independently regulate these phenotypes. We also explore *for'*s plasticity by investigating rover and sitter response to different stressors, specifically isolation, food and sleep deprivation. Finally, we look at networks of mixed rovers and sitters in order to shed light on how these variants may interact with each other in nature. Our findings highlight *for*'s important role in affecting both behavioural elements and social network properties and their behavioural elements in *D. melanogaster*.

4.2 Introduction

Social network analysis (SNA) is a statistical tool used to reveal patterns in groups of interacting individuals (Krause et al., 2009). Most animal species live in a social world, interacting and forming complex relationships with one another. Through social organization these interactions give rise to social networks, in which collective behaviours are often displayed; this can be seen in a variety of organisms, from primates to fish and insects (Alwash et al., 2021; Brent et al., 2013; Croft et al., 2004; Fowler et al., 2009; Jezovit et al., 2020; Schneider et al., 2012). SNA can be defined as the statistical analysis of nodes, with each node representing an individual within a network, connected by edges, which highlight the interactions between individuals (Krause et al., 2009). It provides a conceptual framework and an array of statistical tools to

describe social structure and to explore the interplay between individual behaviour and population structure.

Recent technological advances in machine vision and tracking software have led to increased interest in SNA from researchers in the scientific community, facilitating the use of SNA in studies of multiple species (Branson et al., 2009; Robie et al., 2017; Straw & Dickinson, 2009). For instance, a study investigating monozygotic and dizygotic twins indicated that network measures in humans are likely heritable (Fowler et al., 2009). Similarly, evidence for the heritability of some network measures has been found in rhesus macaques (*Macaca mulatta*) (Brent et al., 2013) and fruit flies (Alwash et al., 2021; Schneider et al., 2012). Schneider et al. (Schneider et al., 2012) pioneered the use of SNA in examining *Drosophila* group structure, yielding the finding that *D. melanogaster* groups form non-random and complex social networks. Consequently, more researchers have since investigated *Drosophila* social networks using SNA, expanding on the field (Alwash et al., 2021; Bentzur et al., 2020; Jezovit et al., 2020; Liu et al., 2018; Pasquaretta, Battesti, et al., 2016; Rooke et al., 2020). For instance, using SNA, social isolation was shown to affect the behavioural elements of *Canton-S* flies, as well as their social network measures (Bentzur et al., 2020; Liu et al., 2018; Schneider et al., 2012) (Chapter 2).

The *Drosophila melanogaster foraging* (*for*) gene is a well-established example of a pleiotropic gene that has been used in the study of behavioural genetics (Anreiter & Sokolowski, 2019). *for* was first characterized by its effect on larval foraging patterns (Sokolowski, 1980). Two natural variants of *for* in *Drosophila* were observed: rovers, which move further from a food patch, and sitters, which tend to stay near a food patch (Sokolowski, 1980). These variants occur at stable frequencies in the natural population of Southern Ontario, at 70% rover to 30% sitter. Differences between these variants were mapped to the left arm of the second chromosome, and *for* was found to encode a cGMP-dependent protein kinase (PKG), a type of signaling molecule (Osborne et al., 1997). At the time of *for*'s characterization, the influence of genetics on behaviour was a relatively new field of study. Thus, *for*'s effect on behaviour was pioneering and has since been investigated extensively in the last three decades.

Soon after *for*'s effect on larval path length was characterized (Sokolowski, 1980; Sokolowski et al., 1997), other differences were observed between the natural polymorphisms and were

attributed to *for*'s pleiotropic nature (Anreiter & Sokolowski, 2019). The influence of *for* in *Drosophila melanogaster* is not limited to food-related behaviours and extends to many other phenotypes, including pupation site selection (Sokolowski, 1985), adult foraging strategies (Pereira & Sokolowski, 1993), sucrose response (Belay et al., 2007), fat storage (Allen et al., 2017), oviposition site preference (Battesti et al., 2014; McConnell & Fitzpatrick, 2017), and dispersal rates (Edelsparre et al., 2014). *for* related phenotypes also include learning and memory (Foucaud et al., 2013; Kaun, Hendel, et al., 2007; Kuntz et al., 2012; Mery et al., 2007; Wang et al., 2008), and neuronal plasticity (Dason et al., 2019, 2020; Peng et al., 2016; Renger et al., 1999).

Various studies have also implicated *for* in affecting social behaviours. *for* orthologues influence social dynamics across species, having been shown to affect the division of labor and social organization in many eusocial insects (Ben-Shahar et al., 2002, 2003; Bockoven et al., 2017; Ingram et al., 2011; Tobback et al., 2008). In flies, *foraging* expression has been implicated in influencing social behaviours such as aggression (Wang & Sokolowski, 2017). Wang & Sokolowski (2017) showed that rover males exhibited higher levels of aggressive behaviour compared to sitter males. When learning, sitter flies seem to be more sensitive to their social environment and learn better when trained and tested in groups, whereas rovers' ability to learn was unaffected by their social environment (Kohn et al., 2013). When placed in groups of mixed strains, aggregation behaviour was found to be dependent on the rover to sitter ratio (Philippe et al., 2016). *for* has also been reported to influence cross-species communication; only sitter flies were affected after exposure to the honeybee queen mandibular pheromone and were found to have smaller ovaries containing fewer eggs compared to rovers (Camiletti et al., 2014).

Recently, Alwash et al. (2021) (Chapter 3) used the social network analysis (SNA) approach and reported that *for* affects how flies interact and their group structure. Differences observed between network phenotypes of rovers, sitters, and genetically manipulated flies that affect *for* dosage, were mainly influenced by the *for* locus. Using these findings as a foundation, we aimed to explore the effect of *for* on behavioural elements and social networks phenotypes further.

The pleiotropic nature of *for* is hypothesized to be influenced by its complex molecular structures and gene products. *for* has four promoters, approximately twenty-one transcripts and nine protein isoforms (Allen et al., 2017). The expression of various gene products in different tissues and at different times is likely the mechanism by which *for* regulates its phenotypes. Promoter specific expression has been confirmed and characterized in both larval and adult stages (Allen et al., 2018; Allen & Sokolowski, 2021). These four promoters seem to drive expression of *foraging* in different tissues and at different periods during development (See table 1) (Allen et al., 2018; Allen & Sokolowski, 2021). Recent work has revealed new insight into the underpinnings of *for* and how it influences specific phenotypes. For instance, adult foraging differences were shown to be a result of differences in promoter 4 expression in the brain and/or ovaries via G9a-mediated epigenetic regulation (Anreiter et al., 2017). G9a-mediated epigenetic regulation did not affect larval foraging behaviour (Anreiter & Sokolowski, 2018). Promoter 1 was shown to regulate larval nociception and larval path length (Allen et al., 2018; Dason et al., 2020; Donlea et al., 2012), while promoter 3 influences larval triglyceride level (Allen et al., 2018). In addition to adult foraging differences, promoter 4 expression also regulates larval food intake (Allen et al., 2018).

Table 4.1 (Table 1). *for*'s different promoter expression patterns across the nervous, gastric and reproductive system in the larval and adult stages.

System	Larval Stage (Allen et al., 2017)	Adult Stage (Allen & Sokolowski, 2021)
Nervous	- Promoter 1: neurons in the central brain and the ventral nerve cord - Promoter 2: midline glia - Promoter 3: surface glia - Promoter 4: neurons in the optic lobe	- Promoter 1: a pair of neurons connecting the suboesophageal zone to the medulla - Promoter 2: neurons in the trachea and air sacs that innervate the brain - Promoter 3: surface and perineural glia - Promoter 4: optic lobes in the outer and inner chiasm glia
Gastric	- Promoter 1: enteroendocrine cells of the midgut - Promoter 2: stem cells of the midguts and in the ureter of the Malpighian tubules. Also	- Promoter 1: no expression - Promoter 2: epithelia cells of the foregut, crop, and cardia in addition to expression in the midgut intestinal stem cells and the

	expressed in the salivary imaginal ring - Promoter 3: enteroendocrine cells of the midgut, midgut enterocytes, muscles of both the midgut and the hindgut, and primary cells of the anterior Malpighian tubule. Also expressed in fat body - Promoter 4: hindgut, the salivary imaginal ring, and the anterior and posterior spiracles	stem cell zone of ureter and lower Malpighian tubule - Promoter 3: a narrow band of enterocytes in the middle region of the midgut, a few anterior enteroendocrine cells, the visceral gut muscle, principal cells of the transitional segment of the Malpighian tubules and the throughout the salivary gland - Promoter 4: epithelia of the hindgut, as well as the rectal ampulla and the salivary duct of the salivary gland
Reproductive		- Promoter 1: in the male, it is expressed seminal vesicle and secondary cells of the accessory glands - Promoter 2: only promoter in female reproductive system, spermatheca and the follicle cells of developing eggs and a small segment of the common oviduct. In the male, it is expressed in the seminal vesicle and secondary cells of the accessory glands and in a small ring of cells at the base of vas deferens where it joins with the ejaculatory duct - Promoter 3: no expression in male or female reproductive system - Promoter 4: in the male, it is expressed seminal vesicle and secondary cells of the accessory glands. It is also expressed in the ejaculatory bulb

Most research done on the *for* gene has utilized its rover and sitter natural variants and a sitter mutant generated on a rover genetic background, but a recent study by Allen et al. (2017) generated transgenic lines, including a *for* knockout (for^{null}). Homozygous for^{null} was viable during larval stages but was lethal during pupal stages and so flies did not survive into

adulthood. These findings indicated that *for* expression was essential and suggested a role for *foraging* during development. Anreiter et al. (2021) investigate this further and mapped the critical stage for lethality using both deletion analysis as well as temperature-restricted knockdowns of *for*. The authors mapped the critical stage to early pupal development and showed that *for* expression during these stages is crucial for normal emergence behaviour from the pupal case and viability. This is likely a result of energy deficiency linked to defects in fat body morphology associated with promoter 3 *for* expression (Anreiter et al., 2021).

To better understand the relationship between the *foraging* gene and social behaviour, we should also consider how *for* is affected by the environment. *for* displays behavioural plasticity, with rovers and sitters distinctively adjusting their behaviour in response to environmental changes. Given *for'*s well-established influence on various food-related phenotypes, it is not surprising that these phenotypes are sensitive to food availability and quality. In chronic food-deprivation conditions, larvae with the rover allele exhibit higher survivorship and faster development when compared to larvae with the sitter allele (Kaun, Riedl, et al., 2007). A similar trend is seen in adult response to food deprivation, with rovers displaying greater changes in their gene expression profiles and metabolic levels compared to sitters and sitter mutants (Kent et al., 2009). Donlea et al. (2012) reported that rovers and sitters display resilience to certain environmental stresses and vulnerability to others. When faced with a learning task, rover flies were more resilient to sleep deprivation than sitters while sitter flies were more resilient to food deprivation (Donlea et al., 2012).

In this paper, we use the SNA approach to further characterize *for'*s influence on social networks by first investigating *for'*s spatiotemporal pattern of expression in regard to its effect on behavioural elements and social network measures of *D. melanogaster.* Using a temperature-restricted knockdown of *for*, we aim to map the critical period during which *for* expression influences social network phenotypes. We also investigate the promoter-specific contribution to the behavioural elements and network phenotypes by rescuing *for* expression in a for^{null} line using promoter-specific Gal4 drivers. Next, we explore *for*'s phenotypic plasticity by testing whether various types of stress differentially affect social behaviour. In particular, we address how adversity interacts with the natural variants of *for* to affect behavioural elements and network phenotypes in *D. melanogaster.* We hypothesized that the natural variants of *for*, rovers

and sitters, will display different levels of sensitivity to the different stress types they are exposed to. Three types of stressors were investigated 1) isolation, 2) food deprivation (after both 24 and 48 hours of food deprivation), and 3) sleep deprivation. Finally, we also examine networks of mixed groups of rovers and sitters at different ratios to investigate the effect of social environment on behavioural elements and social network measures of the group and shed to light on how the rover and sitter variants may interact with each other in nature.

4.3 Methods

4.3.1 Fly strains, crosses, and rearing

Flies used in these experiments were maintained in standard medium containing agar, glucose, sucrose, yeast, cornmeal, wheat germ, soy flour, molasses, propionic acid, and Tegosept in a 12-hr light/dark cycle (LD 12:12) and were kept at either 18°C, 25°C, or 30°C incubators as indicated.

The wild-type allelic variants of the *foraging* (*for*) gene in *D. melanogaster*; rover *(for^R)* and sitter *(for^S)* as previously described (Allen et al., 2017; Anreiter et al., 2017).

In order to investigate the temporal effect of *for* expression on social interactions and social network phenotypes, I used the UAS/GAL-4 system combined with the temperature-sensitive GAL80 to generate five crosses to knockdown *for* expression at different stages of development; 1) *Tub* Knockdown - Ubiquitous GAL4 driver *Tubulin, tub-GAL80^{ts};tub-Gal4* crossed to UAS-*$for^{RNAi\text{-}comma7}$*, 2) *Da* Knockdown - Ubiquitous GAL4 driver *Daughterless, tub-GAL80^{ts};Da-Gal4* crossed to UAS-*$for^{RNAi\text{-}comma7}$*, 3) *Tub.*GAL4 Control - *tub-GAL80^{ts};tub-Gal4* crossed to a homozygous sitter, 4) *Da.*GAL4 Control - *tub-GAL80^{ts};Da-Gal4* crossed to a homozygous sitter, 5) UAS Control - UAS-*$for^{RNAi\text{-}comma7}$* crossed to a homozygous sitter. Both *Tubulin* and *Daughterless* GAL4 drivers were previously shown to significantly and effectively drive RNAi to knockdown *foraging* expression in the whole body and thus were used (Anreiter et al., 2017).

I also used to UAS/GAL-4 system in order to generate nine crosses that have promoter-specific increase of *for* expression in order to investigate the promoter contribution to social network phenotypes: 1) UASPR1 – a *for^{null}* line with a UAS-*for* crossed to *pr1-Gal4* to increase

for expression in tissues that express *for-pr1*, 2) PR1CON- a Gal4 driver control in which *pr1-Gal4* was crossed to homozygous rovers, 3) UASPR2 – a *for*^{null} line with a UAS-*for* crossed to *pr2-Gal4* to increase *for* expression in tissues that express *for-pr2*, 4) PR2CON- a Gal4 driver control in which *pr2-Gal4 was* crossed to homozygous rovers, 5) UASPR3 – a *for*^{null} line with a UAS-*for* crossed to *pr3-Gal4* to increase *for* expression in tissues that express *for-pr3*, 6) PR3CON- a Gal4 driver control in which *pr3-Gal4 was* crossed to homozygous rovers, 7) UASPR4 – a *for*^{null} line with a UAS-*for* crossed to *pr4-Gal4* to increase *for* expression in tissues that express *for-pr4*, 8) PR4CON- a Gal4 driver control in which *pr4-Gal4 was* crossed to homozygous rovers, 9)UAST1CON – a UAS control in which the *for*^{null} line with a UAS-*for* was crossed to homozygous rovers.

4.3.2 Spatiotemporal expression experiments

Three days prior to the experiment, 12-16 virgin female *D. melanogaster* were collected under light anesthesia (CO_2) and placed in vials (25mm diameter, 95 mm height). These vials were kept in constant conditions in a 12L:12D cycle at either 18°C or 30°C for three days (See Figure 1).

4.3.3 Isolation

Females in the late pupal stage were collected three days prior to the experiment. Each pupa was placed in a separate vial and kept in a box with dividers to ensure that flies were fully isolated upon eclosion. 12-16 female pupae were placed in separate vials. These vials were kept at constant conditions in a 12-hr light/dark cycle at 25°C for three days.

4.3.4 Food deprivation

Three days prior to the experiment, 12-16 virgin female flies were collected under light anesthesia (CO_2) and placed in vials. These vials were kept at constant conditions in a 12-hr light/dark cycle at 25°C. When testing 24 hours of food deprivation, food-deprived treatments were transferred into vials containing 4 mL of agar 24 hours before videos were recorded. When testing 48 hours of food deprivation, food-deprived treatments were transferred into vials

containing 4mL of agar 48 hours before videos were recorded. The agar in the food deprivation treatment provided hydration for the flies.

4.3.5 Sleep deprivation

Three days prior to the experiment, 12-16 virgin female flies were collected under light anesthesia (CO_2) and placed in vials. These vials were kept at constant conditions in a 12L:12D, 25°C for two days. The day before the experiment, individual flies were transferred into activity tubes and placed in activity monitors. Sleep-deprived treatments were placed on vortexes and flies were sleep deprived for 8 hours by vortexing them at low setting for 2 seconds/minute. Fly activity was tracked across sequential 1 minute time periods, if no activity was recorded for 5 minutes, the fly was considered to be asleep. On the day of the experiment, both sleep-deprived treatments and control treatments were grouped into their respective vials.

4.3.6 Mixed rover-sitter experiment

Three days prior to the experiment, 12-16 virgin female flies were collected under light anesthesia (CO_2) and placed in vials. The strains were housed in separate vials. These vials were kept at constant conditions in a 12-hr light/dark cycle at 25°C. On the day of the experiment a metal divider was placed in the arena, and rover and sitter flies were placed on opposite sides of the divider according to the ratios being tested. The experimental groups were 9 rovers: 1 sitter (1S:9R), 7 rovers: 3 sitters (7R:3S), 5 rovers: 5 sitters (5R:5S), 3 rovers: 7 sitters (7S:3R), and 1 rover: 9 sitters (1R:9S). Dividers were removed as soon as video recording began to allow the identification of rover and sitter fly identities in the videos. 40 minutes of video footage were recorded, and the first 10 minutes were not included in the analysis to allow time for the flies to acclimatize.

4.3.7 Acquiring SINs

Twelve flies were transferred into a plexiglass arena (60 mm diameter, 2mm height) and covered with a glass lid fifteen minutes before videos were recorded (10 flies for the mixed ratio experiments). All videos were recorded 9.5 hours after lights on. Using fview, an application used to record footage from digital cameras, thirty minutes of video were recorded for all

experiments (except for the mixed ratio experiment) (Straw & Dickinson, 2009). We collected ~20 videos for each treatment group. The videos were then analyzed using an applied machine vision system, Ctrax, to obtain fly identity, orientation, position and trajectories in the arena throughout the 30-minute trial (Branson et al., 2009). An automated method was used to identify values for interaction criteria. This program was used to identify each experimental group's social spacing patterns by generating estimates of the distance, angle, and time parameters for a social interaction to occur (Schneider & Levine, 2014). Finally, MATLAB was then used to import the videos and a program within MATLAB was used as a classifier to identify interactions that occurred between flies based on the three-set criteria (proximity of flies, their angular orientation during the interaction, and the duration of the interaction).

4.3.8 Network measurements

The MATLAB code analyzed four behavioural elements of networks, in addition to four network measures. The behavioural elements of networks were: (1) interaction duration - the mean time, in seconds, flies spend interacting, (2) interaction rate - the mean number of social interactions expressed as interactions per minute, (3) Reciprocation - the mean proportion of social interactions that are reciprocated, and (4) Movement - the average speed for each experimental group, which is the mean locomotor activity of all flies within the arena, calculated by tracking the motion of each fly in all video trials using Ctrax.

The four network measures MATLAB analyzed were: (5) Assortativity - the probability of an individual interacting with another individual of similar degree. Degree is defined as the number of interactions involving an individual. Assortativity is a measure of the homogeneity of the network in the number of the interactions across all individuals (Newman, 2010). Networks with higher assortativity values have a homogenous distribution of interactions. (6) Clustering coefficient - a measure of network interconnectedness. High clustering coefficient individuals are more likely to be connected to a cluster of interconnected neighbors (Newman, 2010). (7) Global efficiency – a measure of how efficient information relay is within the network. Networks with higher global efficiency values require fewer interactions for information relay, and thus there is a more efficient flow of information through the network (Latora & Marchiori, 2001b). (8) Betweenness centrality – indicates the importance of individuals for communication relay and

network cohesion. Individuals with higher betweenness centrality values are more important for network cohesion and information flow (Newman, 2010).

4.3.9 Statistical analyses

Networks were normalized using z-scores to control for degree distribution and allow for the comparison between networks of different *Drosophila* strains. Networks and *z*-scores were generated as described in Schneider et al. (2012).

In order to determine if the network properties between rovers and sitters are different, a two-tailed t-test was performed. For other experiments, a One-way or a Two-way ANOVA test was used to determine whether network properties were significantly different. The Tukey-Kramer method was used as the post-hoc test. Outliers were removed from the data (outliers were calculated as follows: A trial's mean was considered an outlier if it was less than the 25th quartile-(1.5*IQR) or greater than the 75th quartile + (1.5*IQR)). The null hypothesis is that there is no difference between groups and is tested with $\alpha = 0.05$.

4.4 Results

4.4.1 for knockdown during adult stage does not affect behavioural elements and social network measures

To determine if knocking down *for* during adulthood affects behavioural elements and social network measures, I used a temperature-sensitive UAS-GAL4-GAL80 system. Two knockdown crosses were generated, along with their control crosses and wild-type rover and sitter flies (See Methods). Progeny were kept at 18°C till the late pupal stages and then transferred to a 30°C incubator (Fig. 1b). All videos were recorded in a 30°C filming chamber. Table 2 shows the interaction criteria of those crosses and their controls. Figures 2 and 3 show the effect of knocking down *for* in the whole body during adult stages using ubiquitous *Tubulin* and *Daughterless* Gal4 drivers. Overall, knocking down *for* during the adult stages does not affect behavioural elements and social network phenotypes. Interaction duration of both *Tub.* knockdown and *Da.* knockdown did not differ from the Gal4 driver controls (Fig. 2a, 3a). A similar trend for both knockdowns was observed for rates of interaction (Fig. 2b, 3b),

movement (Fig. 2d, 3d), and assortativity (Fig. 2e, 3e). The clustering coefficient and betweenness centrality values of both knockdown lines did not differ from the Gal4 driver or UAS controls (Fig. 2f, 2h, 3f, 3h). Reciprocation levels of *Tub* knockdown were intermediate between their Gal4 and UAS controls but did not differ from sitter reciprocation levels (Fig. 2c), while the reciprocation levels of *Da.* knockdowns are similar to that of its Gal4 control (Fig 3c). For global efficiency values, *Tub* knockdown did not differ from its UAS control, while *Da.* Knockdown did not differ from its Gal4 driver control (Fig. 2g, 3g). The rover-sitter differences were maintained at 30°C, with rovers having shorter interaction duration and being less likely to reciprocate a reaction. Rovers also displayed higher rates of interaction and movement during the 30-minute trial (Fig 2a-d, 3a-d). As previously observed, sitters also form more homogenous networks (Fig 2e, 3e), are more clustered (Fig. 2f, 3f), and form networks in which the flow of information is less efficient when compared to rovers (Fig 2g, 3g). However, unlike networks of rover and sitters filmed at 25°C, networks of rovers and sitters filmed 30°C show significant differences in betweenness centrality values, with sitters forming networks with significantly higher betweenness centrality values (Fig 2h, 3h). These results indicate that knocking down *for* during the adult stage alone in the whole body of flies does not influence the behavioural elements, or social network measures.

Table 4.2 (Table 2). Interaction criteria of the RNAi knockdown crosses, their respective controls, and rovers and sitters. Interaction criteria for each strain were automatically computed for distance, angle and time criteria across the replicates in order to capture interactions objectively across videos.

Strain	Distance (Body lengths)	Angle (º)	Time (Sec)
***Da.*Knockdown**	2.00	65	0.70
***Tub.*Knockdown**	1.75	105	0.75
UASControl	1.75	120	0.95
***Da.*Control**	2.00	65	0.70
***Tub.*Control**	2.00	65	0.70
Rover	2.00	50	0.45
Sitter	1.75	110	0.75

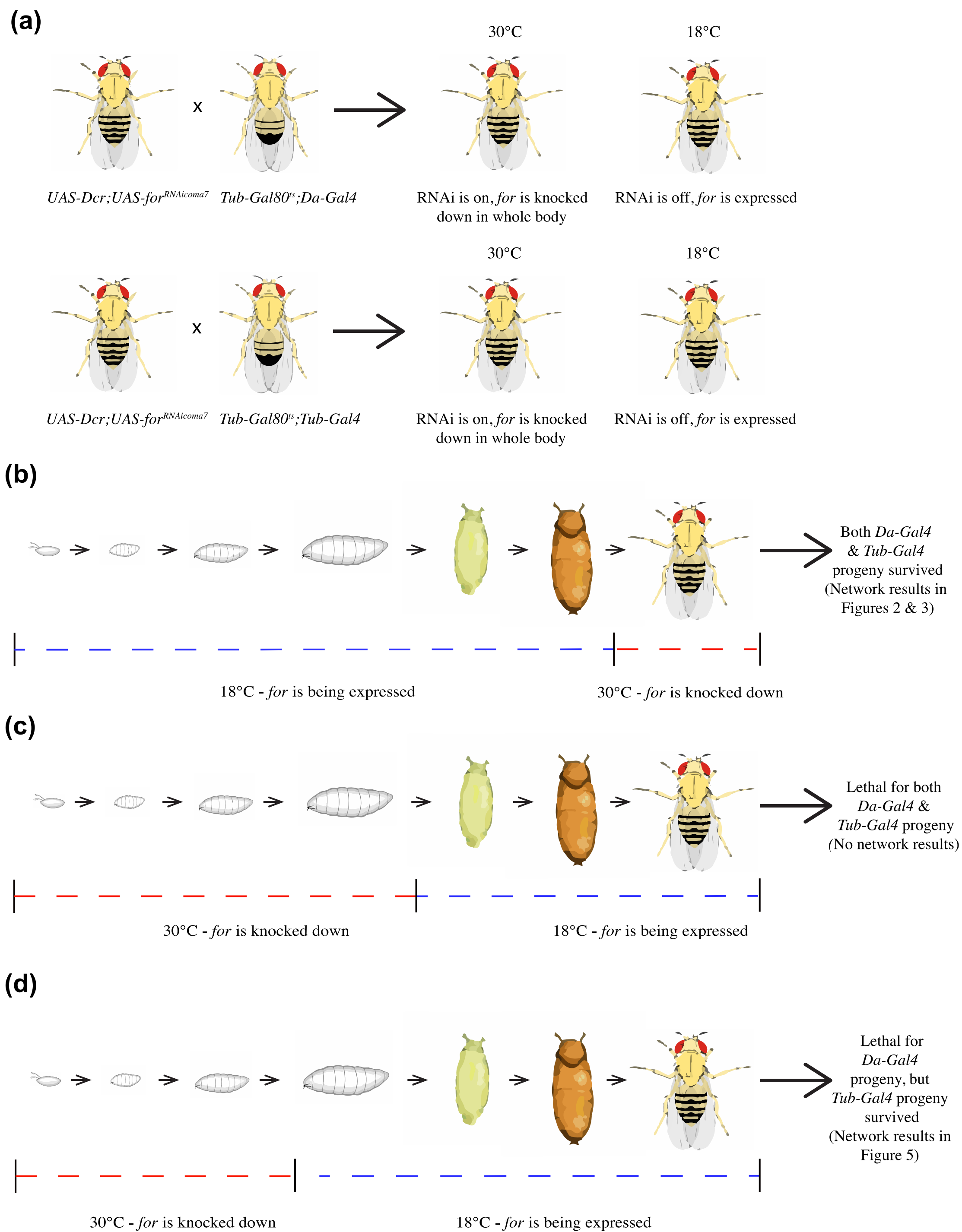


Figure 4.1 (Figure 1). Schematic of the experimental design that is used to temporally knockdown *for* using the temperature sensitive GAL80^{ts} which represses GAL4 expression at 18°C and allows GAL4 expression at 30°C. (a) The two *for*-RNAi lines in which a UAS-

for^{RNAicomma7} was crossed to either the ubiquitous drivers *Da-Gal4* or *Tub-Gal4*. Progeny of these crosses where then either kept at 18°C where the temperature sensitive GAL80^{ts} represses the Gal4 drivers and thus *for* is expressed or kept at 30°C where the temperature sensitive GAL80^{ts} allows Gal4 driver expression and thus RNAi is expressed and *for* is knocked down. (b) The first manipulation performed; both crosses in addition to their UAS and Gal4 driver controls, along with homozygous rovers and sitters were kept at 18°C until the late pupal stage, and then were switched to 30°C (Videos were filmed at 30°C). (c) The second manipulation performed; both crosses in addition to their UAS and Gal4 driver controls, along with homozygous rovers and sitters were kept at 30°C until the late pupal stage, and then were switched to an 18°C incubator, but the progeny of these crosses were not viable. (d) The third manipulation performed; both crosses in addition to their UAS and Gal4 driver controls, along with homozygous rovers and sitters were kept at 30°C until the 3rd instar larval stage, and then were switched to an 18°C incubator (Videos were filmed at 18°C).

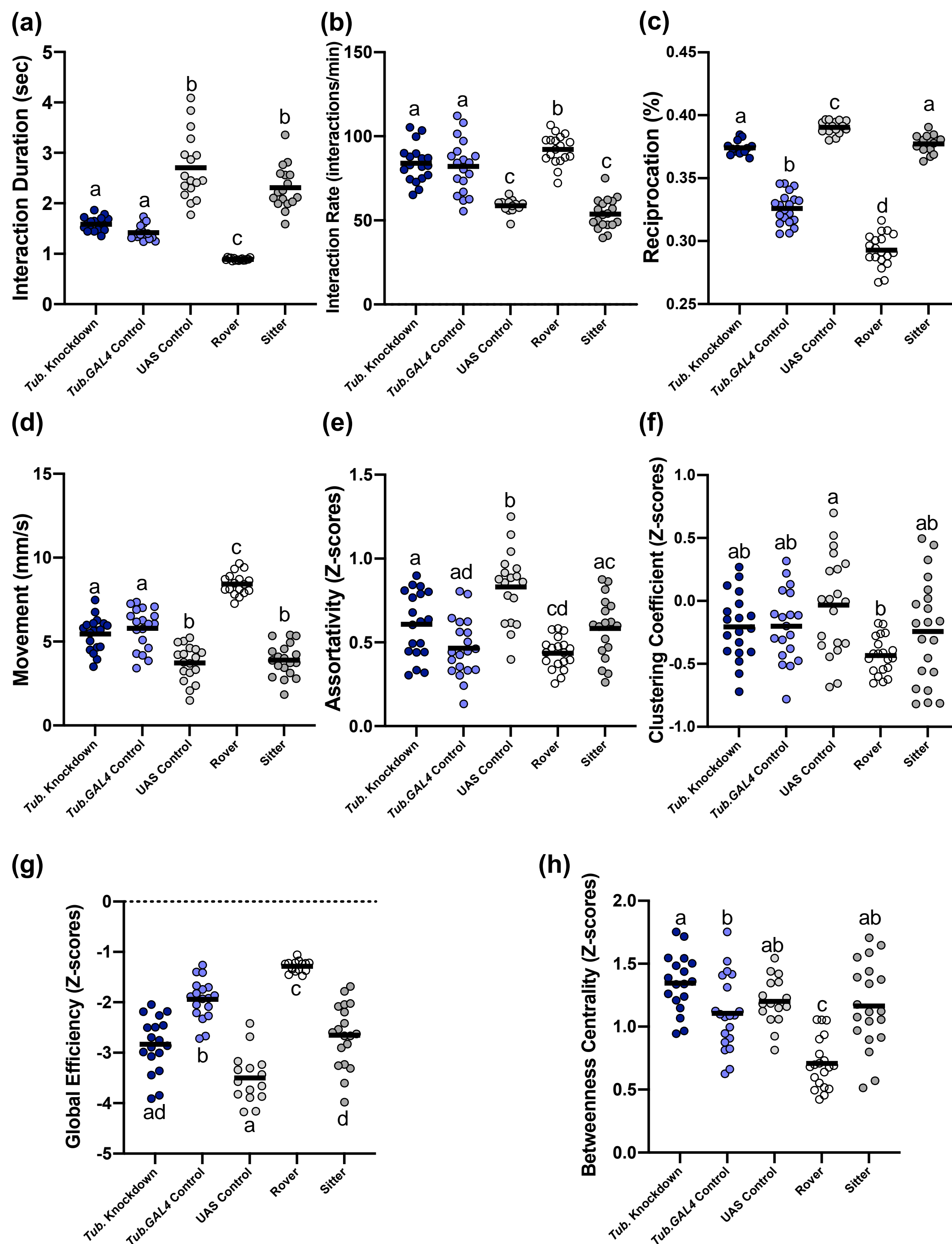


Figure 4.2 (Figure 2). RNAi knockdown of *for* using Tubulin-driver (30°C) during adult stages and its effects on behavioural elements and social networks measures (Manipulation from Figure 1b). (a) Average duration of an interaction of rovers were significantly lower

than other groups ($F_{(4,79)} = 65.36$) ($p \leq 0.0001$) (*Tub.* knockdown n = 17, *Tub. GAL4* control n =17, UAS control n = 17, rover n = 16, sitter n = 17). (b) Rates of an interaction were highest in networks of rover flies ($F_{(4,82)} = 41.82$) ($p \leq 0.0001$) (*Tub.* knockdown n = 18, *Tub. GAL4* control n = 19, UAS control n = 15, rover n = 18, sitter n = 19). (c) Percentage reciprocation of rover networks were significantly lower than other groups tested ($F_{(4,80)} = 309.00$) ($p \leq 0.0001$) (*Tub.* knockdown n = 14, *Tub. GAL4* control n =20, UAS control n = 15, rover n = 19, sitter n = 17). (d) Movement values were unaffected by the *Tub.* Knockdown, and rover flies had significantly higher average movement ($F_{(4,87)} = 62.48$) ($p \leq 0.0001$) (*Tub.* knockdown n = 19, *Tub. GAL4* control n = 19, UAS control n = 18, rover n = 17, sitter n = 19). (e) Networks of UAS controls had highest assortativity values ($F_{(4,89)} = 14.38$) ($p \leq 0.0001$) (*Tub.* knockdown n = 19, *Tub. GAL4* control n =20, UAS control n = 18, rover n = 19, sitter n = 18). (f) No significant differences in clustering coefficient values found due to *Tub.* knockdown ($F_{(4,94)} = 4.009$) ($p \leq 0.01$) (*Tub.* knockdown n = 19, *Tub. GAL4* control n =19, UAS control n = 20, rover n = 20, sitter n = 21). (g) Global Efficiency values were significantly higher than other groups ($F_{(4,80)} = 48.63$) ($p \leq 0.0001$) (*Tub.* knockdown n = 18, *Tub. GAL4* control n =18, UAS control n = 15, rover n = 15, sitter n = 19). (h) Betweenness centrality values were significantly lower in rover networks ($F_{(4,89)} = 16.29$) ($p \leq 0.0001$) (*Tub.* knockdown n = 18, *Tub. GAL4* control n = 20, UAS control n = 16, rover n = 20, sitter n = 20). *a-h* were analyzed with one-way ANOVA to determine if statistical differences exist between the groups. The Tukey-Kramer method was used as a post-hoc test (Methods). Outliers were removed from all the datasets. Bars indicate mean. Letters indicate statistical significance. *e-g* Measurements were standardized using z-scores.

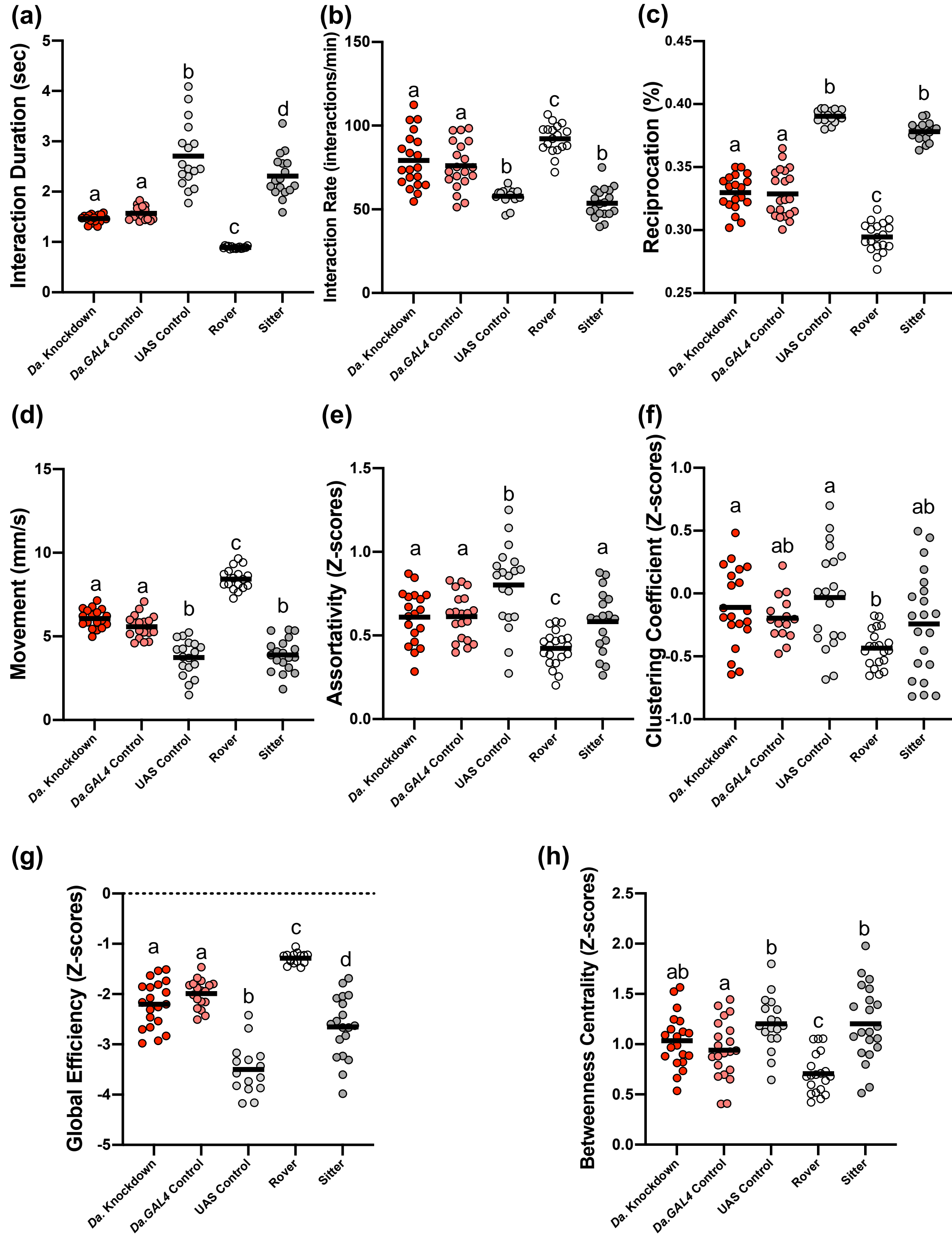


Figure 4.3 (Figure 3). RNAi knockdown of *for* using Daughterless-driver (30°C) during adult stages, behavioural elements and social networks measures (Manipulation from Figure 1b). (a) Average duration of an interaction of rovers were significantly lower than

other groups ($F_{(4,80)} = 67.08$) ($p \leq 0.0001$) (*Da.* knockdown n = 17, *Da. GAL4* control n =18, UAS control n = 17 , rover n = 16, sitter n = 17). (b) Rates of an interaction were highest in networks of rover flies ($F_{(4,86)} = 31.59$) ($p \leq 0.0001$) (*Da.* knockdown n = 20, *Da. GAL4* control n =20, UAS control n = 14 , rover n = 18, sitter n = 19). (c) Networks of sitter flies and UAS controls have a higher percentage reciprocation ($F_{(4,87)} = 159.08$) ($p \leq 0.0001$) (*Da.* knockdown n = 19, *Da. GAL4* control n =21, UAS control n = 15 , rover n = 19, sitter n = 17). (d) Movement values were unaffected by the *Da.* Knockdown, and networks of rover flies have highest movement values ($F_{(4,85)} = 95.50$) ($p \leq 0.0001$) (*Da.* knockdown n = 18, *Da. GAL4* control n =18, UAS control n = 18, rover n = 17, sitter n = 19). (e) Networks of UAS controls have higher assortativity values compared to other groups ($F_{(4,91)} = 11.81$) ($p \leq 0.0001$) (*Da.* knockdown n = 19, *Da. GAL4* control n =20, UAS control n = 19, rover n = 20, sitter n = 18). (f) No significant differences in clustering coefficient values found due to *Da.* knockdown ($F_{(4,91)} = 4.558$) ($p \leq 0.01$) (*Da.* knockdown n = 20, *Da. GAL4* control n =15, UAS control n = 20, rover n = 20, sitter n = 21). (g) Global Efficiency values were unaffected by the *Da.* Knockdown ($F_{(4,82)} = 52.85$) ($p \leq 0.0001$) (*Da.* knockdown n = 20, *Da. GAL4* control n =18, UAS control n = 15, rover n = 15, sitter n = 19). (h) Betweenness centrality values of rover flies were significantly lower ($F_{(4,95)} = 10.25$) ($p \leq 0.0001$) (*Da.* knockdown n = 20, *Da. GAL4* control n =21, UAS control n = 18, rover n = 20, sitter n = 21). *a-h* were analyzed with one-way ANOVA to determine if statistical differences exist between the groups. The Tukey-Kramer method was used as a post-hoc test (Methods). Outliers were removed from all the datasets. Bars indicate mean. Letters indicate statistical significance. *e-h* Measurements were standardized using z-scores.

4.4.2 The developmental effect of for on behavioural elements and social network phenotypes

To investigate the developmental effect of *for*, I first needed to confirm that flies can form networks at 18°C. Table 3 shows interaction criteria for rovers and sitters at 18°C. Figure 4 shows that *D. melanogaster* are capable of forming networks at 18°C and that the rover-sitter differences are maintained. For the four behavioural elements, rover flies tended to have shorter interaction durations (Fig. 4a), were less likely to reciprocate an interaction (Fig. 4c), have higher rates of interaction (Fig. 4c), and move more during the 30-minute trial (Fig. 4d). Sitter networks were also more clustered (Fig. 4f) while there was a more efficient flow of information in rover networks (Fig. 4g). Sitters also formed networks with higher assortativity and betweenness centrality values, however, these differences were not significant (Fig 4e, h).

After verifying that the rover-sitter differences were maintained at 18°C, I was able to generate the knockdown crosses (See methods) and follow the second manipulation design (Fig. 1c). Knocking down *for* using both *Tubulin* and *Daughterless* Gal4 drivers until the pupal stage did

not generate viable progeny and thus, I was not able to test the effect of this knockdown during these stages on social network phenotypes. I proceeded to the next manipulation design in which knockdown crosses were kept at 30°C till the 3rd instar larval (late wandering stage) and then switched to an 18°C incubator (Fig. 1d). Only *Tubulin* knockdown flies were able to survive to adulthood. I acquired and analyzed networks of *Tubulin* knockdown, their Gal4, and UAS controls as well as rovers and sitters in an 18°C filming chamber. Table 4 shows the interaction criteria of these three crosses as well as their rover and sitter controls. Overall, no effect was observed in network phenotypes as a result of the *Tub.* knockdown during these stages of development (Fig. 5). *Tub.* knockdown did not differ from its Gal4 or UAS controls for interaction duration (Fig 5a), reciprocation (Fig. 5c), movement (Fig. 5d), clustering coefficient (Fig. 5f), or global efficiency (Fig. 5e). Rates of interactions of the *Tub.* knockdown flies were significantly higher than that of the UAS control but did not differ from its Gal4 control (Fig. 5b). No significant differences were found between the five groups tested for assortativity (Fig. 5e) or betweenness centrality (Fig. 5h). Together these results show that knocking down *for* in the whole body till wandering larvae stages also did not affect behavioural elements and social networks.

Table 4.3 (Table 3). Interaction criteria of rover and sitters tested at 18°C. Interaction criteria for each strain were automatically computed for distance, angle and time criteria across the replicates in order to capture interactions objectively across videos.

Strain	**Distance (Body lengths)**	**Angle (º)**	**Time (Sec)**
Rover	2.00	120	0.95
Sitter	1.50	125	2.15

Table 4.4 (Table 4). Interaction criteria of the *Tub.* Knockdown and its controls tested at 18°C. Interaction criteria for each strain were automatically computed for distance, angle and time criteria across the replicates in order to capture interactions objectively across videos.

Strain	**Distance (Body lengths)**	**Angle (º)**	**Time (Sec)**
***Tub.*Knockdown**	1.75	130	1.65
UASControl	1.50	130	1.45
***Tub.*Control**	1.50	120	1.65
Rover	1.50	120	0.90
Sitter	1.50	125	0.95

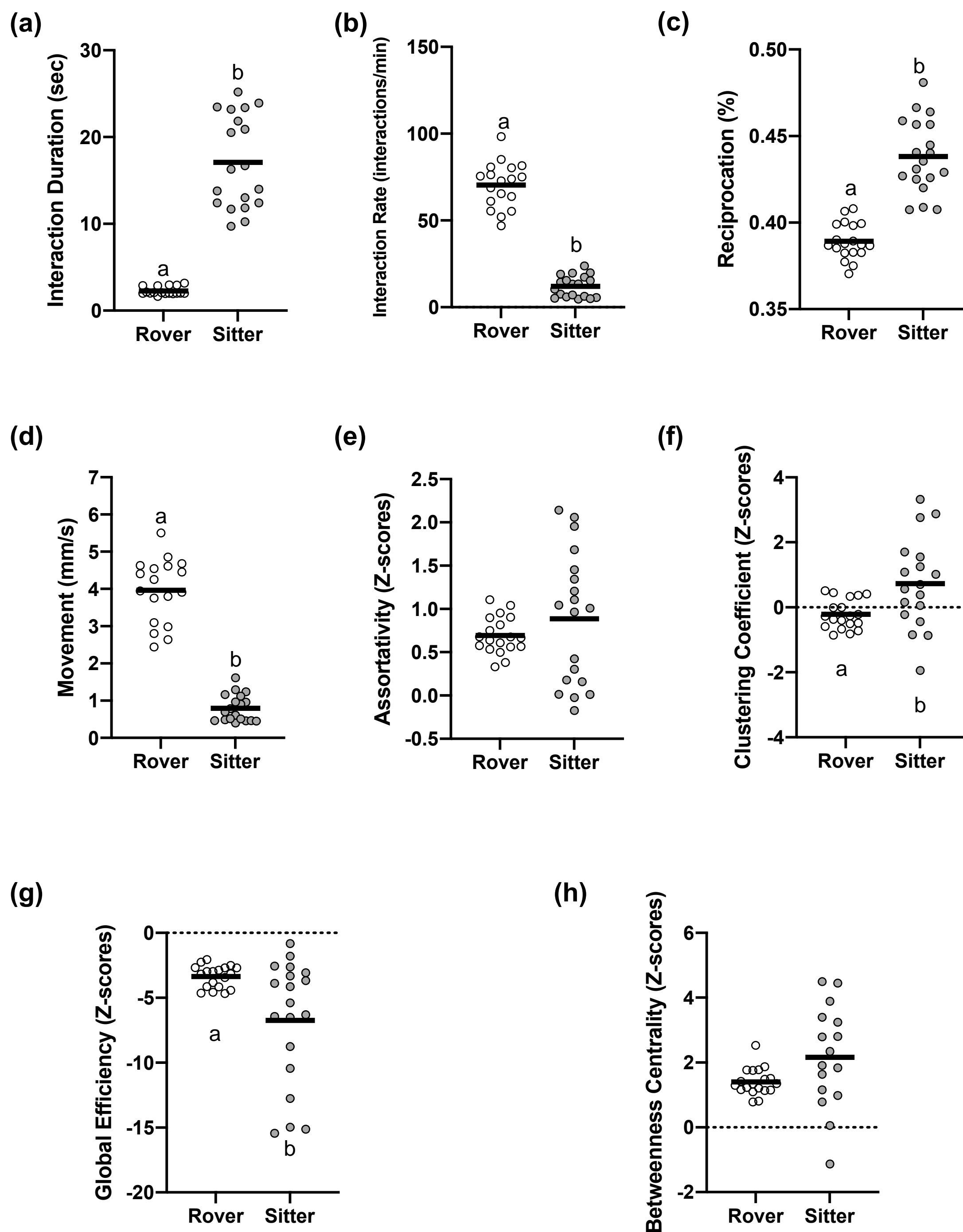


Figure 4.4 (Figure 4). Rover and sitter strains differ in behavioural elements and social network properties (18°C). (a) Average duration of an interaction was significantly lower in rover networks ($t = 11.32$) ($df = 34$) ($p \leq 0.0001$) (rover $n = 17$, sitter $n = 19$). (b) Interaction Rate was

significantly higher in networks of rover flies (t = 17.50) (df = 35) ($p \leq 0.0001$) (rover n = 18, sitter n = 19). (c) Percentage of interactions that were reciprocated by the receiver was higher in sitter flies (t = 9.05) (df = 36) ($p \leq 0.0001$) (rover n = 19, sitter n = 19). (d) Rovers moved significantly more during the trial compared to sitters (t = 14.74) (df = 35) ($p \leq 0.0001$) (rover n = 18, sitter n = 19). (e) Assortativity values did not differ between networks of rovers and sitters (t = 1.06) (df = 36) ($p = 0.2957$) (rover n = 19, sitter n = 19). (f) Clustering coefficient values of sitter flies were significantly higher than rover flies (t = 2.81) (df = 35) ($p \leq 0.01$) (rover n = 19, sitter n = 18). (g) Global Efficiency values were significantly higher in networks of rovers relative to sitter networks (t = 3.02) (df = 36) ($p \leq 0.01$) (rover n = 19, sitter n = 19). (h) Betweenness centrality values was not affected by the natural polymorphism of *for* (t = 2.03) (df = 33) ($p = 0.0504$) (rover n = 19, sitter n = 16). *a-h* were analyzed with two-tailed t-tests (*Methods)*. Outliers were removed from all the datasets. Bars indicate mean. Letters indicate statistical significance. *e-h* Measurements were standardized using z-scores.

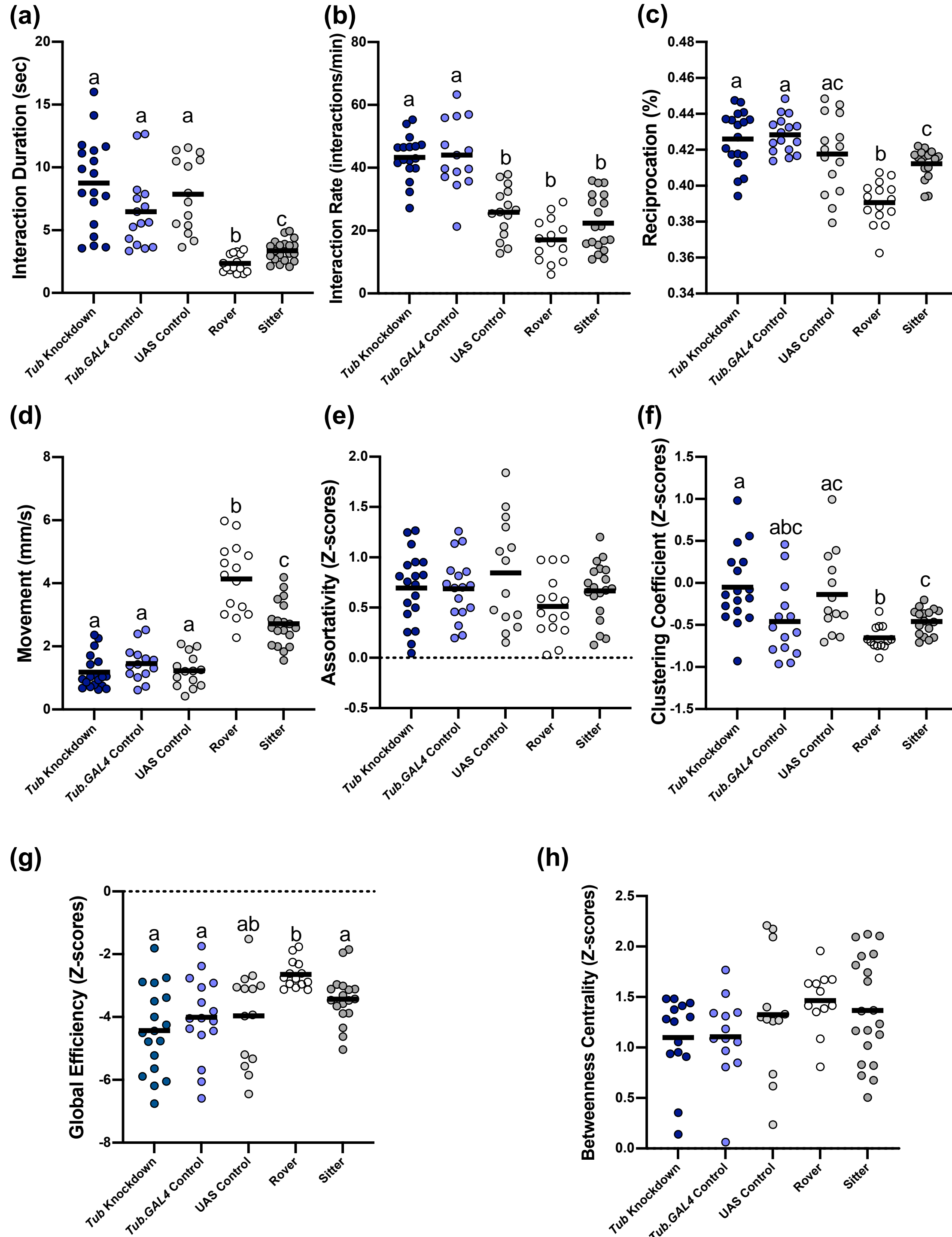


Figure 4.5 (Figure 5). Developmental effect of *for,* behavioural elements, and social networks (Experiments performed at 18°C) (Manipulation from Figure 1d). (a) Average duration of an interaction was not affected by the Tub. Knockdown ($F_{(4,77)} = 20.75$) ($p \leq 0.0001$) (*Tub.*

knockdown n = 18, *Tub*. GAL4 control n =15, UAS control n = 14 , rover n = 15, sitter n = 20). (b) *Tub.* Knockdown and *Tub.GAL4* control had higher interaction rates ($F_{(4,74)} = 32.55$) ($p \leq 0.0001$) (*Tub*. knockdown n = 17, *Tub*. GAL4 control n =14, UAS control n = 15 , rover n = 14, sitter n = 19). (c) Percentage reciprocation was not affected by the *Tub.* Knockdown ($F_{(4,76)} = 17.43$) ($p \leq 0.0001$) (*Tub*. knockdown n = 18, *Tub*. GAL4 control n =16, UAS control n = 15 , rover n = 15, sitter n = 17). (d) Movement values were unaffected by the *Tub.* Knockdown ($F_{(4,76)} = 48.62$) ($p \leq 0.0001$) (*Tub*. knockdown n = 19, *Tub*. GAL4 control n =14, UAS control n = 14 , rover n = 15, sitter n = 19). (e) No significant differences in assortativity were found across the groups tested ($F_{(4,79)} = 1.548$) ($p = 0.1966$) (*Tub*. knockdown n = 19, *Tub*. GAL4 control n =17, UAS control n = 14 , rover n = 15, sitter n = 19). (f) No significant differences in clustering coefficient values found due to *Tub.* Knockdown ($F_{(4,69)} = 6.714$) ($p \leq 0.0001$) (*Tub*. knockdown n = 17, *Tub*. GAL4 control n =14, UAS control n = 13 , rover n = 13, sitter n = 17). (g) Global Efficiency values were unaffected by the *Tub.* Knockdown ($F_{(4,75)} = 5.441$) ($p \leq 0.001$) (*Tub*. knockdown n = 18, *Tub*. GAL4 control n =16, UAS control n = 14 , rover n = 14, sitter n = 18). (h) No differences were found in betweenness centrality values across the five groups ($F_{(4,66)} = 1.630$) ($p = 0.1771$) (*Tub*. knockdown n = 14, *Tub*. GAL4 control n =13, UAS control n = 12 , rover n = 12, sitter n = 20). *a-h* were analyzed with one-way ANOVA to determine if statistical differences exist between the groups. The Tukey-Kramer method was used as a post-hoc test (Methods). Outliers were removed from all the datasets. Bars indicate mean. Letters indicate statistical significance. *e-h* Measurements were standardized using z-scores.

4.4.3 Investigating the differential promoter contribution to behavioural elements and social network phenotypes

for's complexity can be partially attributed to the four different promoters and their spatiotemporal patterns of expression. We investigated whether differences in expression from each of *for's* promoters influences behavioural elements and social network phenotypes. We used a Gal4 driver to drive the expression of each promoter product in *foraging* knockout flies (Fig. 6). Table 5 shows interaction criteria for the groups tested in this experiment and their controls. Overall, the promoter-specific increased expression showed a reduced ability to form networks. In the promoter 1 increased expression, UASPR1, only 5 out of the 20 videos analyzed were able to form networks (Fig. 6a). Promoter 2 and promoter 4 increased expression, UASPR2 and UASPR4, formed networks in 16 out of the 20 videos analyzed (Fig. 6b, d). In Promoter 3 increased expression, UASPR3 formed networks in 4 out of the 20 videos (Fig. 6c)

Figure 7 shows the effect of promoter 1 increased expression. UASPR1 had lower mean number of interactions, reciprocation rates and movement relative to its UAS and GAL4 controls (UAST1CON and PR1CON) as well as rover and sitter controls (Fig. 7b, 7c, 7d). Networks of

UASPR1 also showed the highest values for the three network measures: assortativity, clustering coefficient, and global efficiency values (Fig. 7e, 7f, 7g). Figure 8 shows that promoter 2 products contribute to behavioural elements of a networks. Flies in networks of UASPR2 had the longest interaction duration and rates of reciprocation (Fig. 8a, 8c) and lower rates of interaction and movement when compared to its UAS and GAL4 controls (Fig. 8b, 8d). Figure 9 shows social networks of the promoter 3 increased expression. UASPR3 had the lowest average values for the mean number of interactions, likelihood of flies reciprocating an interaction, and movement (Fig. 9b, 9c, 9d). Flies is a UASPR3 network spend more time interacting during the trial when compared to their UAS and GAL4 controls (Fig. 9a). Networks of UASPR3 had the highest global efficiency values (Fig. 9g). Finally, promoter 4 increased expression results are shown in figure 10. Both interaction duration and reciprocation were significantly higher in networks of UASPR4 (Fig. 10a, 10c). Consistent with promoter 1, 3, and 4 results, movement was significantly lower in UASPR4 groups (Fig. 10d). The mean number of interactions were also significantly lower in networks of UASPR4 (Fig. 10b). Global efficiency of UASPR4 networks was similar to those of sitter networks (Fig. 10g). Overall, these results indicate that all four promoters play a role in network formation and that different combination of *for*'s promoters may be responsible for regulating the different behavioural elements and social network measures observed.

Table 4.5 (Table 5). Interaction criteria of the promoter specific increased expression and their controls tested. Interaction criteria for each strain were automatically computed for distance, angle and time criteria across the replicates in order to capture interactions objectively across videos.

Strain	Distance (Body lengths)	Angle (º)	Time (Sec)
UASPR1	1.25	20	0.400
PR1CON	1.75	55	0.550
UASPR2	1.75	120	1.350
PR2CON	1.75	55	0.550
UASPR3	1.50	5	0.550
PR3CON	1.50	85	0.550
UASPR4	1.50	135	2.125
PR4CON	1.50	90	0.600
UAST1CON	2.00	65	0.600
Rover	2.00	85	0.550
Sitter	1.50	110	0.800

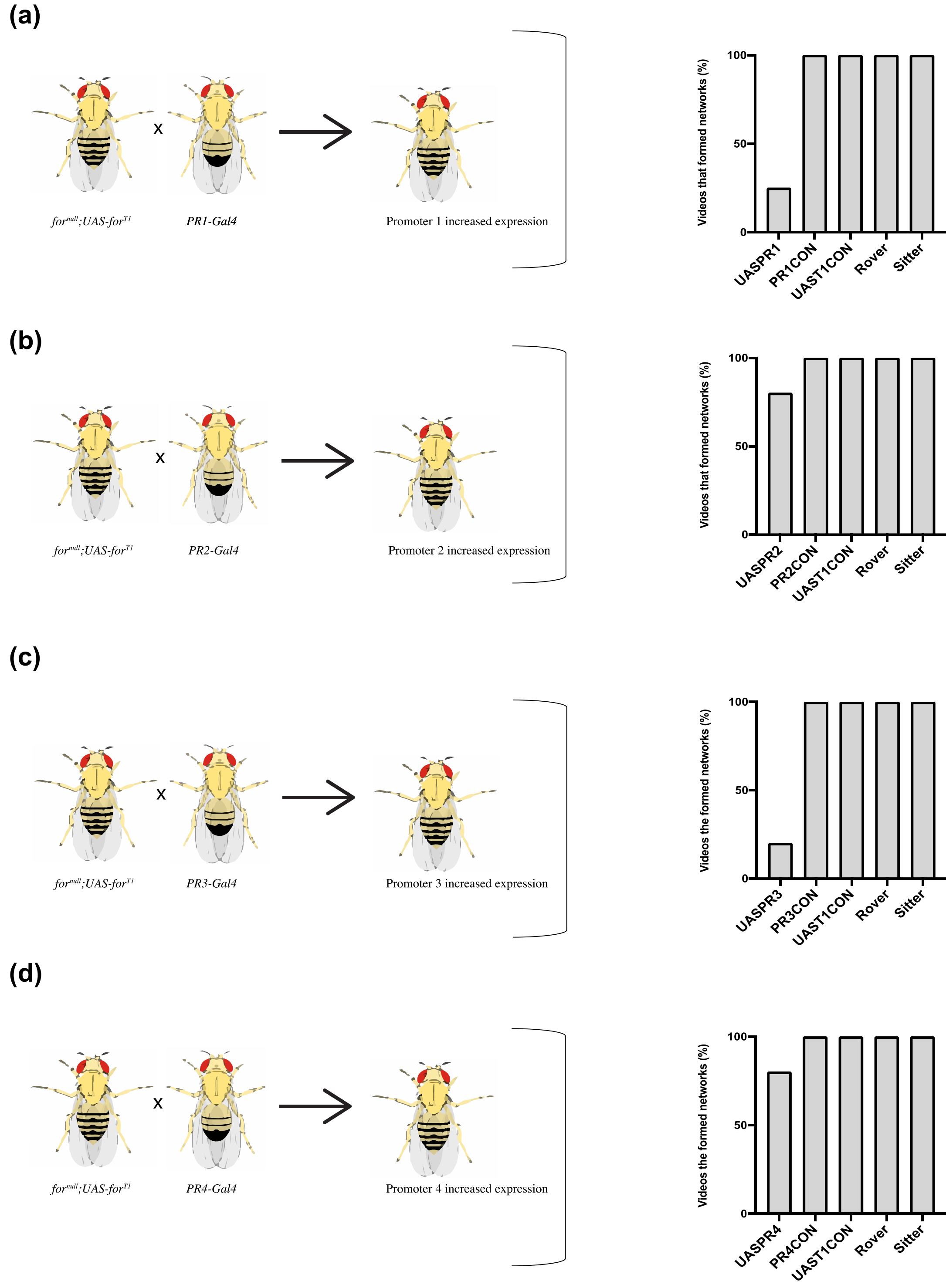


Figure 4.6 (Figure 6). Experimental design of promoter-specific increased experiment. (a) *for*^{null} flies were crossed to *pr1*-Gal4 to generate promoter 1 increased expression progeny. *for* expression was increased in tissues that express promoter 1. The graph shows a reduction in the

ability of the UASPR1 line to form networks with only 5 videos of the 20 analyzed able to form networks. (b) *for*^{null} flies were crossed to *pr2*-Gal4 to generate promoter 2 increased expression progeny. *for* expression was increased in different tissues that express promoter 2. For the UASPR2 line, 16 videos of the 20 analyzed were able to form networks. (c) *for*^{null} flies were crossed to *pr3*-Gal4 to generate promoter 3 increased expression progeny. *for* expression was increased in tissues that express promoter 3. The UASPR3 line shows a great reduction in the ability to form networks with only 4 videos of the 20 analyzed able to form networks. (d) *for*^{null} flies were crossed to *pr4*-Gal4 to generate promoter 4 increased expression progeny. *for* expression was increased in tissues expressing promoter 4. The graph shows a reduction in the ability of the UASPR4 line to form networks with 16 videos of the 20 analyzed able to form networks.

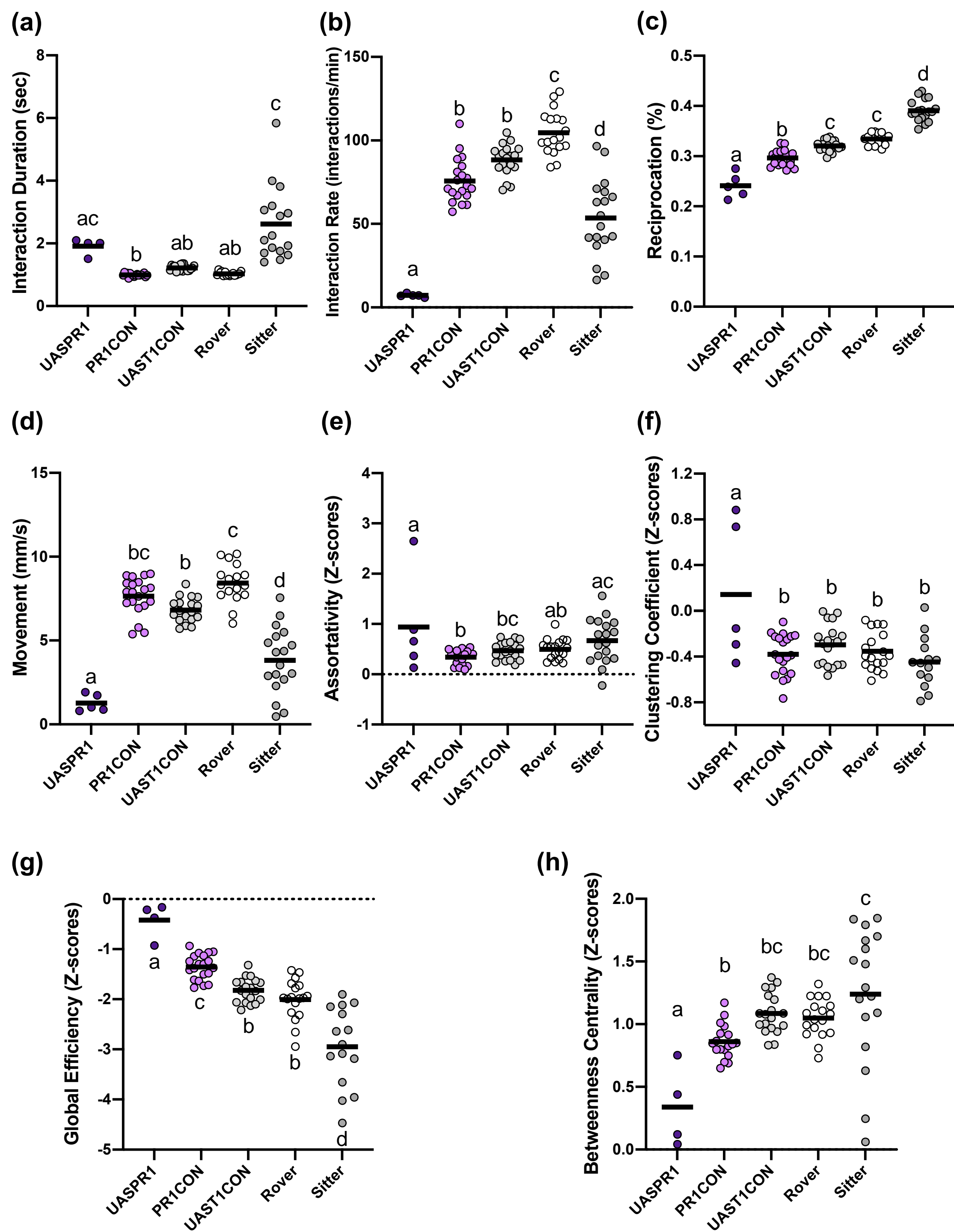


Figure 4.7 (Figure 7). Promoter 1 contribution to behavioural elements and social network measures. (a) Average duration of an interaction did not differ between the UASPR1 and its respective PR1CON and UAST1CON controls ($F_{(4,68)} = 23.64$) ($p \leq 0.0001$) (UASPR1 n = 4,

PR1CON n = 18, UAST1CON n = 19, rover n = 16, sitter n = 16). (b) UASPR1 flies had significantly lower interaction rates relative to all other groups ($F_{(4,75)} = 56.28$) ($p \leq 0.0001$) (UASPR1 n = 5, PR1CON n = 20, UAST1CON n = 19, rover n = 18, sitter n = 18). (c) Percentage reciprocation was lowest in networks of UASPR1 flies ($F_{(4,74)} = 127.0$) ($p \leq 0.0001$) (UASPR1 n = 5, PR1CON n = 20, UAST1CON n = 18, rover n = 18, sitter n = 18). (d) Flies in networks of UASPR1 moved significantly less relative to its controls and rovers and sitters ($F_{(4,73)} = 53.28$) ($p \leq 0.0001$) (UASPR1 n = 5, PR1CON n = 20, UAST1CON n = 18, rover n = 17, sitter n = 18). (e) Assortativity values of networks of UASPR1 were significantly higher than UAST1CON and PR1CON, but did not differ from rovers and sitters ($F_{(4,76)} = 4.139$) ($p \leq 0.01$) (UASPR1 n = 5, PR1CON n = 20, UAST1CON n = 20, rover n = 18, sitter n = 18) (f) Clustering coefficient values were highest in UASPR1 ($F_{(4,71)} = 6.437$) ($p \leq 0.001$) (UASPR1 n = 5, PR1CON n = 20, UAST1CON n = 19, rover n = 18, sitter n = 14) (g) Networks of UASPR1 had the highest values of global efficiency ($F_{(4,71)} = 39.22$) ($p \leq 0.0001$) (UASPR1 n = 4, PR1CON n = 20, UAST1CON n = 19, rover n = 18, sitter n = 15). (h) Networks of UASPR1 had the lowest values of betweenness centrality $F_{(4,71)} = 9.264$) ($p \leq 0.0001$) (UASPR1 n = 4, PR1CON n = 18, UAST1CON n = 19, rover n = 18, sitter n = 17)**.** *a-h* were analyzed with one-way ANOVA to determine if statistical differences exist between the groups. The Tukey-Kramer method was used as a post-hoc test (Methods). Outliers were removed from all the datasets. Bars indicate mean. Letters indicate statistical significance. *e-h* Measurements were standardized using z-scores.

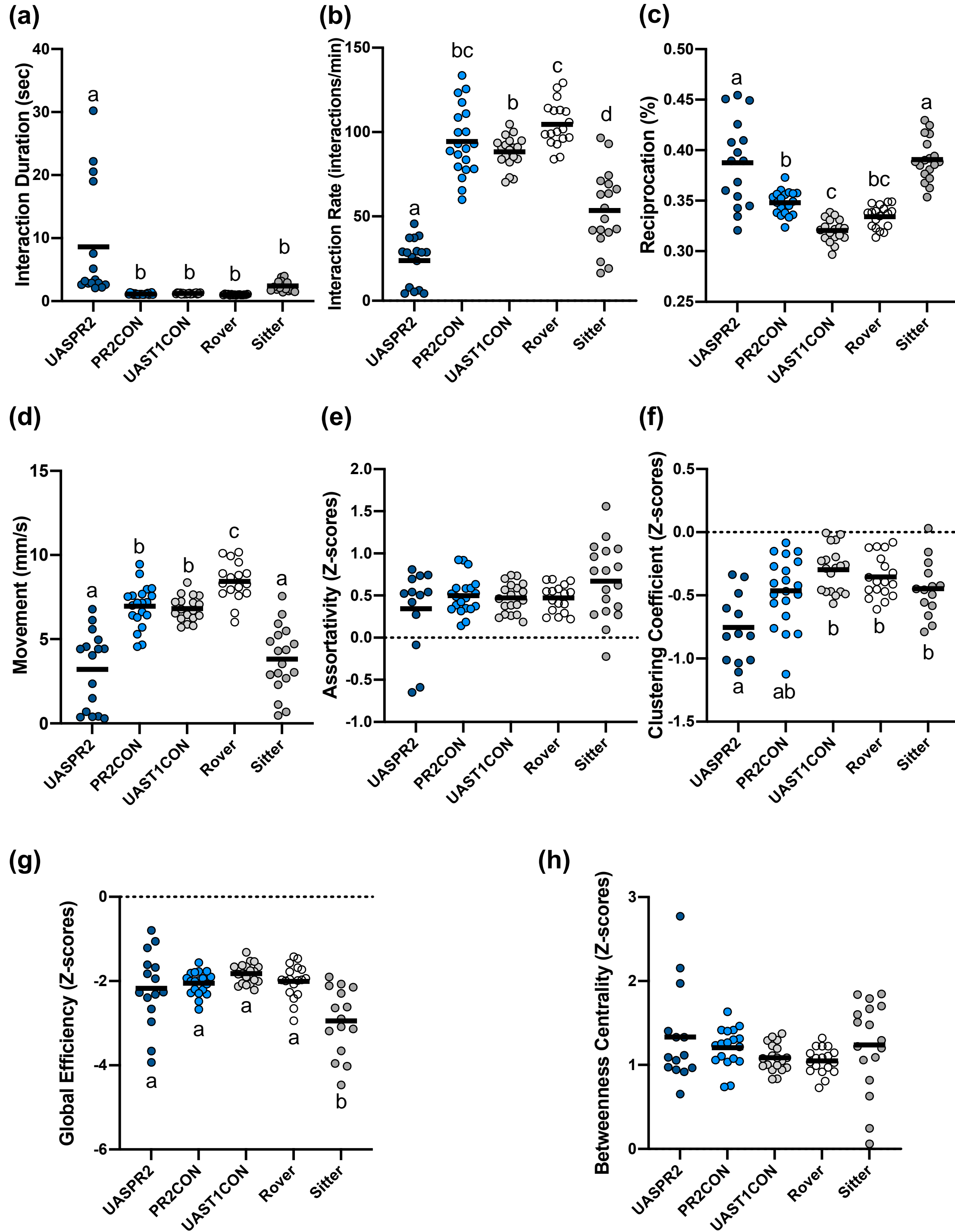


Figure 4.8 (Figure 8). Promoter 2 contribution to behavioural elements social network measures. (a) Average duration of an interaction of UASPR2 were significantly higher than other groups ($F_{(4,81)} = 10.75$) ($p \leq 0.0001$) (UASPR2 n = 15, PR2CON n = 20, UAST1CON n = 19,

rover n = 16, sitter n = 16). (b) Flies in networks of UASPR2 showed significantly lower rates of interaction ($F_{(4,86)} = 67.94$) ($p \leq 0.0001$) (UASPR2 n = 16, PR2CON n = 20, UAST1CON n = 19, rover n = 18, sitter n = 18). (c) Percentage reciprocation of UASPR2 networks were similar to percentage reciprocation of sitters ($F_{(4,77)} = 6.083$) ($p \leq 0.001$) (UASPR2 n = 16, PR2CON n = 20, UAST1CON n = 17, rover n = 17, sitter n = 13). (d) Flies in networks of UASPR2 moved significantly less relative to its controls and were similar to sitters ($F_{(4,84)} = 34.53$) ($p \leq 0.0001$) (UASPR2 n = 16, PR2CON n = 20, UAST1CON n = 18, rover n = 17, sitter n = 18). (e) No significant differences in assortativity values of networks were observed ($F_{(4,84)} = 2.184$) ($p = 0.0777$) (UASPR2 n = 13, PR2CON n = 20, UAST1CON n = 20, rover n = 18, sitter n = 18) (f) Clustering coefficient values of UASPR2 were the lowest relative to other groups tested but did not significantly differ from clustering coefficient values of sitter ($F_{(4,84)} = 5.981$) ($p \leq 0.001$) (UASPR2 n = 12, PR2CON n = 20, UAST1CON n = 19, rover n = 18, sitter n = 16) (g) Networks of sitters had the lowest global efficiency values ($F_{(4,81)} = 9.732$) ($p \leq 0.0001$) (UASPR2 n = 15, PR2CON n = 19, UAST1CON n = 19, rover n = 18, sitter n = 15). (h) Betweenness centrality values did not significantly differ across the five groups tested ($F_{(4,81)} = 1.631$) ($p = 0.1744$) (UASPR2 n = 14, PR2CON n = 18, UAST1CON n = 19, rover n = 18, sitter n = 17). *a-h* were analyzed with one-way ANOVA to determine if statistical differences exist between the groups. The Tukey-Kramer method was used as a post-hoc test (Methods). Outliers were removed from all the datasets. Bars indicate mean. Letters indicate statistical significance. *e-h* Measurements were standardized using z-scores.

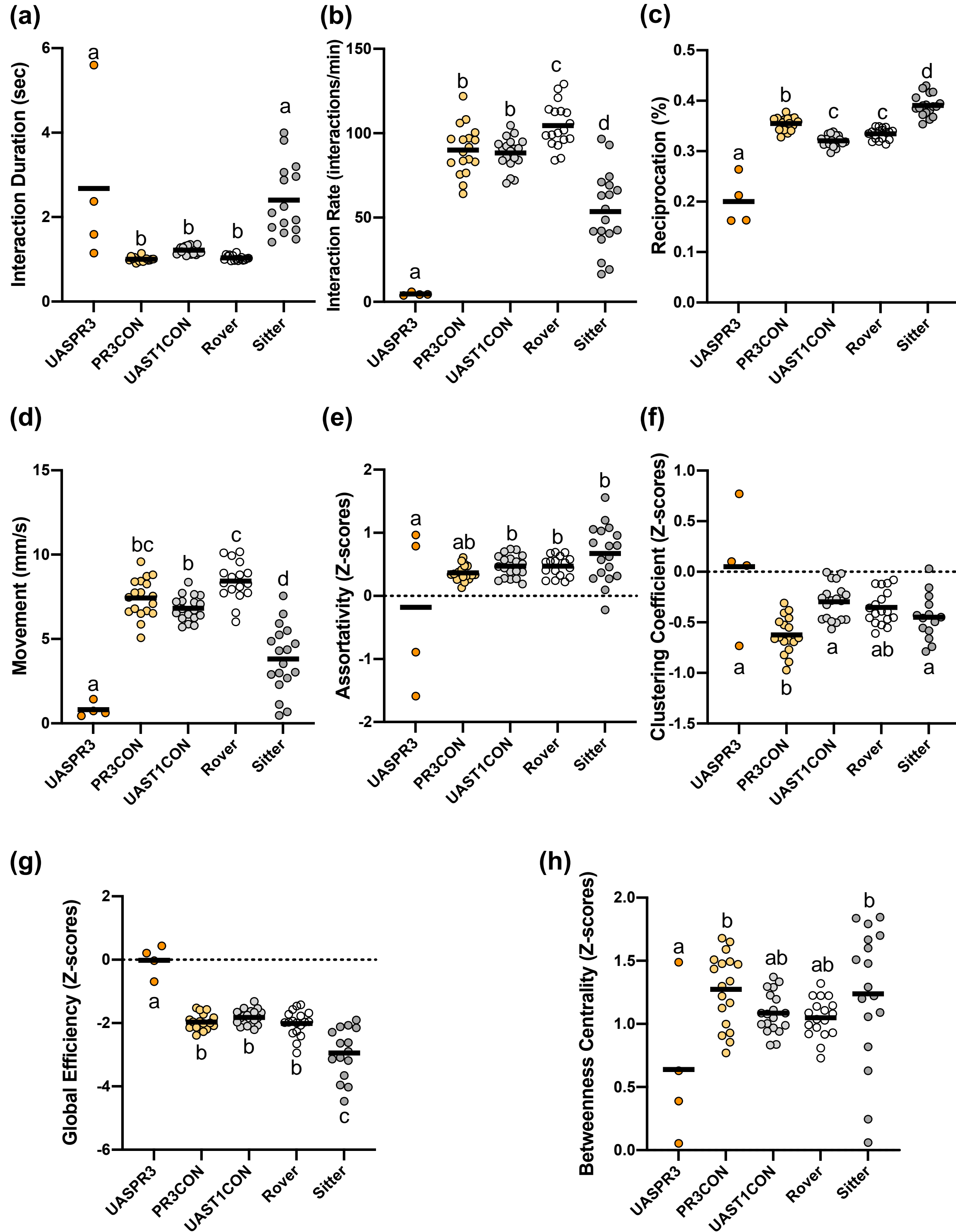


Figure 4.9 (Figure 9). Promoter 3 contribution to behavioural elements and social network measures. (a) Average duration of an interaction of UASPR3 were significantly higher than other groups but are similar to the average duration of interaction of sitter flies ($F_{(4,66)} = 16.88$) ($p \leq$

0.0001) (UASPR3 n = 4, PR3CON n = 16, UAST1CON n = 19, rover n = 16, sitter n = 16). (b) Flies in networks of UASPR3 showed significantly lower rates of interaction ($F_{(4,72)} = 51.85$) ($p \leq 0.0001$) (UASPR3 n = 4, PR3CON n = 18, UAST1CON n = 19, rover n = 18, sitter n = 18). (c) Percentage reciprocation of UASPR3 networks were lowest relative to other groups tested ($F_{(4,71)} = 110.4$) ($p \leq 0.0001$) (UASPR3 n = 4, PR3CON n = 18, UAST1CON n = 17, rover n = 17, sitter n = 13). (d) Flies in networks of UASPR3 moved significantly less relative to all other groups ($F_{(4,70)} = 48.85$) ($p \leq 0.0001$) (UASPR3 n = 4, PR3CON n = 18, UAST1CON n = 18, rover n = 17, sitter n = 18). (e) Networks of UASPR3 flies showed lowest assortativity values but were not significantly different from PR3CON ($F_{(4,73)} = 4.768$) ($p \leq 0.01$) (UASPR3 n = 4, PR3CON n = 18, UAST1CON n = 20, rover n = 18, sitter n = 18). (f) Clustering coefficient values of PR3CON were the lowest relative to other groups tested but did not significantly differ from clustering coefficient values of rovers ($F_{(4,69)} = 5.559$) ($p \leq 0.001$) (UASPR3 n = 4, PR3CON n = 17, UAST1CON n = 19, rover n = 18, sitter n = 16) (g) Networks of UASPR3 had the highest global efficiency values ($F_{(4,68)} = 35.01$) ($p \leq 0.0001$) (UASPR3 n = 4, PR3CON n = 17, UAST1CON n = 19, rover n = 18, sitter n = 15). (h) Betweenness centrality was lower in UASPR3 flies but did not significantly differ from UAST1CON and rover flies ($F_{(4,71)} = 0.4974$) ($p \leq 0.01$) (UASPR3 n = 4, PR3CON n = 18, UAST1CON n = 19, rover n = 18, sitter n = 17). *a-h* were analyzed with one-way ANOVA to determine if statistical differences exist between the groups. The Tukey-Kramer method was used as a post-hoc test (Methods). Outliers were removed from all the datasets. Bars indicate mean. Letters indicate statistical significance. *e-h* Measurements were standardized using z-scores.

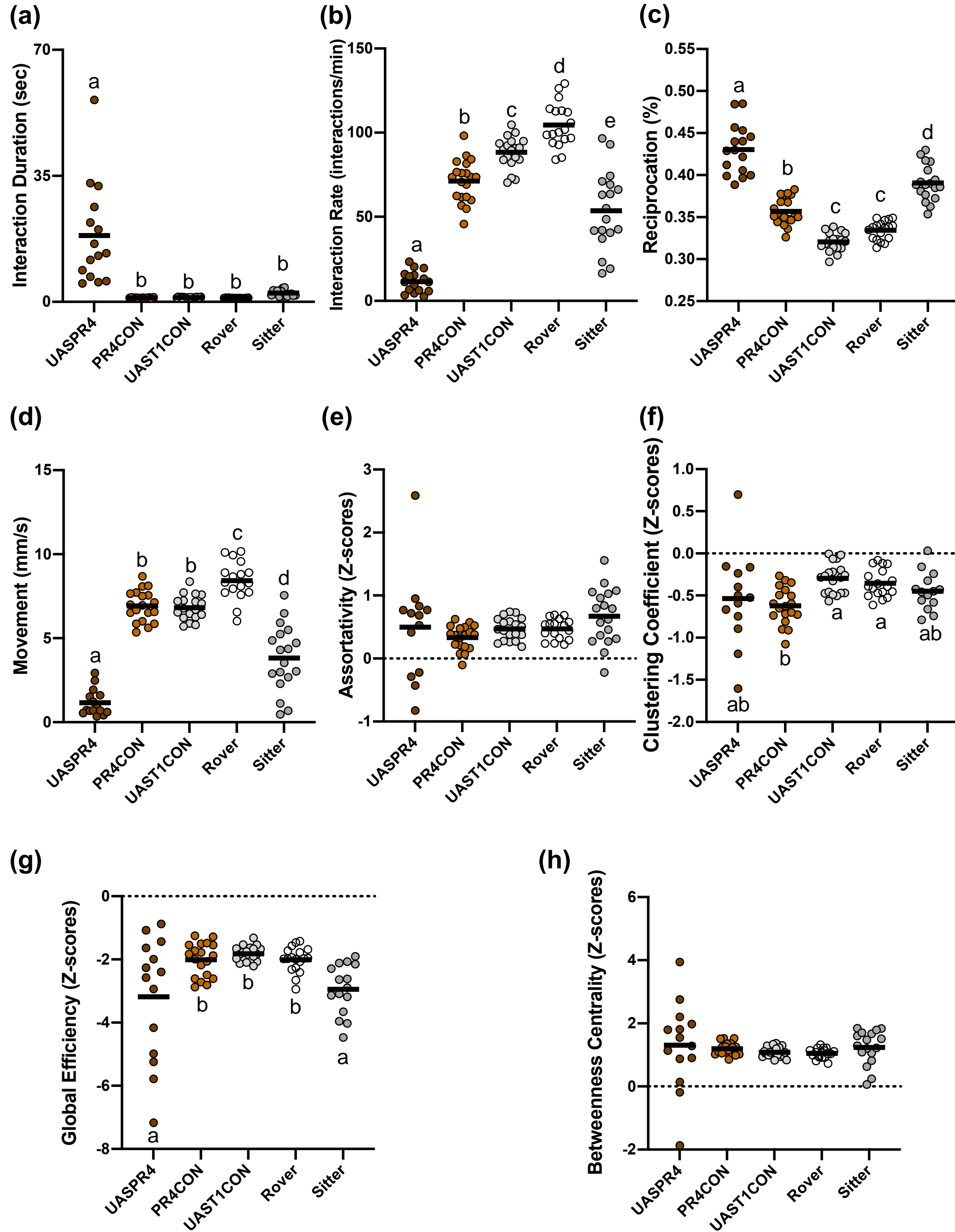


Figure 4.10 (Figure 10). Promoter 4 contribution to behavioural elements and social network measures. (a) Average duration of an interaction of UASPR4 were significantly higher than other groups ($F_{(4,80)} = 25.89$) ($p \leq 0.0001$) (UASPR4 n = 15, PR4CON n = 19, UAST1CON n = 19,

rover n = 16, sitter n = 16). (b) Rates of interaction were highest in networks of rover flies ($F_{(4,86)} = 109.2$) ($p \leq 0.0001$) (UASPR4 n = 12, PR4CON n = 18, UAST1CON n = 17, rover n = 17, sitter n = 13). (c) Percentage reciprocation of UASPR4 networks was the highest relative to other groups tested ($F_{(4,85)} = 94.79$) ($p \leq 0.0001$) (UASPR4 n = 16, PR4CON n = 20, UAST1CON n = 17, rover n = 17, sitter n = 13). (d) Flies in networks of UASPR4 moved significantly less relative to all other groups ($F_{(4,82)} = 92.37$) ($p \leq 0.0001$) (UASPR4 n = 15, PR4CON n = 19, UAST1CON n = 18, rover n = 17, sitter n = 18). (e) Assortativity values did not differ across the groups tested ($F_{(4,83)} = 1.602$) ($p = 0.1815$) (UASPR4 n = 13, PR4CON n = 19, UAST1CON n = 20, rover n = 18, sitter n = 18). (f) Clustering coefficient values of UASPR4 did not differ from other groups ($F_{(4,80)} = 3.480$) ($p \leq 0.05$) (UASPR4 n = 13, PR4CON n = 19, UAST1CON n = 19, rover n = 18, sitter n = 16) (g) Networks of UASPR4 and sitter had the lowest global efficiency values ($F_{(4,80)} = 7.289$) ($p \leq 0.0001$) (UASPR4 n = 14, PR4CON n = 19, UAST1CON n = 19, rover n = 18, sitter n = 15). (h) Betweenness centrality values did not differ across the groups tested ($F_{(4,82)} = 0.4974$) ($p = 0.7377$) (UASPR4 n = 14, PR4CON n = 19, UAST1CON n = 19, rover n = 18, sitter n = 17). *a-h* were analyzed with one-way ANOVA to determine if statistical differences exist between the groups. The Tukey-Kramer method was used as a post-hoc test (Methods). Outliers were removed from all the datasets. Bars indicate mean. Letters indicate statistical significance. *e-h* Measurements were standardized using z-scores.

4.4.4 Effect of isolation on behavioural elements and social network measures

Isolation seems to affect the behavioural elements, while network measures showed resilience in response to isolation stress. Table 6 shows interaction criteria of isolated treatments and their controls consistent with all previous experiments, sitters seem to have a longer interaction duration criterion for an interaction to be defined. Both movement and interaction rate scores were affected by isolation, with isolated groups moving significantly less during the 30 minute-trial and having a lower rate of interaction, rover-sitter differences were, however, maintained in the isolated treatments (Fig. 11b, 11d). For interaction duration, sitter flies seem to be more sensitive to isolation treatment, with isolated sitters spending significantly more time interacting (Fig. 11a). Whereas, for rovers, reciprocation was affected by the isolation treatment, with isolated rovers having a significantly lower reciprocation rate (Fig. 11c). All behavioural elements are affected by both *for* and isolation treatment (Fig. 11a-d). No differences were found between isolated treatments and their controls for assortativity, global efficiency, clustering coefficient, or betweenness centrality (Fig. 11e, 11f, 11g, 11h). Only *for* influences assortativity and global efficiency with no effect of isolation observed (Fig. 11e,11g). Together, these results show that social isolation affects the behavioural elements of interacting flies.

Table 4.6 (Table 6). Interaction criteria of isolated treatment and their controls. Interaction criteria for each strain were automatically computed for distance, angle and time criteria across the replicates in order to capture interactions objectively across videos.

Strain	Distance (Body lengths)	Angle (º)	Time (Sec)
Isolated rover	2.00	90	0.80
Isolated sitter	1.75	120	1.35
Rover	1.75	115	0.60
Sitter	1.50	125	0.80

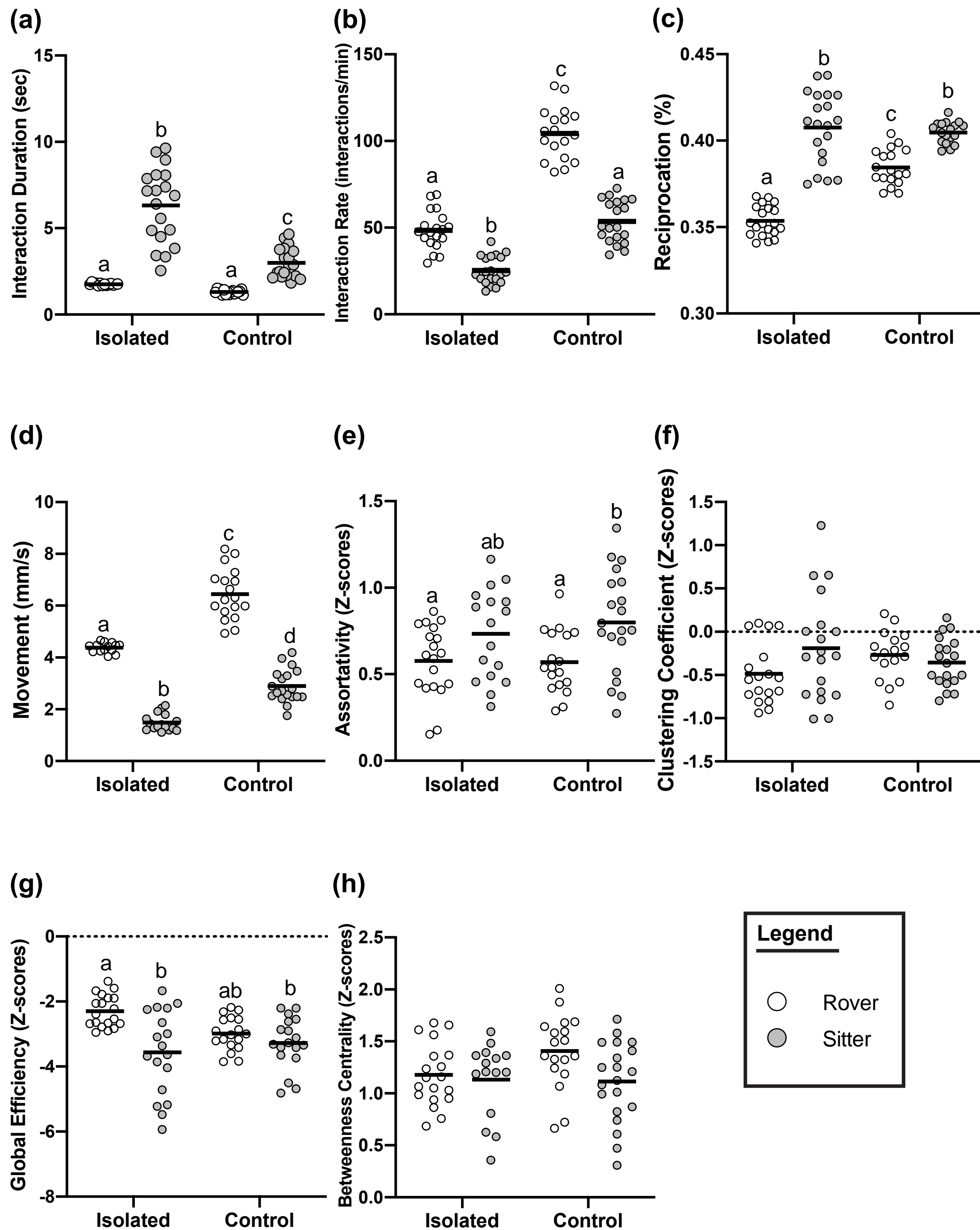


Figure 4.11 (Figure 11). The effect of isolation on behavioural elements and social network measures. (a) Average duration of an interaction was higher in isolated sitters compared to sitters (*for:* ($F_{(1,65)}$ = 109.00) ($p \leq 0.0001$), Isolation: ($F_{(1,65)}$ = 40.03) ($p \leq 0.0001$), *for* x Isolation:

($F_{(1,65)} = 23.09$) ($p \leq 0.0001$)) (Isolated rover n = 14, Isolated sitter n = 19, rover n = 17, sitter n = 19). (b) Rates of interaction of isolated treatments were significantly lower than their rover and sitter controls (*for:* ($F_{(1,73)} = 191.00$) ($p \leq 0.0001$), Isolation: ($F_{(1,73)} = 247.70$) ($p \leq 0.0001$), *for* x Isolation: ($F_{(1,73)} = 26.74$) ($p \leq 0.0001$)) (Isolated rover n = 19, isolated sitter n = 20, rover n = 18, sitter n = 20). (c) Isolation affected percentage reciprocation of rovers, isolated rover had lower reciprocation rate relative to rover (*for:* ($F_{(1,74)} = 130.60$) ($p \leq 0.0001$), Isolation: ($F_{(1,74)} = 13.91$) ($p \leq 0.001$), *for* x Isolation: ($F_{(1,74)} = 43.28$) ($p \leq 0.0001$)) (Isolated rover n = 20, isolated sitter n = 20, rover n = 18, sitter n = 20). (d) Isolated rover and sitter moved less than their respective controls (*for:* ($F_{(1,63)} = 423.90$) ($p \leq 0.0001$), Isolation: ($F_{(1,63)} = 123.00$) ($p \leq 0.0001$), *for* x Isolation: ($F_{(1,63)} = 4.47$) ($p \leq 0.05$)) (Isolated rover n = 14, isolated sitter n = 16, rover n = 18, sitter n = 19). (e) Assortativity values were not affected by isolation, and thus isolated treatments and their control did not differ (*for:* ($F_{(1,70)} = 11.85$) ($p \leq 0.001$), Isolation: ($F_{(1,70)} = 0.29$) ($p = 0.5922$), *for* x Isolation: ($F_{(1,70)} = 0.43$) ($p = 0.5130$)) (Isolated rover n = 19, isolated sitter n = 17, rover n = 18, sitter n = 20). (f) Clustering coefficient values did not differ across groups (*for:* ($F_{(1,69)} = 1.16$) ($p = 0.2843$), Isolation: ($F_{(1,69)} = 0.08$) ($p = 0.7798$), *for* x Isolation: ($F_{(1,69)} = 4.01$) ($p \leq 0.05$)) (Isolated rover n = 18, isolated sitter n = 19, rover n = 17, sitter n = 19). (g) Isolated rover and rover networks had the highest global efficiency values (*for:* ($F_{(1,69)} = 15.73$) ($p \leq 0.001$), Isolation: ($F_{(1,69)} = 1.05$) ($p = 0.3103$), *for* x Isolation: ($F_{(1,69)} = 6.21$) ($p \leq 0.05$)) (Isolated rover n = 19, isolated sitter n = 18, rover n = 18, sitter n = 18). (h) Betweenness centrality values did not differ across the four groups (*for:* ($F_{(1,68)} = 3.86$) ($p = 0.0536$), Isolation: ($F_{(1,68)} = 1.60$) ($p = 0.2104$), *for* x Isolation: ($F_{(1,68)} = 2.25$) ($p = 0.1386$)) (Isolated rover n = 19, isolated sitter n = 15, rover n = 18, sitter n = 20). *a-h* were analyzed with Two-way ANOVA to determine if statistical differences exist between the groups. The Tukey-Kramer method was used as a post-hoc test (Methods). Outliers were removed from all the datasets. Bars indicate mean. Letters indicate statistical significance. *e-h* Measurements were standardized using z-scores.

4.4.5 Effect of food deprivation on behavioural elements and social network measures

The effect of food deprivation on behavioural elements and social network phenotypes was also investigated. Networks were acquired both after 24 hours and 48 hours of food deprivation. These experiments were done independently, and thus different rover-sitter controls were used. Tables 7 and 8 show interaction criteria of groups tested after 24 and 48 hours of food deprivation respectively. Figure 12 shows the effect of 24 hours of food deprivation on behavioural element and social network phenotypes. Both interaction duration and reciprocation of sitter networks were affected after 24 hours of food deprivation, with food-deprived sitters spending more time interacting relative to their control and were more likely to reciprocate an interaction (Fig. 12a, 12c). *for* seems to significantly influence all network phenotypes except for betweenness centrality (Fig. 12a-g). Although there are no significant differences between rover and sitter flies for betweenness centrality, rover flies subjected to 24 hours of food deprivation

have significantly higher betweenness centrality values compared to sitter controls (Fig. 12h). Next, we tested the effect of 48 hours of food deprivation on social network phenotypes, figure 13 shows the results of this experiment. Opposite to differences in reciprocation difference after 24 hours of food deprivations (Fig. 12c), only reciprocation values of rover flies were affected after 48 hours of food deprivation, with food-deprived rovers having significantly higher reciprocation rates compared to their controls (Fig. 13c). Food-deprived rovers also showed higher rates of interaction when compared to their controls, while food-deprived sitters were intermediate between rover and sitter controls (Fig. 13b). Both rates of interaction and reciprocation are influenced by both *for* and 48 hours of food deprivation (Fig. 13b, 13c). After both 24 and 48 hours of food deprivation, movement was unaffected by 48 hours of food-deprivation alone but there was a significant effect of *for* and an interaction between *for* and food-deprivation (*for* x food-deprivation) (Fig. 13d). Additionally, both interaction duration and assortativity were only affected by *for* expression and were higher in sitter flies (Fig. 13a, 13e). Clustering coefficient values of food deprived treatments were lower when compared to control, however, these differences were not significant (Fig. 13f). Two-way ANOVA results indicate that both *for* expression and food deprivation significantly influence global efficiency and betweenness centrality (Fig. 13g, 13h).

Table 4.7 (Table 7). Interaction criteria of food deprived treatment and their controls after 24 hours of food deprivation. Interaction criteria for each strain were automatically computed for distance, angle and time criteria across the replicates in order to capture interactions objectively across videos.

Strain	Distance (Body lengths)	Angle (°)	Time (Sec)
Rover-FD	1.50	120	0.55
Sitter-FD	1.50	127.5	0.90
Rover	1.50	115	0.55
Sitter	1.50	120	0.85

Table 4.8 (Table 8). Interaction criteria of food deprived treatment and their controls after 48 hours of food deprivation. Interaction criteria for each strain were automatically computed for distance, angle and time criteria across the replicates in order to capture interactions objectively across videos.

Strain	Distance (Body lengths)	Angle (°)	Time (Sec)
Rover-FD	1.75	115	0.55
Sitter-FD	1.50	120	0.80
Rover	1.50	115	0.60
Sitter	1.25	115	0.75

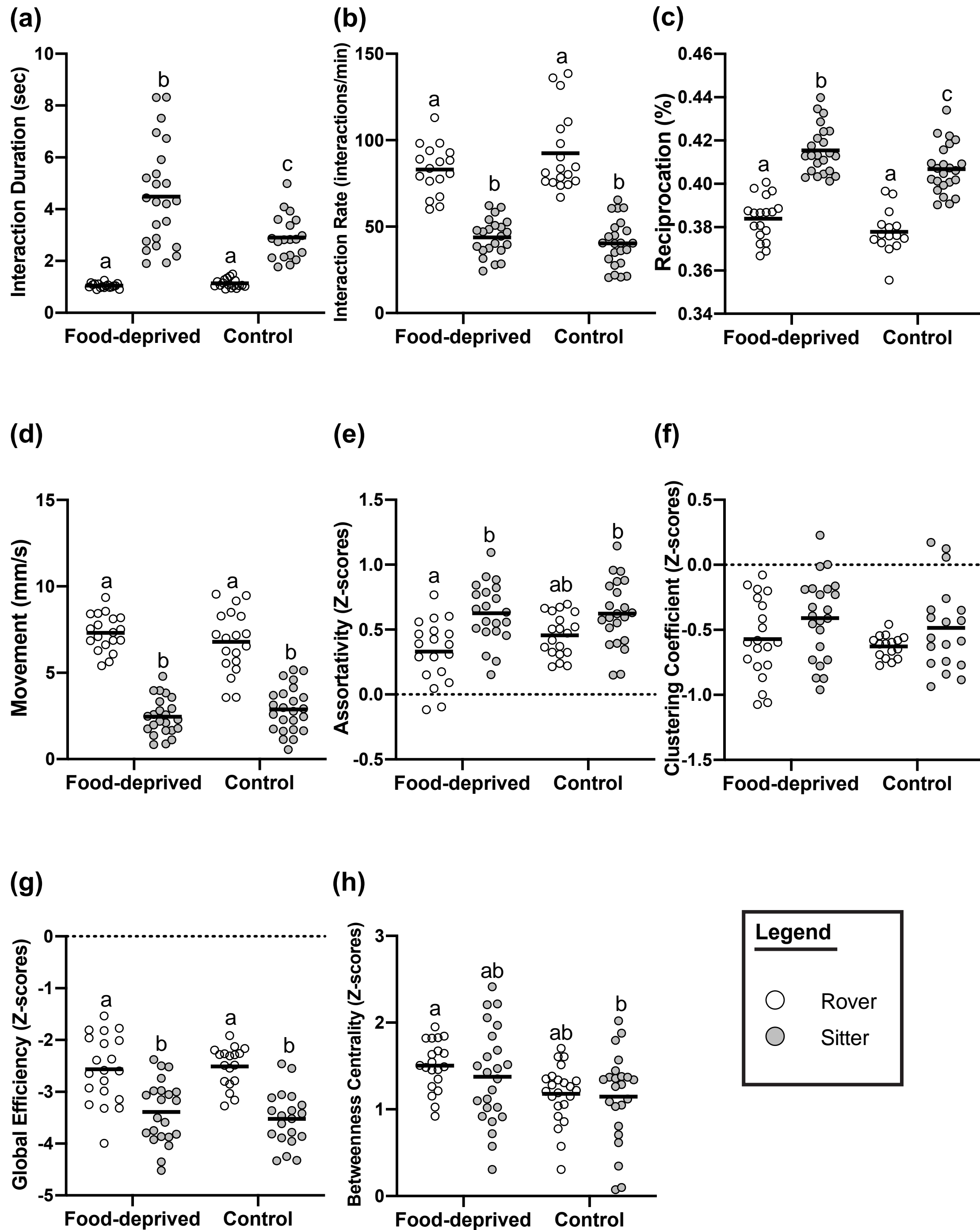


Figure 4.12 (Figure 12). The effect of 24 hour of food deprivation on behavioural elements and social network measures. (a) Average duration of an interaction was higher in food deprived

sitters compared to sitters (*for:* ($F_{(1,74)} = 91.39$) ($p \leq 0.0001$), Food-deprived: ($F_{(1,74)} = 7.51$) ($p \leq 0.01$), *for* x Food-deprived: ($F_{(1,74)} = 9.45$) ($p \leq 0.01$)) (rover-FD n = 18, sitter-FD n = 24, rover n = 17, sitter n = 19). (b) Rates of interaction did not differ between the food deprived treatments and their controls (*for:* ($F_{(1,76)} = 166.60$) ($p \leq 0.0001$), Food-deprived: ($F_{(1,76)} = 0.67$) ($p = 0.4166$), *for* x Food-deprived: ($F_{(1,76)} = 3.29$) ($p = 0.0739$)) (rover-FD n = 17, sitter-FD n = 22, rover n = 18, sitter n = 23). (c) Food deprivation did not affect percentage reciprocation of rover or sitter flies (*for:* ($F_{(1,75)} = 151.80$) ($p \leq 0.0001$), Food-deprived: ($F_{(1,75)} = 8.90$) ($p \leq 0.01$), *for* x Food-deprived: ($F_{(1,75)} = 0.28$) ($p = 0.5969$)) (rover-FD n = 18, sitter-FD n = 23, rover n = 15, sitter n = 23). (d) Food deprived rover and rover flies moved more during the trials (*for:* ($F_{(1,81)} = 227.70$) ($p \leq 0.0001$), Food-deprived: ($F_{(1,81)} = 0.02$) ($p = 0.8759$), *for* x Food-deprived: ($F_{(1,81)} = 2.64$) ($p = 0.1083$)) (rover-FD n = 19, sitter-FD n = 23, rover n = 19, sitter n = 24). (e) Assortativity values were not significantly affected by food deprivation (*for:* ($F_{(1,77)} = 20.71$) ($p \leq 0.0001$), Food-deprived: ($F_{(1,77)} = 1.50$) ($p = 0.2252$), *for* x Food-deprived: ($F_{(1,75)} = 0.28$) ($p = 0.5969$)) ($F_{(3,77)} = 7.875$) ($p \leq 0.0001$) (rover-FD n = 19, sitter-FD n = 21, rover n = 19, sitter n = 22). (f) Clustering coefficient values did not differ across groups (*for:* ($F_{(1,74)} = 5.51$) ($p \leq 0.05$), Food-deprived: ($F_{(1,74)} = 1.04$) ($p = 0.3118$), *for* x Food-deprived: ($F_{(1,74)} = 0.02$) ($p = 0.8923$)) (rover-FD n = 20, sitter-FD n = 23, rover n = 16, sitter n = 19). (g) Food deprived rover and rover networks had the highest global efficiency values (*for:* ($F_{(1,76)} = 53.99$) ($p \leq 0.0001$), Food-deprived: ($F_{(1,76)} = 0.11$) ($p = 0.7420$), *for* x Food-deprived: ($F_{(1,76)} = 0.56$) ($p = 0.4556$)) ($F_{(3,76)} = 18.09$) ($p \leq 0.0001$) (rover-FD n = 20, sitter-FD n = 22, rover n = 18, sitter n = 20). (h) Betweenness centrality values did not differ across the four groups tested (*for:* ($F_{(1,86)} =0.75$) ($p = 0.3903$), Food-deprived: ($F_{(1,86)} = 8.83$) ($p \leq 0.01$), *for* x Food-deprived: ($F_{(1,86)} = 0.25$) ($p = 0.6156$)) (rover-FD n = 20, sitter-FD n = 24, rover n = 23, sitter n = 23). *a-h* were analyzed with Two-way ANOVA to determine if statistical differences exist between the groups. The Tukey-Kramer method was used as a post-hoc test (Methods). Outliers were removed from all the datasets. Bars indicate mean. Letters indicate statistical significance. *e-h* Measurements were standardized using z-scores.

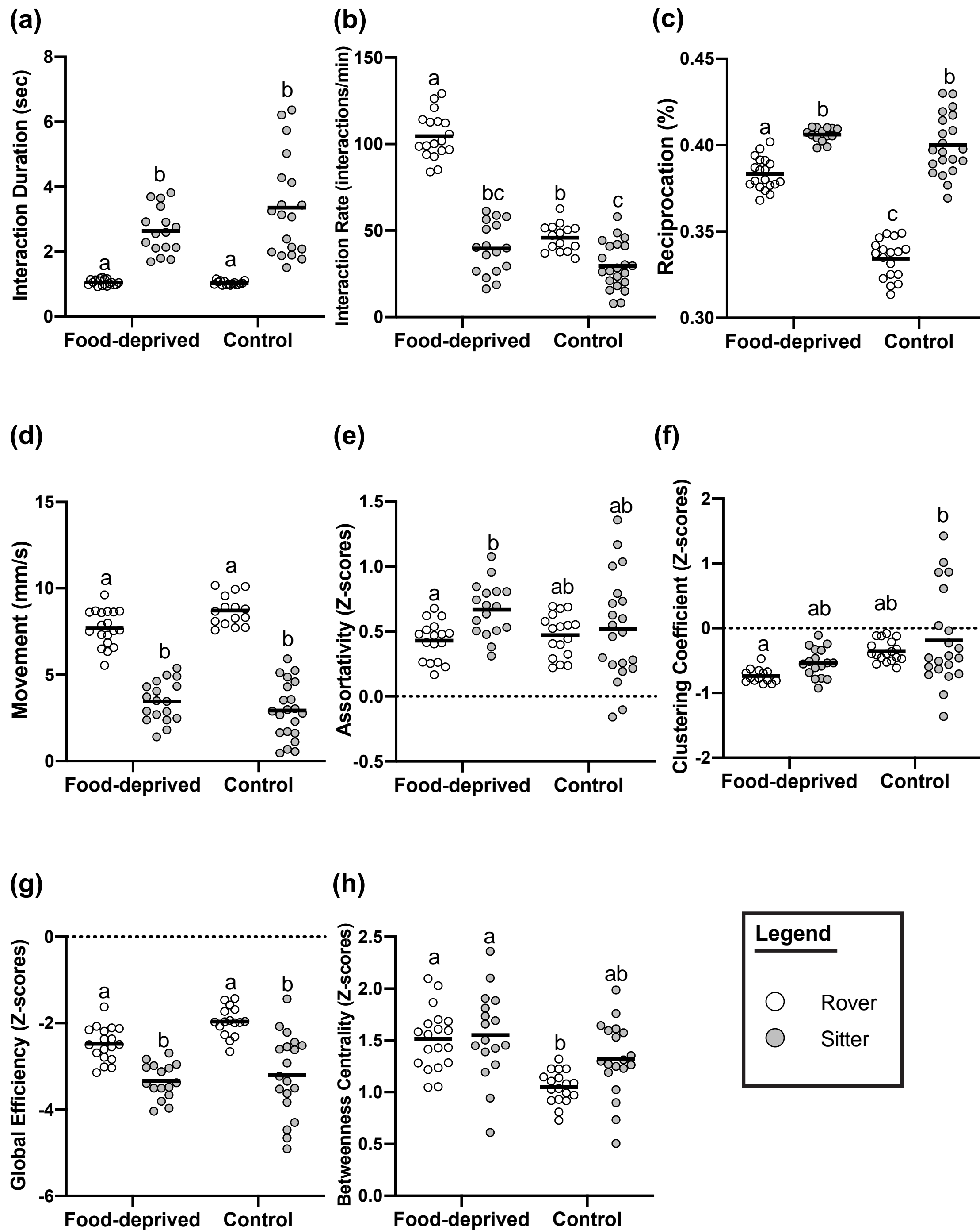


Figure 4.13 (Figure 13). The effect of 48 hour of food deprivation on behavioural elements social network measures. (a) Average duration of an interaction was higher in food deprived sitters and sitters (*for:* ($F_{(1,65)} = 82.88$) ($p \leq 0.0001$), Food-deprived: ($F_{(1,65)} = 2.62$) ($p = 0.11$), *for*

x Food-deprived: ($F_{(1,65)} = 3.01$) ($p = 0.0877$)) (rover-FD n = 18, sitter-FD n = 16, rover n = 16, sitter n = 19). (b) Rates of interaction of food deprived rovers were significantly higher than their controls (*for:* ($F_{(1,68)} = 178.70$) ($p \leq 0.0001$), Food-deprived: ($F_{(1,68)} = 127.40$) ($p \leq 0.0001$), *for* x Food-deprived: ($F_{(1,68)} = 63.88$) ($p \leq 0.0001$)) (rover-FD n = 18, sitter-FD n = 17, rover n = 15, sitter n = 22). (c) Food deprivation rover had higher percentage reciprocation compared to rover (*for:* ($F_{(1,68)} = 239.80$) ($p \leq 0.0001$), Food-deprived: ($F_{(1,68)} = 92.75$) ($p \leq 0.0001$), *for* x Food-deprived: ($F_{(1,68)} = 56.28$) ($p \leq 0.0001$)) (rover-FD n = 19, sitter-FD n = 14, rover n = 18, sitter n = 21). (d) Food deprivation treatments did not differ from their respective controls (*for:* ($F_{(1,69)} = 291.90$) ($p \leq 0.0001$), Food-deprived: ($F_{(1,69)} = 0.70$) ($p = 0.4051$), *for* x Food-deprived: ($F_{(1,69)} = 6.83$) ($p \leq 0.05$)) (rover-FD n = 19, sitter-FD n = 18, rover n = 15, sitter n = 21). (e) Assortativity values did not differ between the food deprived treatments and their controls (*for:* ($F_{(1,68)} = 5.27$) ($p \leq 0.05$), Food-deprived: ($F_{(1,68)} = 0.76$) ($p = 0.3869$), *for* x Food-deprived: ($F_{(1,68)} = 2.35$) ($p = 0.1301$)) ($F_{(3,72)} = 2.036$) ($p = 0.1165$) (rover-FD n = 17, sitter-FD n = 17, rover n = 17, sitter n = 21). (f) Clustering coefficient values was not affected by food deprivation (*for:* ($F_{(1,66)} = 3.19$) ($p = 0.0788$), Food-deprived: ($F_{(1,66)} = 12.25$) ($p \leq 0.001$), *for* x Food-deprived: ($F_{(1,66)} = 0.04$) ($p = 0.8515$)) (rover-FD n = 14, sitter-FD n = 17, rover n = 18, sitter n = 21). (g) Food deprived rover and rover networks had the highest global efficiency values (*for:* ($F_{(1,65)} = 52.73$) ($p \leq 0.0001$), Food-deprived: ($F_{(1,65)} = 5.18$) ($p \leq 0.05$), *for* x Food-deprived: ($F_{(1,65)} = 1.72$) ($p = 0.1938$)) (rover-FD n = 18, sitter-FD n = 16, rover n = 16, sitter n = 19). (h) Food deprived rovers had higher betweenness centrality values when compared to the rover controls (*for:* ($F_{(1,69)} = 4.10$) ($p \leq 0.05$), Food-deprived: ($F_{(1,69)} = 21.49$) ($p \leq 0.0001$), *for* x Food-deprived: ($F_{(1,69)} = 2.33$) ($p = 0.1319$)) (rover-FD n = 19, sitter-FD n = 17, rover n = 18, sitter n = 19). *a-h* were analyzed with Two-way ANOVA to determine if statistical differences exist between the groups. The Tukey-Kramer method was used as a post-hoc test (Methods). Outliers were removed from all the datasets. Bars indicate mean. Letters indicate statistical significance. e-h Measurements were standardized using z-scores.

4.4.6 Effect of sleep deprivation on behavioural elements and social network measures

The effect of sleep deprivation on social networks was investigated by sleep depriving flies the night before videos were recorded for 8 hours. Figure 4 shows the sleep duration of sleep-deprived rovers and sitters relative to their controls and confirms that sleep-deprived conditions show no sleep during the 8 hours of sleep deprivation. Table 9 shows the interaction criteria of sleep-deprived treatments and their controls. Figure 5 shows the effect of sleep deprivation on behavioural elements and social network phenotypes, only behavioural elements of networks were affected by sleep deprivation treatments. Sleep deprivation affected sitter flies average interaction duration and percentage reciprocation, increasing both measures (Fig. 5a, 5c). Whereas sleep-deprived rover flies had significantly higher rates of interaction when compared to its control (Fig. 5b). No differences were found between the sleep-deprived treatments and

their controls for the four network measures (Fig. 5e, 5f, 5g, 5h). Similar to responses to social isolation, sleep deprivation seems to affect the behavioural elements of interacting flies.

Table 4.9 (Table 9). Interaction criteria of sleep deprived treatments and their controls. Interaction criteria for each strain were automatically computed for distance, angle and time criteria across the replicates in order to capture interactions objectively across videos.

Strain	Distance (Body lengths)	Angle (°)	Time (Sec)
Rover-SD	1.50	125	0.80
Sitter-SD	1.50	125	1.10
Rover	1.50	115	0.60
Sitter	1.25	115	0.75

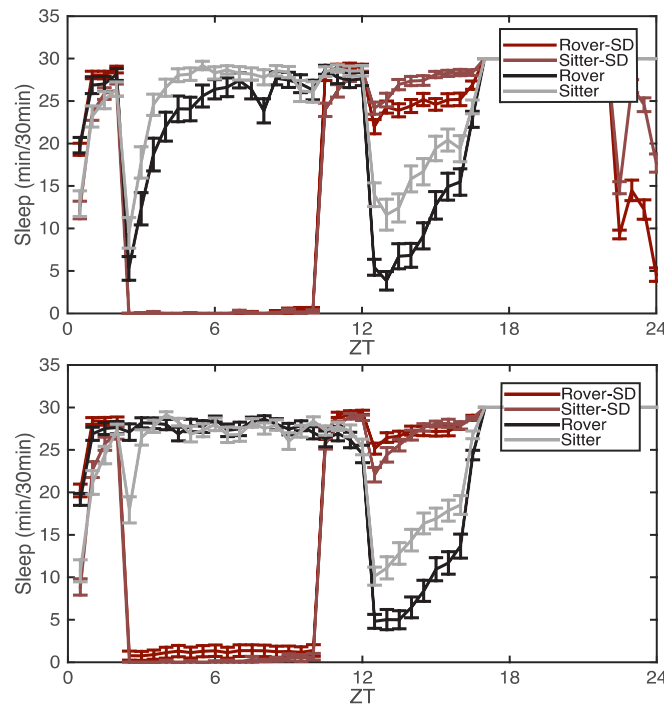


Figure 4.14 (Figure 14). Sleep duration of sleep deprived treatments compared to their controls. The two graphs are replicate experiments and confirm that the sleep deprived treatment (Rover-SD and Sitter-SD were sleep deprived for 8 hours (ZT 2 - ZT 10), whereas Rover and Sitters (rover and sitter controls) show regular sleep duration during these 8 hours, this is observed in both panels.

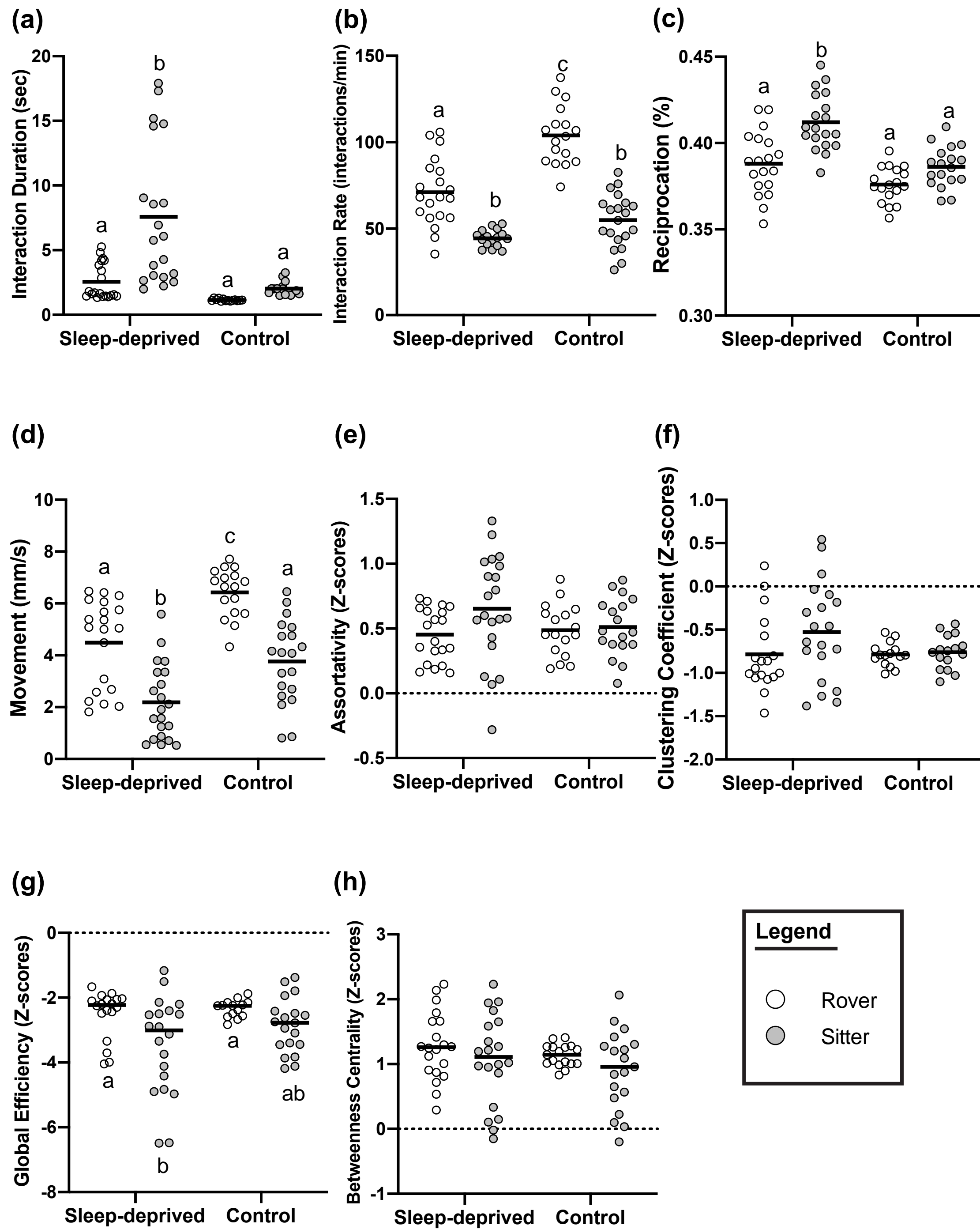


Figure 4.15 (Figure 15). The effect of sleep deprivation on behavioural elements and social network phenotypes. (a) Average duration of an interaction was significantly higher in sleep deprived sitters relative to sitters (*for:* ($F_{(1,66)} = 16.09$) ($p \leq 0.001$), Sleep-deprived: ($F_{(1,66)}$ =

22.59) ($p \leq 0.0001$), *for* x Sleep-deprived: ($F_{(1,66)} = 7.95$) ($p \leq 0.01$)) (rover-SD n = 20, sitter-SD n = 20, rover n = 15, sitter n = 16). (b) Sleep deprived rover had higher rates of interaction compared to their rover control sitters (*for:* ($F_{(1,69)} = 107.10$) ($p \leq 0.0001$), Sleep-deprived: ($F_{(1,69)} = 35.02$) ($p \leq 0.0001$), *for* x Sleep-deprived: ($F_{(1,69)} = 9.43$) ($p \leq 0.01$)) (rover-SD n = 20, sitter-SD n = 16, rover n = 17, sitter n = 20). (c) Sleep deprived sitter had higher percentage reciprocation compared to their sitter controls sitters (*for:* ($F_{(1,70)} = 25.16$) ($p \leq 0.0001$), Sleep-deprived: ($F_{(1,70)} = 31.07$) ($p \leq 0.0001$), *for* x Sleep-deprived: ($F_{(1,70)} = 4.03$) ($p \leq 0.05$)) (rover-SD n = 19, sitter-SD n = 19, rover n = 18, sitter n = 18). (d) Sleep deprived treatments moved less during the trails compared to rovers and sitters (*for:* ($F_{(1,74)} = 55.64$) ($p \leq 0.0001$), Sleep-deprived: ($F_{(1,74)} = 27.87$) ($p \leq 0.0001$), *for* x Sleep-deprived: ($F_{(1,74)} = 0.30$) ($p = 0.5877$)) (rover-SD n = 20, sitter-SD n = 21, rover n = 17, sitter n = 20). (e) Assortativity values did not differ between the sleep deprived treatment and their controls (*for:* ($F_{(1,72)} = 2.92$) ($p = 0.0916$), Sleep-deprived: ($F_{(1,72)} = 0.69$) ($p = 0.4098$), *for* x Sleep-deprived: ($F_{(1,72)} = 1.78$) ($p = 0.1867$)) (rover-SD n = 20, sitter-SD n = 21, rover n = 17, sitter n = 18). (f) Clustering coefficient values did not differ across groups (*for:* ($F_{(1,65)} = 2.14$) ($p = 0.1488$), Sleep-deprived: ($F_{(1,65)} = 1.50$) ($p = 0.2250$), *for* x Sleep-deprived: ($F_{(1,65)} = 1.42$) ($p = 0.2378$)) (rover-SD n = 18, sitter-SD n = 20, rover n = 15, sitter n = 16). (g) Sleep deprived conditions did not differ from their controls (*for:* ($F_{(1,67)} = 9.82$) ($p \leq 0.01$), Sleep-deprived: ($F_{(1,67)} = 2.54$) ($p = 0.1160$), *for* x Sleep-deprived: ($F_{(1,67)} = 0.77$) ($p = 0.3828$)) (rover-SD n = 18, sitter-SD n = 20, rover n = 14, sitter n = 19). (h) Betweenness centrality did not differ across the four groups (*for:* ($F_{(1,72)} = 2.81$) ($p = 0.0978$), Sleep-deprived: ($F_{(1,72)} = 1.431$) ($p = 0.2356$), *for* x Sleep-deprived: ($F_{(1,72)} = 0.01$) ($p = 0.9352$)) (rover-SD n = 20, sitter-SD n = 20, rover n = 17, sitter n = 19). *a-h* were analyzed with Two-way ANOVA to determine if statistical differences exist between the groups. The Tukey-Kramer method was used as a post-hoc test (Methods). Outliers were removed from all the datasets. Bars indicate mean. Letters indicate statistical significance. *e-h* Measurements were standardized using z-scores.

4.4.7 Effect of mixed groups on behavioural elements and social network measures

To test how rovers and sitters interact with each other in groups, we tested them at different ratios: 1S9R (1 sitter: 9 rovers), 7R3S (7 rovers: 3 sitters), 5R5S (5 rovers: 5 sitters), 7S3R (7 sitters: 3 rovers), and 1R9S (1 rover: 9 sitters) as well as controls of 10R (10 rovers) and 10S (10 sitters). Table 10 shows interaction criteria of the different mixed groups, as the number of sitter flies in the arena increases, so does the time needed for an interaction to be defined. This is consistent with previous results of rover and sitter networks. Figure 16 shows the effect of mixed groups on behavioural elements and social network phenotypes, overall, as the number of sitter individuals increases within the group network phenotypes seem to behave more like sitter networks. For both interaction duration and reciprocation, there seems to be a constant increasing trend in values as the networks have more sitter flies, indicating that as sitter numbers increase within the network, flies interact longer and are more likely to reciprocate an interaction (Fig.

16a, 16c). Conversely, as the number of sitters within a network increased, the average interaction rate decreased with 1R9S and 10S networks having the lowest interaction rate (Fig. 16b). The same effect is observed for movement and global efficiency, whereas as sitter numbers increase, both movement and global efficiency values decrease (Fig. 16d, 16g). Networks consisting of 1 rover and 9 sitters, 1R9S, had the highest assortativity and clustering coefficient values and thus formed more homogenous and interconnected networks (Fig. 16e, 16f). No trend differences between groups were found for betweenness centrality (Fig. 16h). Overall, these results suggest that flies may maintain their individual behavioural and social network phenotypes and thus networks increasingly behave like sitters as more sitters are present within the network.

Table 4.10 (Table 10). Interaction criteria rover and sitter in mixed networks of 10 flies. Interaction criteria for each strain were automatically computed for distance, angle and time criteria across the replicates in order to capture interactions objectively across videos.

Strain	**Distance (Body lengths)**	**Angle (º)**	**Time (Sec)**
10R	1.75	110	0.65
1S9R	1.50	115	0.60
7R3S	1.25	120	0.65
5R5S	1.50	120	0.80
7S3R	1.25	130	0.80
1R9S	1.50	120	0.95
10S	1.75	115	0.95

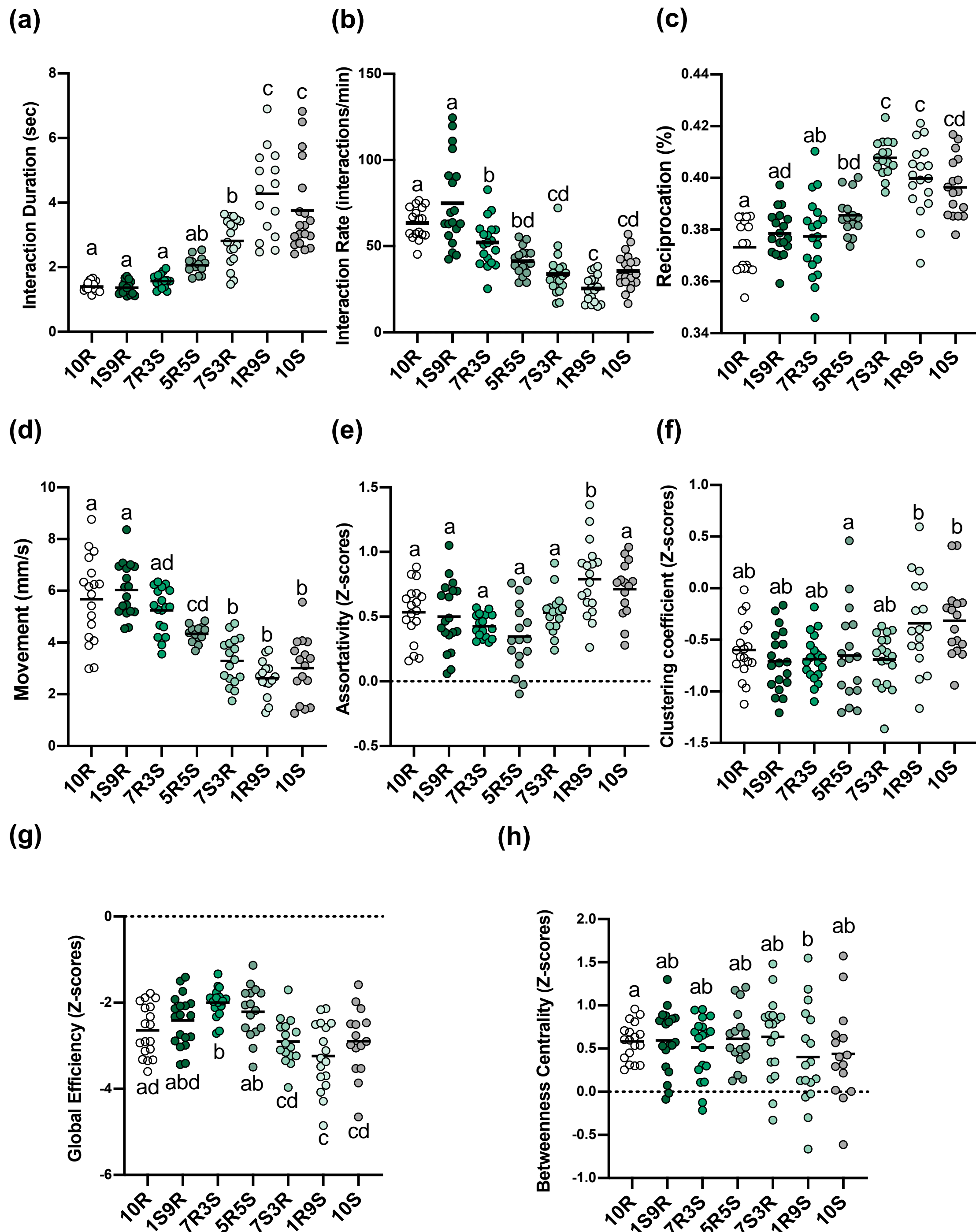


Figure 4.16 (Figure 16). Networks of mixed rover and sitter at different ratios. (a) Average duration of an interaction was highest at networks of 1R9S but did not differ from average duration of networks of 10S ($F_{(6,107)} = 34.68$) ($p \leq 0.0001$) (10R n = 16, 1S9R n = 17, 7R3S n =

16, 5R5S n = 13, 7S3R n = 18, 1R9S n =15, 10S n = 17). (b) Interaction Rate was highest in networks of 10R and 1S9R ($F_{(6,118)} = 29.27$) ($p \leq 0.0001$) (10R n = 17, 1S9R n = 18, 7R3S n = 18, 5R5S n = 17, 7S3R n = 18, 1R9S n =19, 10S n = 18). (c) Percentage of interactions that were reciprocated by the receiver decreased as the number of rover flies in the network increased, with networks of 10R having the lowest reciprocation percentage ($F_{(6,114)} = 23.28$) ($p \leq 0.0001$) (10R n = 15, 1S9R n = 17, 7R3S n = 17, 5R5S n = 11, 7S3R n = 15, 1R9S n =17, 10S n = 20). (d) Networks with more rovers had higher average movement and networks of 10R, 1S9R and 7R3S moved significantly more during the trial ($F_{(6,107)} = 54.42$) ($p \leq 0.0001$) (10R n = 13, 1S9R n = 18, 7R3S n = 17, 5R5S n = 13, 7S3R n = 17, 1R9S n =15, 10S n = 17). (e) Assortativity values of networks of 1R9S were significantly higher than other groups ($F_{(6,109)} = 10.30$) ($p \leq 0.0001$) (10R n = 16, 1S9R n = 18, 7R3S n = 17, 5R5S n = 17, 7S3R n = 13, 1R9S n =16, 10S n = 18). (f) Clustering coefficient values of 1R9S networks flies were higher relative to other groups ($F_{(6,115)} = 3.91$) ($p \leq 0.01$) (10R n = 16, 1S9R n = 19, 7R3S n = 18, 5R5S n = 17, 7S3R n = 18, 1R9S n =16, 10S n = 18). (g) Global Efficiency values lowest in networks 1R9S ($F_{(6,114)} = 9.74$) ($p \leq 0.05$) (10R n = 17, 1S9R n = 19, 7R3S n = 13, 5R5S n = 15, 7S3R n = 14, 1R9S n =17, 10S n = 19). (h) Betweenness centrality did not differ between the five groups ($F_{(6,107)} = 2.94$) ($p \leq 0.05$) (10R n = 15, 1S9R n = 18, 7R3S n = 18, 5R5S n = 15, 7S3R n = 16, 1R9S n =16, 10S n = 18). *a-h* were analyzed with two-tailed t-tests (*Methods)*. Outliers were removed from all the datasets. Bars indicate mean. Letters indicate statistical significance. *e-h* Measurements were standardized using z-scores.

4.5 Discussion

Building on the findings in Chapter 3 (Alwash et al., 2021) that *for* influences behavioural elements and social network phenotypes, we first set out to characterize the spatiotemporal expression of *for* that leads to differences in the behavioural elements and social network phenotypes observed. We attempted to map the critical period in which *for* expression influences social networks. We found that the rover-sitter differences were maintained at both 18°C and 30°C, the temperatures used in the conditional knockdowns. Next, we show that knocking down *for* in adults does not affect behavioural elements or social network measures (Fig. 2 and 3). Knocking down *for* during development, specifically until the wandering larvae stage also did not affect behavioural elements or social network measures (Fig. 5). We also found differences in behavioural elements and social network measures when *for* cDNA was driven using different *for*'s promoters-GAL4's. This helped us better understand promoter-specific contributions of *for* to the phenotypic differences observed (Fig. 7, 8, 9, and 10). Overall, our findings lead us to hypothesize that the critical period of *for* expression in regard to behavioural elements and social network measures may be restricted to stages of metamorphosis. Additionally, that a combination of *for*'s different promoters may regulate the different behavioural elements and

social network phenotypes of interest. Next, we focused on how the natural variants of *for* respond to various types of stress, specifically we examined how social isolation (Fig. 11), food deprivation (Fig. 12 & 13), and sleep deprivation (Fig. 15) affect social networks. We show that mainly the network measures were resilient to these stressors (Fig. 11, 12, 13, 15). However, all four behavioural elements of the network were affected by social isolation (Fig. 11). Similar to social isolation, sleep deprivation also influences all four behavioural elements without affecting the network measures (Fig. 15). For 24-hours of food deprivation, we see an effect on one of the behavioural elements of interest, specifically the duration of interactions (Fig. 12). Whereas, after 48-hours of food deprivation, one of the behavioural elements and one of the social network measures are affected, specifically both reciprocation and betweenness centrality (Fig. 13). Finally, we investigate how the natural variants of *for* interact with each other in a group setting (Fig. 16). Results from this experiment suggest that rover and sitter flies maintain their individual network phenotypes when placed in mixed groups. Taken together, these experiments shed light on *for's* influence on social interactions and measures and characterizes it further. More detail is provided below.

for expression is essential during development. Homozygous *for*^{null} mutants of *D. melanogaster* die during pupal stages, while PKG1 mutant mice die as juveniles (Allen et al., 2017; Pfeifer et al., 1998). We aimed to map the critical period of *for* expression in relation to social network phenotypes using a temperature-restricted knockdown of *for*. Knocking down *for* using ubiquitous drivers *Daughterless* and *Tubulin* during adult stages did not affect social network phenotypes (Fig. 2 & 3). Thus, we theorized that differences in *for* expression during development result in the rover-sitter differences observed. *for's* PKG has previously been implicated in several developmental phenotypes, including neuronal development and plasticity, in addition to wing development (Dason et al., 2019, 2020; Peng et al., 2016; Renger et al., 1999; Schleede & Blair, 2015). We next investigated this by knocking down *for* up until the early pupal stages in the whole body. This manipulation did not generate viable adults, and despite pupae being able to develop with normal morphology after pupation, flies were unable to successfully emerge from the pupal case. A recent study confirmed this and mapped pupal lethality to promoter 3 expression associated with a possible energy deficiency due to defects in fat body morphology (Anreiter et al., 2021). Knocking down *for* until the wandering larval stage using

the *Tubulin* driver produced viable adults and allowed us to investigate whether expression of *for* during these developmental stages affects social networks. Similar to the results of knocking down *for* during adult stages, knocking down *for* until the wandering larvae stage did not affect behavioural elements or social network phenotypes (Fig. 5). Altogether, these findings suggest that the critical period of *for* expression that regulates social behaviour is localized to the pupal stage during metamorphosis, similar to the critical period of lethality. However, we were unable to overcome this lethality in order to test the effect of knocking down *for* during this period. Future studies characterizing the expression of *for* during metamorphosis may highlight tissues of interest and help us further understand the pathway by which *for* regulates social behaviour. Additionally, this is the first experiment that looks at the effect of temperature on social networks, we acquired and analyzed networks of rovers and sitters at 18°C, 25°C, and 30°C and show that rover-sitter differences are maintained across these different temperatures. It is, however, interesting to note that we see a significant difference between rovers and sitters betweenness centrality scores at 30°C only (Figure 2, 3). These results indicate that an increase in temperature may influence the number of individuals within the networks that are important for information relay and network cohesion.

Next, using promoter-specific Gal4 drivers to increase *for* expression, we aimed to map the behavioural elements and social network phenotypes to the different promoters. The pleiotropic nature of *for* is accomplished by a combination of factors: the regulation of its multiple gene products, its spatiotemporal expression patterns, the different substrates it binds to, and the molecular pathway it is involved in. All different promoter increased expression show a reduction in the ability to form networks, with the greatest reduction in promoter 1 (UASPR1) and promoter 3 (UASPR3) (Fig. 6). Promoter 2 (UASPR2) and promoter 4 (UASPR4) specific *for* increased expression have a smaller reduction in the number of networks formed and thus more closely resemble the number of networks formed by the rover and sitter wild-type flies when compared to the other two promoter-specific *for* increased expression (UASPR1 & UASPR3), these results suggest that even though promoters 2 and 4 play bigger roles in network formation but a combination of all four promoter products is required for normal social network formation observed in wild-type flies. Increased expression of all four promoters-specific *for* products have a significantly lower rate of interactions when compared to their controls (Fig. 7b,

8b, 9b, and 10b). Additionally, promoter-specific expression in the *for* knockout line seems to influence behavioural and social network phenotypes, showing that a combination of the different promoters' expression patterns may be responsible for the behavioural elements and network phenotypes observed and that these phenotypes may not be linked and are independently regulated. All of *for*'s promoters play a role in influencing the behavioural elements of a network (Fig. 7a-d, 8a-d, 9a-d, 10a-d). Although *for*'s promoter 3 expression pattern was previously thought to play a role for viability past the pupal stage (Anreiter et al., 2021), our findings suggest that rescuing *for* via promoter 3 expression does not influence most social network phenotypes. Global efficiency measures were affected by increased expression of promoters 1, 3, and 4 (Fig. 7g, 9g, 10g) while the other three social network measures are only influenced by promoter 1 (Fig. 7e, 7f, 7h).

This experiment, in addition to providing further evidence of *for*'s molecular complexity playing a major role in its pleiotropic effect, also shows that certain behavioural and network measures may be independently regulated. In the future, driving the expression of *for* promoter-specific products in the *for* null line may enable us to discern the tissue or combination of tissues responsible for regulating these different phenotypes. For instance, *for*'s promoter 2 products are expressed in some neurons in the CNS, as well as an array of tissues in the gastric system and both the female and male reproductive systems (Allen & Sokolowski, 2021). Driving expression of promoter 2 in these specific tissues or a combination of them will help us determine the tissue-specific expression patterns or combination of tissue-specific *for* expression responsible for regulating these phenotypes. Thus, this would provide insight into the tissues in which *for*'s PKG expression is responsible for the different social network properties. Interestingly, the network measures of promoter 3 increased expression flies have z-scores values close to zero, indicating that these networks were close to random networks. This is an interesting effect considering that promoter 3 is the only constitutive *for* promoter while the other three promoters are regulated (Allen et al., 2017). Social networks are comprised of complex phenotypes, and thus our finding that multiple promoters are responsible for the regulation of both behavioural elements and network measures is not surprising. Overall, these findings shed light on the spatiotemporal regulation of *for* expression in regard to social networks. We hypothesize that the critical period for *for* expression and social behaviour occurs during metamorphosis and that a combination of

different promoter expression in distinct tissues regulates the phenotypes observed in adult social networks.

Additionally, we explore *for'*s plasticity in relation to social networks. Previous studies have found evidence for differential sensitivity to stressors between the rover and sitter variants, and suggest that these differences act as a trade-off in different environments and may explain why both rovers and sitter are maintained in nature (Donlea et al., 2012; Mery et al., 2007). We focus on three stressors, isolation, food, and sleep deprivation. Prior social isolation has been shown to have adverse effects on flies and influences several social behaviours (Ruan & Wu, 2008; Zhou et al., 2008). Studies have also shown that sitters are more sensitive to their social environment and are more likely to aggregate (Camiletti et al., 2014; Foucaud et al., 2013; Kohn et al., 2013; Philippe et al., 2016). The isolation treatment increased the time spent interacting for sitter flies compared to the sitter controls (Fig. 11a) and decreased the proportion of reciprocation for rover flies compared to the rover controls (Fig. 11c). Movement values of both rovers and sitters decreased significantly in the isolated treatments (Fig. 11d). The four behavioural elements of the network were affected by both *for* and isolation stress. Previous studies have explored the effect of isolation on social network phenotypes of *Canton-S* flies. Our results differ from some of these findings, for instance re-analyzing Schneider et al. (2012)'s finding with the automated social space criteria (described in the *Methods*) showed that both interaction rates and reciprocation decreases in isolated treatments, while interaction duration and movement were unaffected (Chapter 2). In addition, global efficiency of isolated flies increases and betweenness centrality values decrease. In contrast, our findings here show movement decreasing and the average times spent interacting increasing along with a similar decrease in rates of interaction and reciprocation (Fig. 11a - 11d) and isolation did not seem to affect any of the network measures (Fig. 11e - 11h). This may be related to the use of different methods and strains in our study and suggests that strain affects the response to isolation.

Quality and availability of food have also been shown to influence social behaviour. For instance, a relationship has been reported between food availability and aggressive behaviours in *D. melanogaster* (Baxter et al., 2015; Lim et al., 2014; Ueda & Kidokoro, 2002). Food deprivation is of particular interest considering *for*'s role in regulating many food-related behaviours (Allen et al., 2017; Anreiter & Sokolowski, 2019; Osborne et al., 1997). In fact, *for*'s

influence in relation to response to food deprivation or low-quality nutrition has been widely investigated (Burns et al., 2012; Donlea et al., 2012; Kaun et al., 2008; Kaun, Riedl, et al., 2007; Kent et al., 2009; Scheiner et al., 2004). Thus, we set out to see if rovers and sitters respond differentially to these stressors. We food deprived flies for 24 hours, and in a second experiment 48 hours, before video recording. Overall, both rovers and sitters show resilience to food deprivation, even after 48 hours of food deprivation (Fig. 12, 13). In response to 24 hours of food deprivation, sitters increase the time spent interacting and the likelihood of reciprocating an interaction, while rovers were unaffected (Fig. 12). In response to 48 hours of food deprivation, sitters were unaffected while rovers increased rates of interaction and reciprocation. Additionally, a decrease in variance is observed after 48 hours of food deprivation in sitter flies for interaction duration, assortativity, clustering, and global efficiency (Fig. 13).

Like isolation and food deprivation, sleep plays a role in influencing social behaviour (Beckwith & French, 2019; Kayser et al., 2014, 2015). We found that sleep deprivation seems to show a greater effect on the behavioral elements compared to food deprivation. However, similar to the other types of stress we measured in this chapter, the effect observed was restricted to behavioural elements of networks (Fig. 15). Sleep-deprived sitters spent more time interacting and had higher rates of interaction when compared to sitter controls (Fig. 15a, 15b). Both rover and sitter percentage reciprocation and movement were affected by sleep deprivation, with sleep-deprived treatments increasing their likelihood to reciprocate an interaction and move less when compared to their controls (Fig. 15c, 15d). In a previous study by Donlea et al. (2012) investigated *for*'s resiliency to both sleep and food deprivation and showed that in a learning task, there seems to be a trade-off where rovers were more resilient to sleep deprivation and sitters were more resilient to food deprivation. Results from our social network experiments do not show this trade-off indicating that previously described resilience may be phenotype dependent.

When comparing stressors, both isolation and sleep deprivation stress influence the four behavioural elements of the network, and some similarities are seen in flies' responses to the two different stressors. In response to both isolation and sleep deprivation, stressed sitter treatments increase the time they spend interacting (Fig. 11a, 15a), while both rover and sitter flies move less during the trials (Fig. 11d, 15d). Interaction rate also decreases in response to isolation and

food deprivation, however, the decrease in interaction rate of sitters is not significant in response to sleep deprivation (Fig. 11b, 15b). The opposite effect is seen in reciprocation measures, in response to isolation, rovers are less likely to reciprocate an interaction relative to the control while sitters were not affected, however in response to sleep deprivation, sitter flies were more likely to reciprocate interactions compared to controls and rover flies were unaffected. When considering food deprivation, 24 and 48 hours of food deprivation seem to have different effects on social networks. For interaction duration, responses to 24 hours of food deprivation were similar to those observed for isolation and sleep deprivation, after 24 hours of food deprivation, sitter flies spend more time interacting when compared to sitter controls while rovers were unaffected (Fig. 12a). After 48 hours of food deprivation, the interaction rate of rover flies increases, this effect is opposite to what was seen in response to isolation and sleep deprivation (Fig. 13b). For reciprocation, different responses are seen after 24 and 48 hours of food deprivation, with sitter increasing their reciprocation after 24 hours of food deprivation and rovers increasing their reciprocation after 48 hours of food deprivation. Altogether, these stress experiments show that there are some similarities in responses to different stressors, suggesting a similar regulation for certain phenotypes. However, a differential sensitivity trade-off across all stressors was not observed for the behavioural elements and social network phenotypes. For instance, in response to isolation, sleep deprivation, and 24 hours of food deprivation, stressed sitter flies increase the time they spend interacting. This is also observed in response to isolation and sleep deprivation for movement and frequency of interaction. These findings add to the knowledge accumulated on *for*'s plasticity in response to environmental cues, a central feature of the *for* gene.

In nature, the *for* variants are present in the same environment and thus may interact with each other. One outstanding question is how rovers and sitters interact with each other. Most experiments investigate homogenous groups of rovers and sitters. One exception is the exploration of the aggregation behaviour of mixed groups of rover and sitter flies (Philippe et al., 2016). Therefore, we acquired networks of mixed rover-sitter groups at different ratios. We tested three specific ratio combinations: (1) 9:1 to investigate how flies interact when there is a minority group, (2) 7:3 to explore how *for* variants interact at the proportion in which they are found in nature (Southern Ontario) and the reverse of that proportion, and (3) 5:5 to investigate

how they would interact when present in equal proportions. Mainly, our findings show that as more sitters are in the network, network phenotypes increasingly resemble sitter phenotypes suggesting that different variants maintain their individual phenotypes. For interaction criteria, as the number of sitters in a network increases, so does the interaction time criteria (Table 9). This is also clearly observed in behavioural elements of the networks and global efficiency, where there is a linear relationship as the number of sitter flies increase (Fig. 16a-d, 16g). For both assortativity and clustering, this trend is less obvious, and networks containing 9, 7, 5, and 3 rovers show similar levels for these network measures (Fig. 16e, 16f). These results suggest that individual flies may retain their phenotypes and do not change their behaviour in the presence of the other strain. This is supported by findings from a previous study that looked at aggregation behaviour in groups of rovers and sitters. The aggregation behaviour of each line was found to be affected by the presence of the other strain; however, the individual aggregation behaviour was maintained by each line. Future experiments identifying the behavioural elements and social network measures of individual rover and sitter flies within a mixed group can aid in us better understanding how these alleles interact with each other and if they maintain or alter their individual phenotypes in response to the presence of a different alleles in the group.

Overall, rover-sitter differences seem to be consistent across experiments even when filmed at different temperatures and different group sizes, demonstrating the resilience and stability of *for*'s influence on social network phenotypes. These results are consistent with previously published results from chapter 3 to a large extent (Alwash et al., 2021).

It was previously determined that *for* influences social behaviour (Chapter 3) (Alwash et al., 2021). The results reported here support the hypothesis that spatiotemporal regulation of *for* influences social behaviour in *D. melanogaster.* The complex molecular structure of *for* is linked to its pleiotropic nature (Allen et al., 2018; Anreiter et al., 2017; Anreiter & Sokolowski, 2019). Previous studies of *for* promotors indicate that there is variable control over spatial expression patterns as well as temporal patterns throughout the life span of the fly (Allen et al., 2018; Allen & Sokolowski, 2021; Anreiter et al., 2021). We narrow down the stage at which *for* influences social behaviour to the pupal stage. The effects on behavioural and social network phenotypes reported here could be the consequence of expression during metamorphosis in various individual tissues or combinations of tissues. Given that the expression of *for* is not

yet characterized during metamorphosis, it is still unclear what tissue expression of the different promoter products is responsible for the different social network phenotypes observed. This will be interesting to address in the future to better understand social interactions and group structures and the underpinning of sociality. Additionally, our findings characterize *for*'s influence further by investigating its plasticity in relation to social network phenotypes and, finally, exploring how the two strains interact with each other and their social networks. Taken together, these findings explore *for*'s role on social interaction and group structure further and characterize it, while also shedding light on the genetic underpinnings of sociality.

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Chapter 5:

Final Discussion and Conclusion

5.1 Overall Discussion and Conclusion of Thesis

Drosophila aggregate, creating a socially complex environment (Jiang et al., 2020; Wertheim et al., 2005; Shorrocks, 1972). Within this environment, flies interact with conspecifics and display many social behaviours, including foraging in groups, aggression, courtship, and mating (Krupp et al., 2008; Sokolowski, 2010; Spieth, 1974). Social isolation has adverse effects in flies, with socially isolated flies previously reported to display more aggressive behaviour and have shorter life spans (Ruan & Wu, 2008; Zhou et al., 2008). Additionally, several studies have suggested that fitness-enhancing information is transferred between individuals during social interactions (Pasquaretta, Battesti, et al., 2016), including information regarding predatory threat (Ferreira & Moita, 2020; Kacsoh et al., 2015), oviposition site choice (Battesti et al., 2014; Pasquaretta, Battesti, et al., 2016; Pasquaretta, Klenschi, et al., 2016), foraging decisions (Lihoreau, Clarke, et al., 2016), and mate choice (Loyau et al., 2012). Thus, *Drosophila* provides a wealth of social behaviours that can be investigated to provide insights into the social life of *Drosophila*, as well as other social insects, and more complex organisms.

The development of the SNA approach has shed light on the social life of several species, from primates to insects. As discussed in chapter 1, recent technological advances have allowed researchers to examine the social networks of insects (Branson et al., 2009; Robie et al., 2017; Straw & Dickinson, 2009). In that chapter, I briefly review studies that used the SNA approach to explore the social life of insects and group-level phenomena, such as social organization and transmission. This draws attention to the value of using the SNA approach to understand group-level behaviour in insects, with a deliberate emphasis on *Drosophila.*

The employment of the SNA approach in *Drosophila* has proved to be a valuable model for studying social behaviour and group structure. Chapter 2 provided a more elaborate dive into the SNA approach in *Drosophila by* highlighting similarities and differences in methodology across these studies and the variety of questions the authors aimed to address. Pioneering work done by Jonathan Schneider (Schneider et al., 2012) showed that *Drosophila* form non-random social networks. Later, several studies relied on this method in order to address a diverse array of

questions about social networks in *Drosophila* (Alwash et al., 2021; Bentzur et al., 2020; Jezovit et al., 2020; Liu et al., 2018; Pasquaretta, Battesti, et al., 2016; Rooke et al., 2020). However, still relatively little is known about the genetic underpinnings of sociality.

The *Drosophila foraging* gene (*for)* provides researchers with a prime example of a gene with natural alleles that regulate behavioural pleiotropy. *for* is a pleiotropic gene that influences multiple developmental and physiological phenotypes (Review in (Anreiter & Sokolowski, 2019)). This gene also has major effects on behaviours in the fly and other organisms, including humans (Alwash et al., 2021; Ben-Shahar et al., 2003; Ingram et al., 2005; Lucas et al., 2010; Philippe et al., 2016; Sokolowski et al., 2017; Struk et al., 2019; Tobback et al., 2008). Although *for* is best known for underlying the differences in the behavioural strategies of foraging between rover and sitter larvae, its effect has been extended to different social behaviours. These social behaviours include aggregation (Philippe et al., 2016), aggression (S. Wang & Sokolowski, 2017), social learning (Battesti et al., 2014; Foucaud et al., 2013; Kohn et al., 2013; Pasquaretta, Battesti, et al., 2016), and cross-species communication (Camiletti et al., 2014). In other species, *for* also plays a similar role in regulating sociality (Ben-Shahar et al., 2002, 2003; Bockoven et al., 2017; Ingram et al., 2005, 2011; Lucas et al., 2010; Tobback et al., 2008). These findings, along with *for'*s pleiotropic nature and the vast amount of knowledge available on this gene, made it of great interest in investigating the underpinnings of sociality. Thus, the *for* gene provides a new avenue for studying how natural genetic variation influences social behaviour. Using a natural polymorphism to study social behaviour provides us with more ecologically relevant insights, as both variants are maintained in nature and are clearly advantageous. In my thesis, I aimed to mainly address the influence of the *for* gene on social behaviour by utilizing the SNA approach.

Deciphering the genetic underpinnings of social behaviour is a difficult task. My thesis contributed to the understanding of how complex social networks are influenced by genetic differences, by providing examples of how *for* influences behavioural elements and social networks measures. My findings show that the *for* gene influences social networks (Chapter 3) (Alwash et al., 2021), expanding on our limited knowledge of the genetic underpinnings of sociality. Although some studies have investigated how certain genes affect social networks

(Schneider et al., 2012; Rooke et al., 2020; Bentzur et al., 2020), this is the first study that aims to characterize the effect of a specific gene on social networks. Knockdown and increased expression manipulations of *for* at different life stages and using different promoters emphasized the complexity of the *for* gene and the manner in which it influences the rover-sitter differences observed in adult social networks (Chapter 4). Results of these experiments showed that the rover-sitter differences in behavioural elements and social networks phenotypes are likely driven by *for* expression in different tissues or a combination of tissues during metamorphosis. These results may hint at the presence of a critical period during which social behaviour circuitry is determined. Additionally, rover-sitter differences exhibit plastic responses to the environmental cues (Chapter 4). These results suggest that social behaviour is heritable and is also affected by the environment and thus these variations in phenotypes related to the social network arise from interdependencies between genes and the environment.

Elucidating how the *foraging* gene influences social behaviour is a valuable endeavor. In this study, I have identified clear effects of *for*'s influence on social networks and provided insight into the group-level behaviour of the *for* variants in nature. This research is instrumental in understanding the genetic underpinnings of sociality, as well as how natural genetic variations can give rise to variations in complex phenotypes. This provides critical insight into how *for* regulates social behaviour further characterizes its pleiotropic nature. These investigations may have implications for deciphering the mechanisms underlying *for's* regulation of social behaviour. While I have demonstrated a role for the *foraging* gene on social networks, my findings also reveal several related questions that need to be addressed in the future, perhaps through the experiments suggested throughout this chapter. Questions concerning why humans and other animals display social behaviour have occupied researchers for centuries. There is some conservation of *for*'s regulation, and already some studies are utilizing *for* orthologs of more complex species, including humans. This thesis has implications not only for studies in *D. melanogaster* and other insects but also a wide variety of taxa with more complex systems such as mammals. I anticipate that research in this direction will not only help us to further understand the link between *for* and social behaviour but also in the potential discovery of other key components and novel genes involved in the regulation of social behaviour.

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Appendix

A.1 Contributions

The qPCR experiments were performed with the help of Amara Rasool, all other experiments were performed by Nawar Alwash

A.2 Methods

A.2.1 Fly strains, crossing and rearing

To assess whether 1 copy of the rover or sitter allele is enough to show the rover-sitter difference, I tested heterozygous animals of the *for* genetic null line *(*for^0*)* crossed to for^S and for^R in order to generate a strain with only one copy of *for* containing either the rover or the sitter allele (A. M. Allen et al., 2017).

Additional lines were used to investigate differences in the genetic background between for^R and for^S. In addition to R1 line previously discussed, a line called R3 was generated in a similar method which has a sitter *foraging* allele backcrossed (9x's) into a sitter genetic background. The R1-W, R3-W, and R4-W were generated in a similar method as the R1 and R3 lines with the difference of having a white eye mutation on chromosome 1. The line called R1-W has a rover *foraging* allele backcrossed (9x's) into a sitter genetic background, while R3-W line has a sitter *foraging* allele backcrossed (9x's) into a sitter genetic background and the R4-W has a sitter *foraging* allele backcrossed (9x's) into a rover genetic background.

A.2.2 Reverse transcription quantitative PCR

Total RNA was extracted from virgin adult female flies using Qiagen RNeasy Mini kit. Following RNA extraction, cDNA was synthesized using the iScript cDNA synthesis kit. RT-qPCR was preformed using universal SYBR green supermix and gene-specific primers.

A.3 Results and discussion

As previously discussed, due to *for'*s pleiotropic nature and its influence on an array of social behaviours in *Drosophila* and other species, I set out to replicate the rover-sitter differences in social interaction networks (presented in Fig. 4.1, S4.1). I acquired and analyzed networks of rover and sitter flies for both females and males and show that *for* influences social interactions between flies and their group-level behaviours (Figure A1, A2). Sitter flies formed networks in which individuals spend, on average, more time interacting and were more likely to reciprocate interactions; as for network measures, sitters formed more homogeneous and clustered networks. Whereas rovers had higher rates of interactions and moved more during these trials, there was also a more efficient flow of information in their networks. These findings fit into a broader paradigm of *for* influencing social behaviour in *D. melanogaster.* These rover-sitter differences were robust across all experiments presented in this thesis and were similar in females and males. Table A1 shows interaction criteria of the rover and sitter strains. The parameters are inter-related, so differences represent trends that are not tested as independent measures.

Rover and sitter flies differ in the behavioural elements and their network phenotypes (Fig. A1 and A2). This was also replicated (Table 4.1 and S4.1, Fig. 1 and S4.1). In both females and males, sitter interact for a significantly longer interaction duration, have lower rates of interaction and are more likely to reciprocate an interaction. The relationship for these three behavioural elements were significant in both males and females, and in two independent experiments (Fig. 4.1a - c, A1a - c, A2a - c, S4.1a - c). Rover flies also moved more on average when compared to sitter flies during the 30-minute trial, and these differences were present in both females and males (Differences were significant in Fig. 4.1d, A2d, S4.1d, trend was observed in A1d but was not significant). Similarly, social network measures followed the same trend in both males and females in the two independent experiments. Sitter females also form more homogenous networks with higher assortativity values (Fig. 4.1e and A1e). Moreover, in both females and males, sitter tend to form more clustered networks, but these differences are not significant in males (Fig. 4.1f, A1f, A2f, S4.1f). The flow of information was more efficient in networks of rovers, and thus they had higher global efficiency values, these differences are again only significant in females (Fig. 4.1g, A1g, A2g, S4.1g). Finally, no significant differences were found between rovers and sitter in betweenness centrality values (Fig. 4.1h, A1h, A2h, S4.1h).

Curiously, differences between rover and sitter males were less pronounced than in females (Figure A2, S4.1). When investigating the size of the effect of *for* on these phenotypes, this is true for most behavioural elements and network phenotypes (See Table A5). A possible explanation for this variation, which is replicated in chapter 4, is that female flies may gain more value from social interactions. This may be due to their increased energy input on decision-making processes such as choosing a mate or a food patch to lay eggs. Social information transferred between flies during these interactions may decrease the energy load required to make these decisions while still enhancing their fitness. These findings are worth further exploration in order to explain the variation in rover-sitter differences between females and males.

Using RT-qPCR, I show differences in *for* expression between the two strains (Fig. A7). Whole body RT-qPCR of virgin female rover and sitter flies showed that rover had significantly lower mRNA expression of *for*-common (Fig. A7a), *for*-PR1 (Fig. A7b), *for*-PR3 (Fig. A7d), *for*-PR4 (Fig. A7e). No differences in expression were found in *for*-PR2 (Fig. A7c).

Additionally, crossing the for^{null} line to homozygous rover or sitter flies showed that the rover-sitter difference is evident even with only one copy of an allele, demonstrating the strength of these phenotypes (Figure A3). I also crossed the for^{null} to both homozygous rovers and sitter to create two lines, the first with one copy of rover *foraging* allele and the second with one copy of sitter *foraging* allele. Interaction criteria of these two lines are indicated in Table A2. Networks of these lines show that one copy of the rover or sitter allele is sufficient to show the rover-sitter difference (Fig. A3). For behavioural elements, one copy of *for* was sufficient to show the rover-sitter differences. *1for(sitter)* shows significantly higher interaction duration, percentage reciprocation, and significantly lower rates of interaction and movement (Fig A3a – d). Assortativity values were significantly higher in *1for(sitter)* (Fig. A3e). No significant differences were found in clustering coefficient or global efficiency measures, but they followed the same trends previously observed in homozygous rovers and sitters (Fig. A3f and g). RT-qPCR was performed on *1for*(rover) and *1for*(sitter lines) with rover and sitter as controls (Fig. A8). Similar to what was observed in Figure A7, *1for*(rover) had lower mRNA expression of *for*-common (Fig. A8a), *for*-PR1 (Fig. A8b), *for*-PR3 (Fig. A8d), *for*-PR4 (Fig. A8e), but these differences were not significant while the rover-sitter differences were maintained.

Figure A9 shows RT-qPCR of heterozygotes. Rover/sitter heterozygotes show intermediate level of mRNA expression relative to rover and sitter controls for both *for*-common and *for*-PR4 (Fig. A9a, e). mRNA expression of *for*-PR1 and *for*-PR2 in heterozygotes were significantly higher than rovers (Fig. A9b, c) and no significant differences were found between the three groups in *for*-PR3 mRNA expression (Fig. A9d).

I also replicated the results from the *for* dosage experiment shown in Figure 4.3. Interaction criteria of these two lines are indicated in Table A3. Similar to previous findings, a *for* gene-dosage effect is observed here (Fig. A4), with average interaction duration and reciprocation decreasing as *for* copy number increases (Fig. A4a, c). Whereas we see the opposite effect in rates of interaction and movement, with averages of both measures increasing as *for* copy number increases (Fig. A4b, d). For the four social network measures, trends seem to replicate ones from previous experiment with assortativity decreasing and clustering coefficient and global efficiency increasing as *for* copy number increases (Fig. A4e, f, and g). Betweenness centrality also follows the same trend, however, Figure A4h shows significant difference between *1for* and the other groups tested for betweenness centrality, whereas in figure 3h these differences were not significant. Figure A10 shows mRNA expression levels of the lines tested in the *for* dosage experiment, as *for* copy number increases, *for* expression levels also increase with *2for* having the highest expression levels.

Animal behaviour is among the most complex phenotypes regulated by genetic background. In the case of the *for* gene, this is even more complex, as the rover and sitter lines uses in the present study don't only differ in the *for* locus but the entirety of the second chromosome. Thus, after initially showing that rover and sitter flies form networks with different phenotypes, I wanted to verify if the differences I observed are due specifically to the *for* locus. In addition to the lines presented in chapter 4, I also tested three more R-lines presented in the appendix (Figure A5, A6). Similar to the R1 strain, the R3 strain has a sitter allele backcrossed (9 generations) into a sitter-like genetic background. The R3 strain formed networks that had intermediate average interaction duration, reciprocation and movement values when compared to rover and sitters (Fig. A5a, c, and d). Average rates of interaction of the R3 flies were similar to those of rover flies (Fig. A5b). Networks of the R3 strain were more homogenous when compared to their rover and sitter controls (Fig. A5e). For clustering and global efficiency,

scores of the R3 strain were similar to those of sitters but significantly differed from rovers (Fig. A5f and g). The R3 line also formed networks with significantly higher betweenness centrality values (Fig. A5h). Results of the lines in figure A6, however, did not seem to resemble previously discussed lines. These lines carried the white eye mutation, which has been reported to lead to a decreased life span, in addition to having both neurological and vision defects (Ferreiro et al., 2018). Although I was unable to use these lines to further investigate the effect of chromosome 2 background differences on the rover-sitter social behavioural and network measures, results from these lines suggest that the white eye mutation plays a role in social interaction networks. Taken together, these results establish a role for the *foraging* locus on social interaction networks and in influencing both behavioural and network measures.

Figure A11 shows RT-qPCR of the R1 and R3 line along with the rover and sitter controls. No significant differences were found in mRNA expression levels between the R1 and R3 lines and the rover-sitter controls groups for *for*-common, *for*-PR1, *for*-PR2, *for*-PR3 or *for*-PR4 (Fig. A11a-e). Greater variance is also observed in the expression levels of R3 line in *for*-common, *for*-PR1 and *for*-PR4 (Fig. A11a, c, e). Three additional lines were tested for this experiment, the R1-W, R3-W and R4-W were white mutant strains. The R1-W similar to the R1 line previously discussed has a sitter allele backcrossed (9 generations) into a sitter-like genetic background, while the R3-W has a sitter allele backcrossed (9 generations) into a sitter-like genetic background, and finally the R4-W strain had a has a sitter allele backcrossed (9 generations) into a rover-like genetic background. Figure A6 shows social network phenotypes of these lines. Overall, the three lines did not seem to differ in their network phenotypes, but the rover-sitter differences were maintained (Fig. A6). Similar to these findings, RT-qPCR shows that no differences were found between the three lines in mRNA expression (Fig. A12)

Furthermore, RT-qPCRs of the lines discussed previously were performed (Figure A7 – A12). These qPCRs considered the expression of the mRNA products of the four promoters using promoter-specific primers, along with a common *for* primer, capturing the expression of all isoforms of *for*. While it was originally thought that the rover-sitter phenotypic differences were due to higher PKG activity in rover heads, recent findings indicate that the relationship is more complicated. Promoter 4 expression in adult sitters was higher than in rovers, whereas when

investigating promoter 1 expression in larvae, rover flies show higher levels of mRNA expression in the central nervous system (Allen et al., 2018; Anreiter et al., 2017). For the qPCR performed for this thesis, all primers showed higher mRNA expression of *for* in the whole body of virgin female adult sitter flies. A similar trend was observed with lines that have one copy of the rover or sitter alleles, but the differences between the two lines were not significant. Levels of mRNA expression in heterozygote flies showed intermediate levels for only the common *for* promoter and promoter 4 products. While those of the transgenic lines used in the *for* dosage experiment showed increasing levels of *for* expression as *for* copy number increases. Finally, qPCRs were performed for R-lines as well. Although I was unable to fully characterize *for* expression in relation to social networks, these are interesting preliminary results and require further investigation to determine the pattern of *for* expression in different tissues and how this relates to the differences observed in social interaction networks. mRNA levels of *for* fluctuated during the day in foraging ants (Ingram et al., 2011; Lei et al., 2019), so time-specific dissections in flies are also worth exploring since this has not been reported in *Drosophila* (Keegan et al., 2007; McDonald & Rosbash, 2001; Wijnen et al., 2006). In addition, the transcriptional regulation of promoters that underlie the rover-sitter differences must also be investigated. Understanding the *for* expression pattern and how this relates to social interaction networks would take us a step closer to understanding the tissues and eventually the molecular pathways by which sociality is regulated and maintained.

Table A1. Interaction criteria for rover and sitter (female and male flies). Interaction criteria for each strain were automatically computed for distance, angle and time criteria across the replicates in order to capture interactions objectively across videos.

Strain	**Distance (Body lengths)**	**Angle (º)**	**Time (Sec)**
Rover (Female)	2.00	75	1.05
Sitter (Female)	1.50	122.5	4.80
Rover (Male)	2.25	60	0.30
Sitter (Male)	2.00	45	0.60

Table A2. Interaction criteria of lines of *for* carrying one copy of either the rover or sitter *foraging* allele. Interaction criteria for each strain were automatically computed for distance, angle and time criteria across the replicates in order to capture interactions objectively across videos.

Strain	**Distance (Body lengths)**	**Angle (º)**	**Time (Sec)**
1for(rover)	1.75	75	0.60
1for(sitter)	1.75	85	0.70

Table A3. Interaction criteria of lines of *for* used in the gene-dosage experiments and their rover and sitter controls (Replicated). Interaction criteria for each strain were automatically computed for distance, angle and time criteria across the replicates in order to capture interactions objectively across videos.

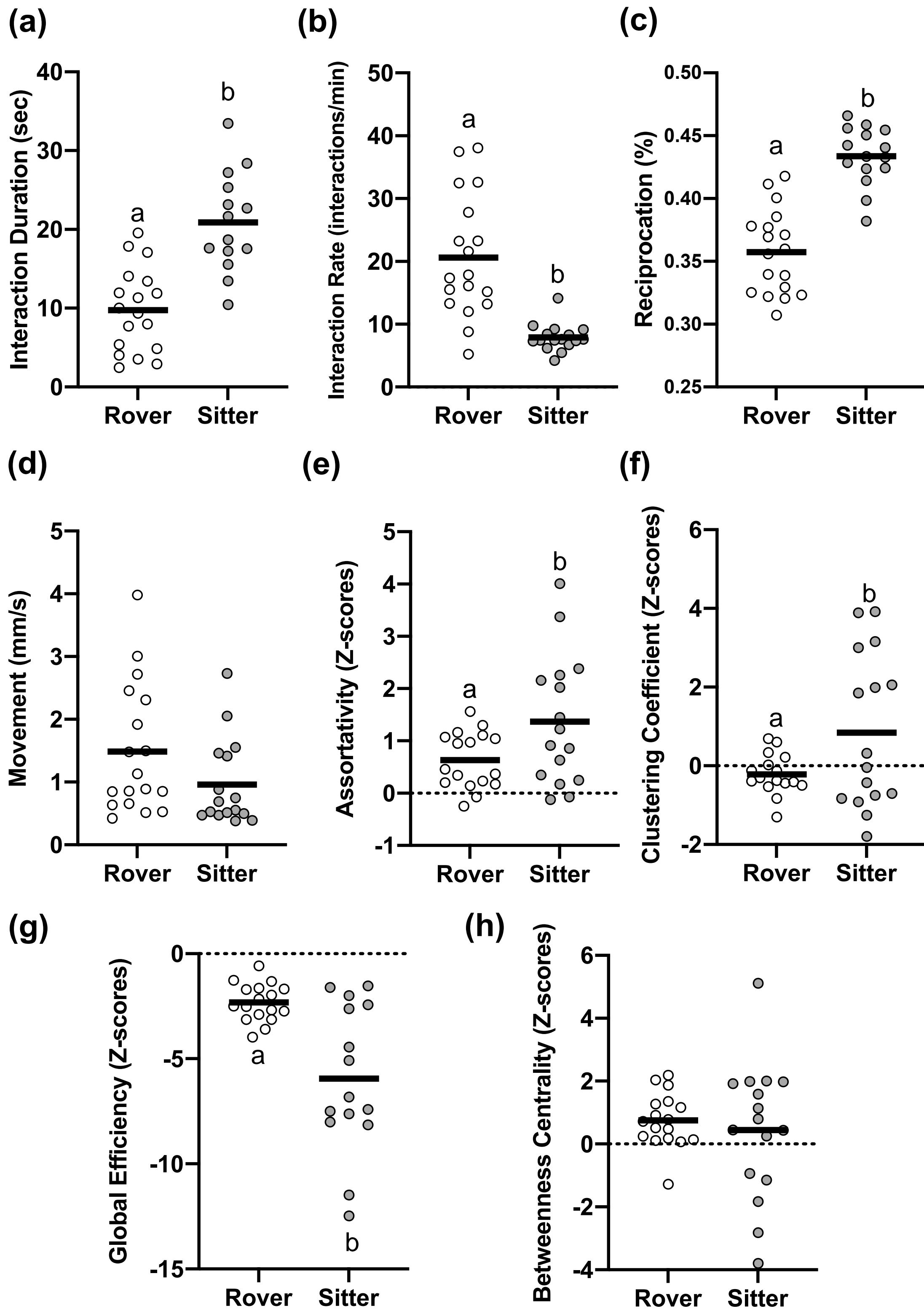
Strain	**Distance (Body lengths)**	**Angle (º)**	**Time (Sec)**
1for	1.75	135	0.95
2for	2.00	65	0.75
4for	2.00	65	0.70
Rover	1.50	115	0.55
Sitter	1.50	120	0.85

Table A4. Interaction criteria of the background lines and the rover and sitter controls. Interaction criteria for each strain were automatically computed for distance, angle and time criteria across the replicates in order to capture interactions objectively across videos.

Strain	**Distance (Body lengths)**	**Angle (º)**	**Time (Sec)**
Rover	1.50	115	0.75
Sitter	1.50	120	0.55
R3	1.75	120	0.85
R1-W	1.50	110	0.80
R3-W	1.50	110	0.65
R4-W	1.50	120	0.80

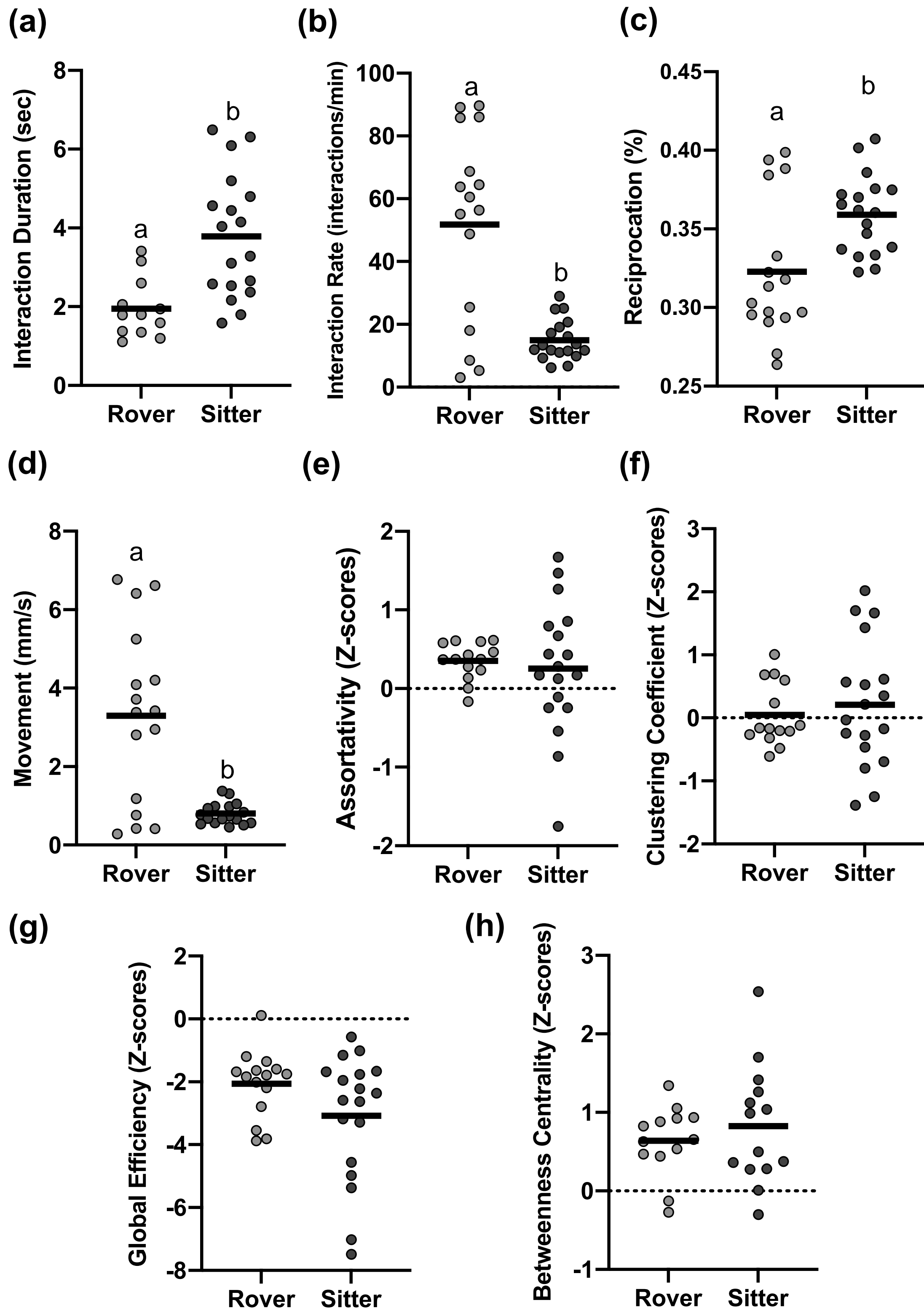
Table A5. Difference between males and females in the effect size of *for* on behavioural elements and social network phenotypes in networks of rovers and sitters

Network Measure	**R^2 (rover-sitter female, Figure A1)**	**R^2 (rover-sitter male, Figure A2)**	**R^2 (rover-sitter female, Figure 4.1)**	**R^2 (rover-sitter male, Figure S4.1)**
Interaction Duration	0.496	0.337	0.590	0.564
Rate of Interaction	0.452	0.437	0.628	0.292
Reciprocation	0.641	0.217	0.436	0.787
Movement	0.086	0.402	0.682	0.709
Assortativity	0.138	0.006	0.117	0.035
Clustering Coefficient	0.127	0.010	0.037	0.063
Global Efficiency	0.363	0.091	0.524	0.556
Betweenness Centrality	0.009	0.024	0.015	0.000



(a)
Interaction Duration (sec)
40
30
20
10
0
a
b
Rover
Sitter
(b)
Interaction Rate (interactions/min)
50
40
30
20
10
0
a
b
Rover
Sitter
(c)
Reciprocation (%)
0.50
0.45
0.40
0.35
0.30
0.25
a
b
Rover
Sitter
(d)
Movement (mm/s)
5
4
3
2
1
0
Rover
Sitter
(e)
Assortativity (Z-scores)
5
4
3
2
1
0
-1
a
b
Rover
Sitter
(f)
Clustering Coefficient (Z-scores)
6
4
2
0
-2
a
b
Rover
Sitter
(g)
Global Efficiency (Z-scores)
0
-5
-10
-15
a
b
Rover
Sitter
(h)
Betweenness Centrality (Z-scores)
6
4
2
0
-2
-4
Rover
Sitter

Figure A1. Rover and sitter strains differ in social behaviours and network properties (Females). (a) Average duration of an interaction was significantly different between rover and sitter flies, with rovers having significantly shorter interactions ($t = 5.43$) ($df = 30$) ($p \leq 0.0001$) (rover $n = 18$, sitter $n = 14$). (b) Interaction Rate was significantly higher in rover flies ($t = 5.14$) ($df = 32$) ($p \leq 0.0001$) (rover $n = 18$, sitter $n = 16$). (c) Percentage of interactions that were reciprocated by the receiver was higher in sitter flies ($t = 7.45$) ($df = 31$) ($p \leq 0.0001$) (rover $n = 18$, sitter $n = 15$). (d) Rovers move more during the trial compared to sitters but differences were not significant ($t = 1.73$) ($df = 32$) ($p = 0.0941$) (rover $n = 18$, sitter $n = 16$). (e) Sitters form networks with significantly higher assortativity values ($t = 2.23$) ($df = 31$) ($p \leq 0.05$) (rover $n = 17$, sitter $n = 16$). (f) Clustering coefficient values were higher in sitter networks ($t = 2.52$) ($df = 30$) ($p \leq 0.05$) (rover $n = 16$, sitter $n = 16$). (g) Global Efficiency values were significantly higher in networks of rovers relative to sitter networks ($t = 4.13$) ($df = 30$) ($p \leq 0.001$) (rover $n = 17$, sitter $n = 15$). (h) Betweenness centrality values did not differ between networks of rovers and sitters ($t = 0.54$) ($df = 31$) ($p = 0.5953$) (rover $n = 17$, sitter $n = 16$). *a-h* were analyzed with two-tailed t-tests (*Methods)*. Outliers were removed from all the datasets. Bars indicate mean. Letters indicate statistical significance. *e-h* Measurements were standardized using z-scores.



(a)
Interaction Duration (sec)
8
6
4
2
0
a
b
Rover
Sitter
(b)
Interaction Rate (interactions/min)
100
80
60
40
20
0
a
b
Rover
Sitter
(c)
Reciprocation (%)
0.45
0.40
0.35
0.30
0.25
a
b
Rover
Sitter
(d)
Movement (mm/s)
8
6
4
2
0
a
b
Rover
Sitter
(e)
Assortativity (Z-scores)
2
1
0
-1
-2
Rover
Sitter
(f)
Clustering Coefficient (Z-scores)
3
2
1
0
-1
-2
Rover
Sitter
(g)
Global Efficiency (Z-scores)
2
0
-2
-4
-6
-8
Rover
Sitter
(h)
Betweenness Centrality (Z-scores)
3
2
1
0
-1
Rover
Sitter

Figure A2. Rover and sitter strains differ in social behaviours and network properties (Males). (a) Average duration of an interaction was different between rover and sitter flies (rover n = 12, sitter n = 18), with rovers having significantly shorter interactions (t = 3.77) (df = 28) ($p \leq 0.001$). (b) Interaction Rate was significantly higher in rover flies (t = 4.98) (df = 32) ($p \leq 0.0001$) (rover n = 16, sitter n = 18). (c) Percentage of interactions that were reciprocated by the receiver was higher in sitter flies (t = 2.98) (df = 32) ($p \leq 0.01$) (rover n = 16, sitter n = 18). (d) Rovers moved significantly more during the trial compared to sitters (t = 4.56) (df = 31) ($p \leq 0.0001$) (rover n = 26, sitter n = 17). (e) Assortativity values did not differ between networks of male rovers and sitter (t = 0.41) (df = 30) ($p = 0.6844$) (rover n = 14, sitter n = 18). (f) Clustering coefficient values did not differ between rover and sitter networks (t = 0.55) (df = 30) ($p = 0.5857$) (rover n = 14, sitter n = 18). (g) Global Efficiency values did not significantly differ between rovers and sitters (t = 1.76) (df = 31) ($p = 0.0878$) (rover n = 15, sitter n = 18). (h) Betweenness centrality values did not differ between rovers and sitters (t = 0.78) (df = 25) ($p = 0.4435$) (rover n = 13, sitter n = 14). *a-h* were analyzed with two-tailed t-tests (*Methods)*. Outliers were removed from all the datasets. Bars indicate mean. Letters indicate statistical significance. *e-h* Measurements were standardized using z-scores.

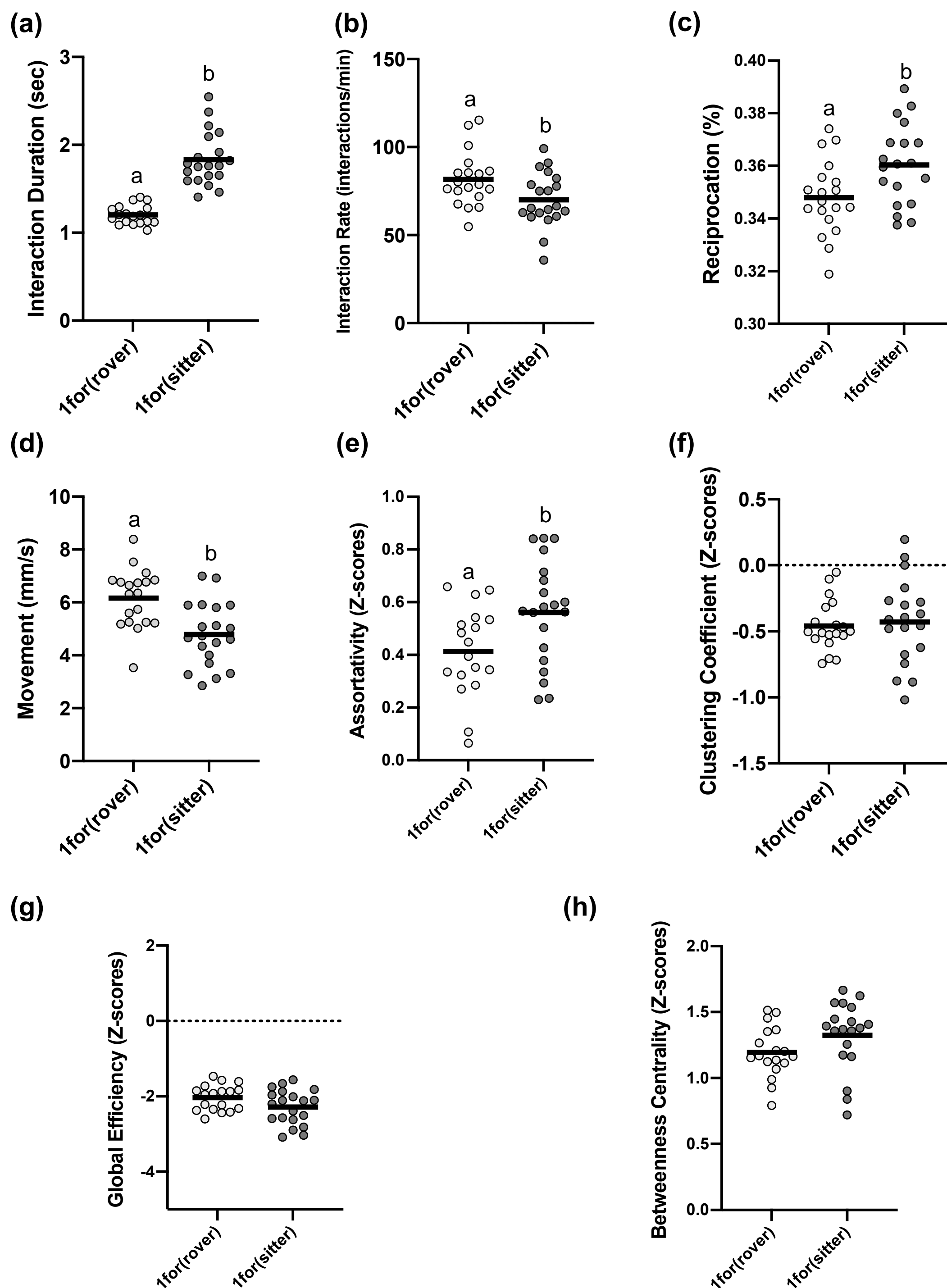
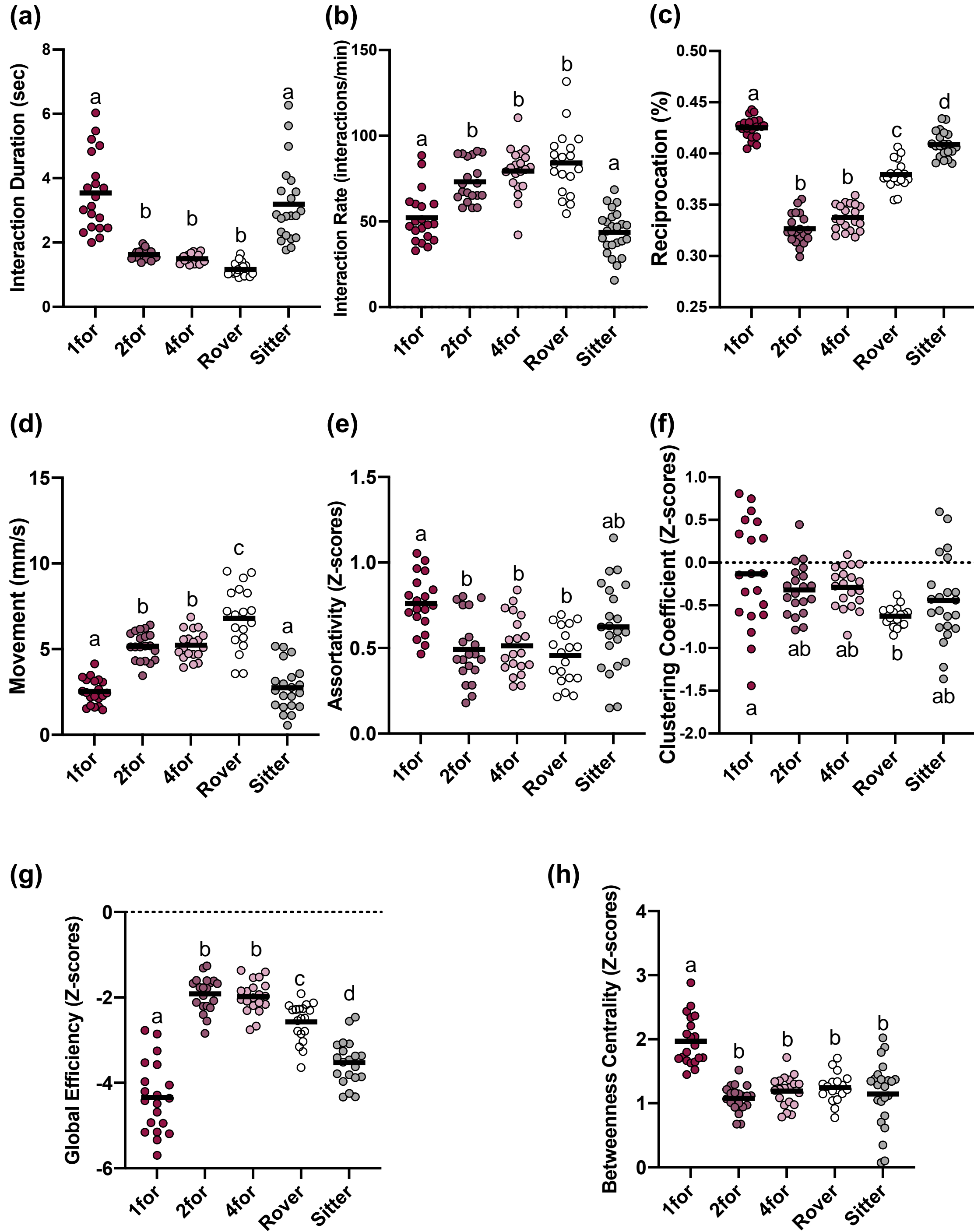


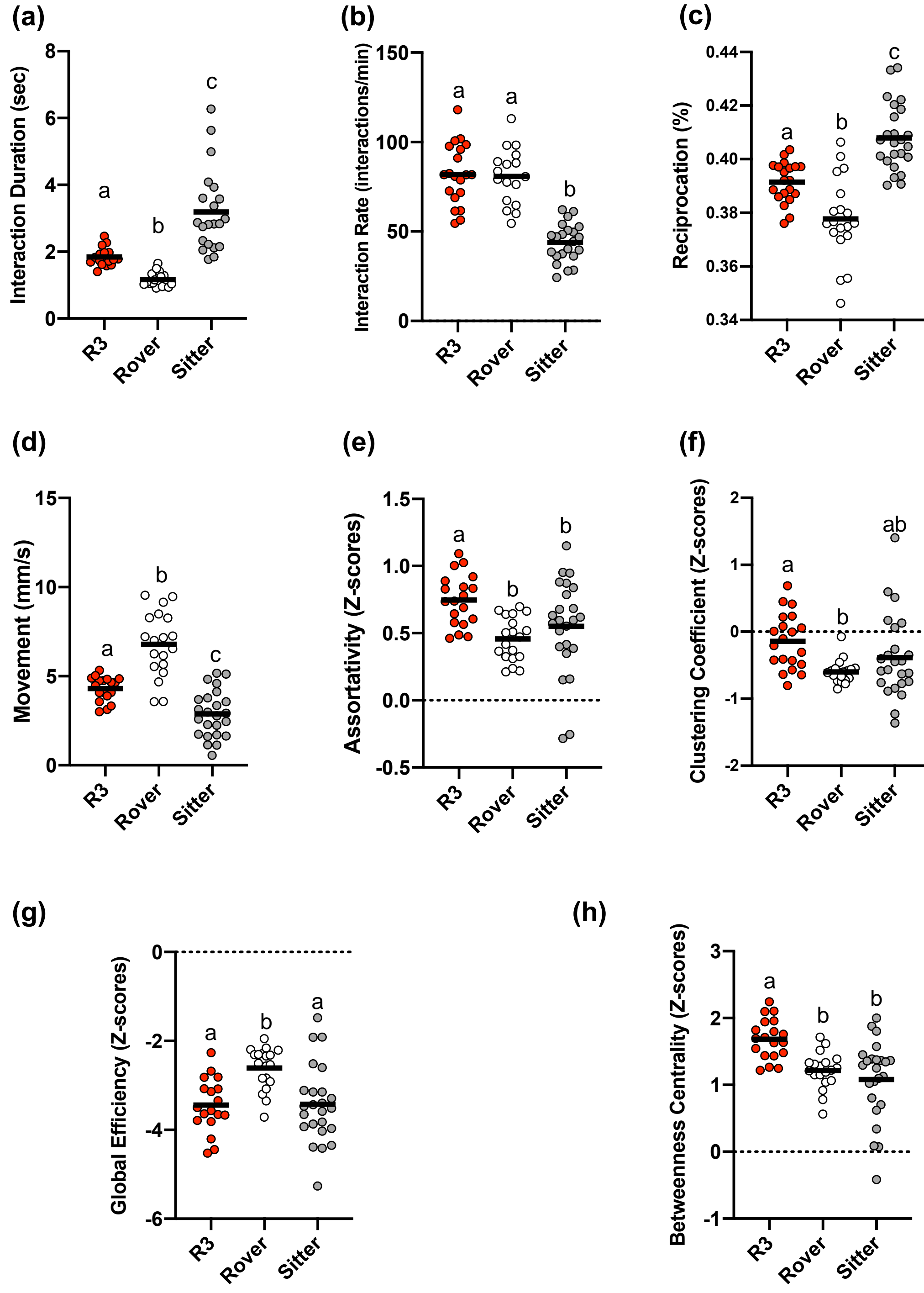
Figure A3. 1 copy of *for* shows the rover-sitter differences. (a) Average duration of an interaction was significantly higher in networks of *1for*(sitter) (t = 8.30) (df = 36) ($p \leq 0.0001$) (*1for*(rover) n = 18, *1for*(sitter) n = 20). (b) Interaction rate was significantly higher in

1for(rover) (t = 2.34) (df = 37) ($p \leq 0.05$) (*1for*(rover) n = 19, *1for*(sitter) n = 20). (c) Percentage of interactions that were reciprocated by the receiver were significantly higher in networks of *1for*(sitter) (t = 2.61) (df = 36) ($p \leq 0.05$) (*1for*(rover) n = 19, *1for*(sitter) n = 29, rover n = 18, sitter n = 21). (d) Rover-sitter differences in movement of flies during the trial were maintained between *1for*(rover) and *1for*(sitter) flies (t = 3.70) (df = 37) ($p \leq 0.001$) (*1for*(rover) n = 19, *1for*(sitter) n = 20). (e) Assortativity values of the networks were lower in networks of *1for*(rover) (t = 2.47) (df = 36) ($p \leq 0.05$) (*1for*(rover) n = 18, *1for*(sitter) n = 20). (f) Clustering coefficient value of networks did not differ between networks of *1for*(rover) and *1for*(sitter) flies (t = 0.34) (df = 36) ($p = 0.7382$) (*1for*(rover) n = 19, *1for*(sitter) n = 19). (g) Global efficiency did not differ between networks of *1for*(rover) and *1for*(sitter) flies (t = 1.88) (df = 36) ($p = 0.0679$) (*1for*(rover) n = 18, *1for*(sitter) n = 20). (h) Betweenness centrality values did not differ between *1for*(rover) and *1for*(sitter) flies (t = 1.70) (df = 35) ($p = 0.0972$) (*1for*(rover) n = 18, *1for*(sitter) n = 19). *a-h* were analyzed with two-tailed t-tests (*Methods)*. Outliers were removed from all the datasets. Bars indicate mean. Letters indicate statistical significance. *e-h* Measurements were standardized using z-scores.



(a)
Interaction Duration (sec)
1for
2for
4for
Rover
Sitter
(b)
Interaction Rate (interactions/min)
(c)
Reciprocation (%)
(d)
Movement (mm/s)
(e)
Assortativity (Z-scores)
(f)
Clustering Coefficient (Z-scores)
(g)
Global Efficiency (Z-scores)
(h)
Betweenness Centrality (Z-scores)

Figure A4. *for* copy number affects social behaviour and social networks of *Drosophila melanogaster* (Replicated). (a) Average duration of an interaction decreased as *for* copy number increased, and sitter had significantly higher average interaction duration compared to rover ($F_{(4,93)} = 36.04$) ($p \leq 0.0001$) (*1for* n = 20, *2for* n = 20, *4for* n = 19, rover n = 18, sitter n = 21). (b) Interaction rate was lower in networks of 1*for* and sitter flies ($F_{(4,98)} = 30.70$) ($p \leq 0.0001$) (*1for* n = 20, *2for* n = 20, *4for* n = 20, rover n = 19, sitter n = 24). (c) Percentage of interactions that were reciprocated by the receiver decreased as *for* copy number increased and were highest in networks of 1*for* ($F_{(4,94)} = 222.00$) ($p \leq 0.0001$) (*1for* n = 20, *2for* n = 20, *4for* n = 20, rover n = 18, sitter n = 21). (d) Movement of flies during the trial increased as *for* copy number increased, rover moved more on average during trials compared to sitter ($F_{(4,94)} = 47.03$) ($p \leq 0.0001$) (*1for* n = 20, *2for* = 20, *4for* n =19, rover n = 19, sitter n = 21). (e) As *for* copy number increased, assortativity values of the networks decreased, assortativity was significantly higher in sitter and 1*for* ($F_{(4,95)} = 7.71$) ($p \leq 0.0001$) (*1for* n = 19, *2for* n = 20, *4for* n =20, rover n = 19, sitter n = 22). (f) Clustering coefficient value of networks decreased as *for* copy number increased ($F_{(4,95)} = 3.98$) ($p \leq 0.01$) (*1for* n = 20, *2for* n = 20, *4for* n =20, rover n = 18, sitter = 22). (g) Global efficiency values increased as *for* copy number increased ($F_{(4,94)} = 73.94$) ($p \leq 0.0001$) (*1for* n = 20, *2for* n = 22, *4for* n = 18, rover n = 20, sitter = 17). (h) Betweenness centrality values were highest in networks of 1*for* ($F_{(4,97)} = 22.69$) ($p \leq 0.0001$) (*1for* n = 20, *2for* n = 20, *4for* n = 20, rover n = 18, sitter = 23). *a-h* were analyzed with one-way ANOVA. The Tukey-Kramer method was used as the post-hoc test (*Methods)*. Outliers were removed from all the datasets. Bars indicate mean. Letters indicate statistical significance. *e-h* Measurements were standardized using z-scores.



(a)
Interaction Duration (sec)
8
6
4
2
0
a
b
c
R3
Rover
Sitter
(b)
Interaction Rate (interactions/min)
150
100
50
0
a
a
b
R3
Rover
Sitter
(c)
Reciprocation (%)
0.44
0.42
0.40
0.38
0.36
0.34
a
b
c
R3
Rover
Sitter
(d)
Movement (mm/s)
15
10
5
0
a
b
c
R3
Rover
Sitter
(e)
Assortativity (Z-scores)
1.5
1.0
0.5
0.0
-0.5
a
b
b
R3
Rover
Sitter
(f)
Clustering Coefficient (Z-scores)
2
1
0
-1
-2
a
b
ab
R3
Rover
Sitter
(g)
Global Efficiency (Z-scores)
0
-2
-4
-6
a
b
a
R3
Rover
Sitter
(h)
Betweenness Centrality (Z-scores)
3
2
1
0
-1
a
b
b
R3
Rover
Sitter

Figure A5. Rover-sitter allelic variants, social behaviour and social networks (R3 line). (a) Average duration of an interaction differed with sitters having significantly longer interactions ($F_{(2,53)} = 34.65$) ($p \leq 0.0001$) (R3 n = 17, rover n = 18, sitter n = 21). (b) R3 and Rover flies had higher interaction rates than sitter flies ($F_{(2,56)} = 46.17$) ($p \leq 0.0001$) (R1 n = 20, rover n = 17, sitter n = 22). (c) R3 flies had intermediate reciprocity when compared to rover and sitter flies ($F_{(2,60)} = 33.08$) ($p \leq 0.0001$) (R3 n = 20, rover n = 19, sitter n = 24). (d) Rover moved significantly more than both R3 flies and sitters ($F_{(2,58)} = 44.46$) ($p \leq 0.0001$) (R1 n = 18, rover n = 19, sitter n = 24). (e) Assortativity values of R3 lines were significantly higher than those of rovers or sitters ($F_{(2,59)} = 6.25$) ($p \leq 0.01$) (R1 n = 19, rover n = 19, sitter n = 24). (f) Clustering coefficient values of R3 flies were significantly higher than those of rover flies ($F_{(2,60)} = 4.84$) ($p \leq 0.01$) (R3 n = 20, rover n = 19, sitter n = 24). (g) Global Efficiency values were significantly higher in rovers relative to sitter and R3 ($F_{(2,58)} = 9.24$) ($p \leq 0.001$) (R3 n = 18, rover n = 19, sitter n = 24). (h) Betweenness centrality values of R1 line was significantly higher but did not differ between rovers and sitter ($F_{(2,59)} = 10.88$) ($p \leq 0.0001$) (R3 n = 19, rover n = 19, sitter n = 24). *a-h* were analyzed with one-way ANOVA to determine if statistical differences exist between the groups. The Tukey-Kramer method was used as a post-hoc test (Methods). Outliers were removed from all the datasets. Bars indicate mean. Letters indicate statistical significance. *e-h* Measurements were standardized using z-scores.

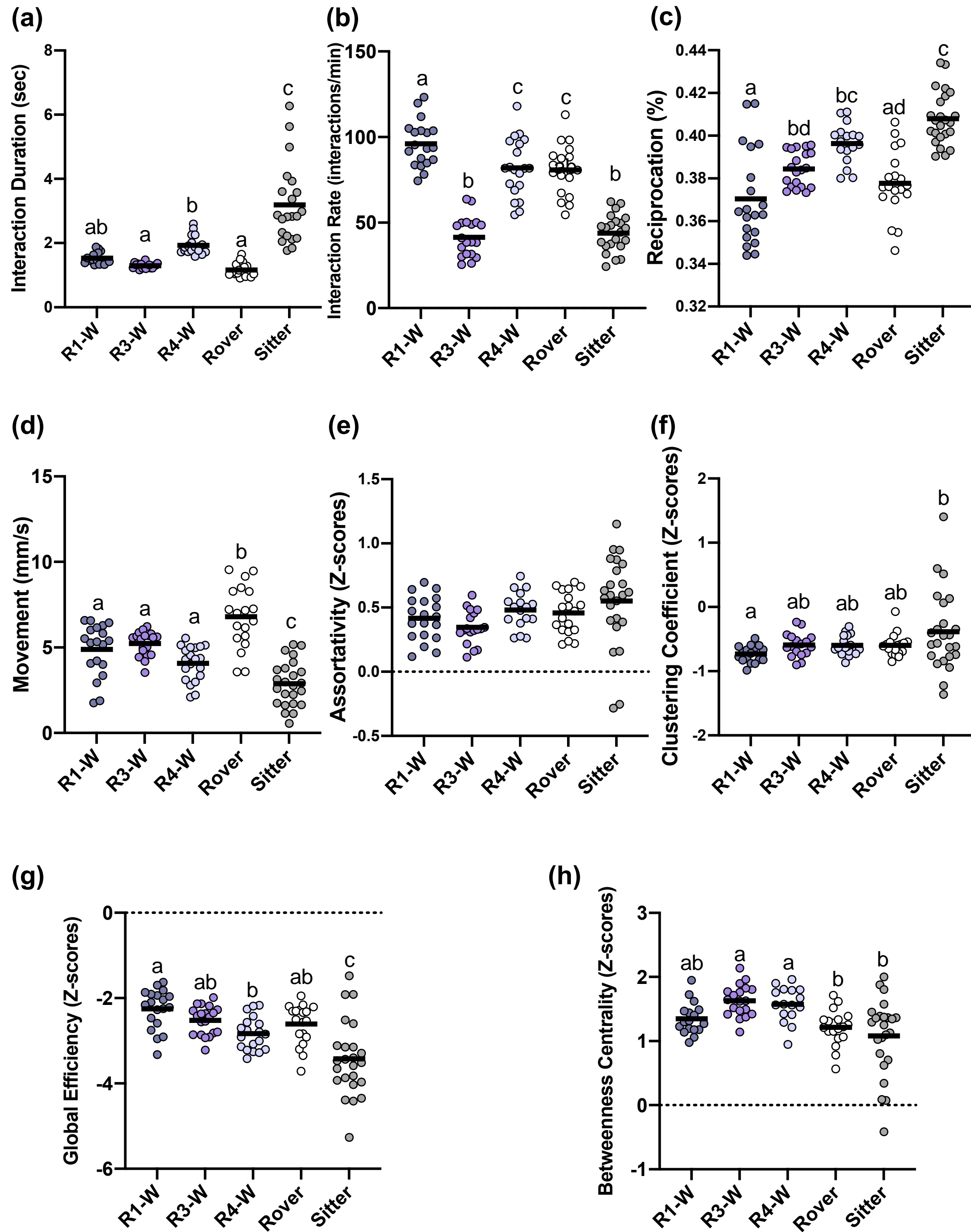


Figure A6. Rover-sitter allelic variants, social behaviour and social networks (White mutant lines). (a) Average duration of an interaction was highest in networks of sitter flies

($F_{(4,86)}$ = 34.93) ($p \leq 0.0001$) (R1-W n = 17, R3-W n = 17, R4-W n = 18, rover n = 18, sitter n = 21). (b) R1-W flies had significantly higher rates of interactions relative to other groups tested ($F_{(4,92)}$ = 63.19) ($p \leq 0.0001$) (R1-W n = 19, R3-W n = 19, R4-W n = 20, rover n = 17, sitter n = 22). (c) Sitter and R4-W flies had higher reciprocity percentage ($F_{(4,95)}$ = 22.92) ($p \leq 0.0001$) (R1-W n = 20, R3-W n = 20, R4-W n = 17, rover n = 19, sitter n = 24). (d) Rovers moved significantly more during the trial compared to other groups tested ($F_{(4,96)}$ = 24.97) ($p \leq 0.0001$) (R1-W n = 19, R3-W n = 19, R4-W n = 20, rover n = 19, sitter n = 24). (e) No significant differences in assortativity were found ($F_{(4,92)}$ = 2.45) ($p = 0.052$) (R1-W n = 19, R3-W n = 18, R4-W n = 17, rover n = 19, sitter n = 24). (f) No significant differences in clustering coefficient values were found between the three transgenic lines ($F_{(4,90)}$ = 2.78) ($p \leq 0.05$) (R1-W n = 17, R3-W n = 17, R4-W n = 18, rover n = 18, sitter n = 24). (g) Global Efficiency values were significantly lower in sitter flies ($F_{(2,60)}$ = 7.09) ($p \leq 0.01$) (R1-W n = 19, R3-W n = 20, R4-W n = 19, rover n = 19, sitter n = 24). (h) Betweenness centrality values of transgenic lines did not differ ($F_{(4,90)}$ = 7.76) ($p \leq 0.0001$) (R1-W n = 17, R3-W n = 19, R4-W n = 16, rover n = 19, sitter n = 24). *a-h* were analyzed with one-way ANOVA to determine if statistical differences exist between the groups. The Tukey-Kramer method was used as a post-hoc test (Methods). Outliers were removed from all the datasets. Bars indicate mean. Letters indicate statistical significance. *e-h* Measurements were standardized using z-scores.

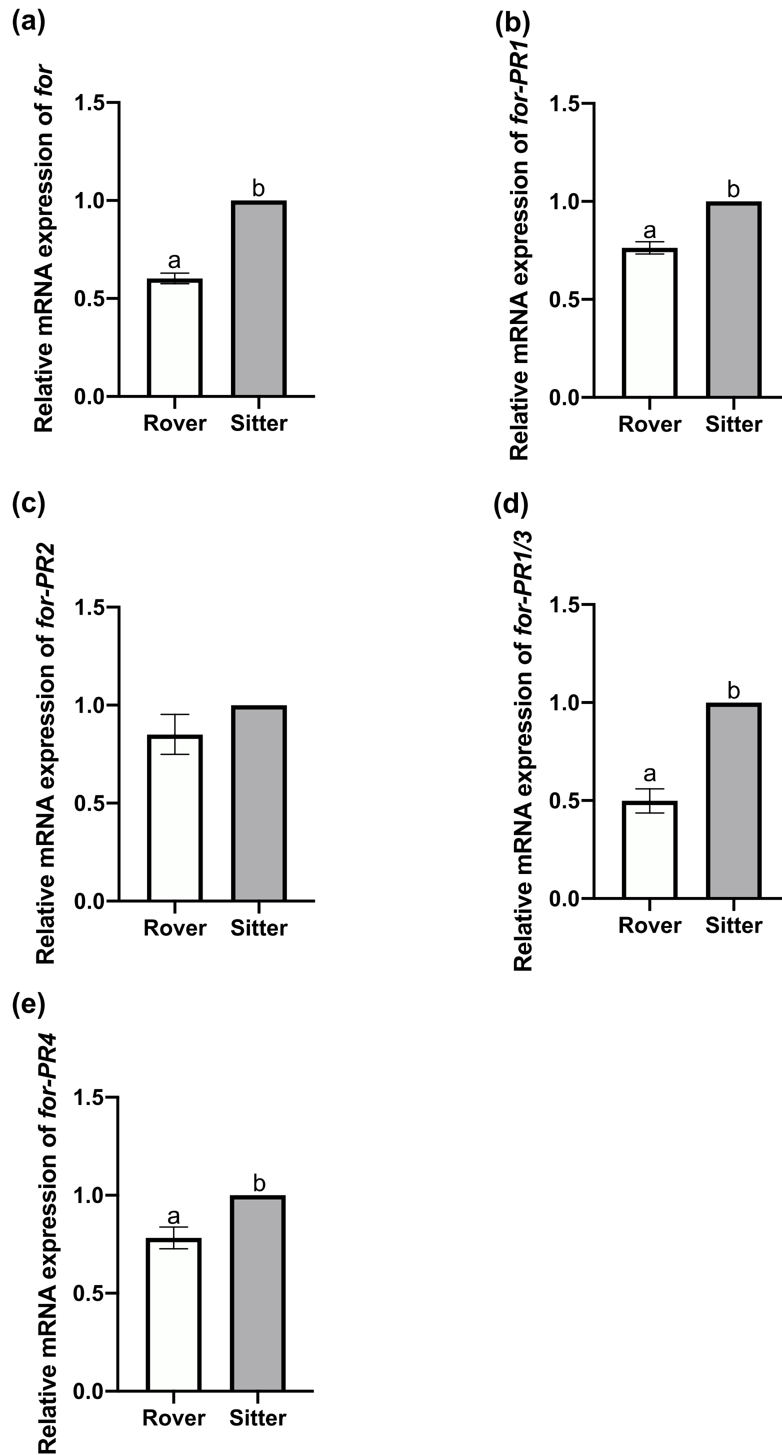


Figure A7. *for* gene expression in whole adult flies of *D. melanogaster* quantified using RT-qPCR. (a) RT-qPCR results *for* products driven by *for*-common promoter differed between rover and sitter strains with sitter having significantly higher expression (n = 7 for RT-qPCR) (t =

14.5) (df = 12) ($p \leq 0.0001$). All ΔΔCqs were calculated relative to mean ΔCq value of sitter. (b) Rover had lower expression of *for*-PR1 compared to sitter expression (n=8 for RT-qPCR) (t = 7.59) (df = 14) ($p \leq 0.0001$). All ΔΔCqs were calculated relative to mean ΔCq value of sitter control. (c) Rover and sitter did not differ in *for*-PR2 expression (n = 7 for RT-qPCR) (t = 1.46) (df = 12) ($p = 0.1706$). All ΔΔCqs were calculated relative to mean ΔCq value of sitter. (d) RT-qPCR results of rover and sitter strains showed differences in *for*-PR1/3 expression, with the sitter strain showing higher expression (n = 9 for RT-qPCR) (t = 8.08) (df = 16) ($p \leq 0.0001$). All ΔΔCqs were calculated relative to mean ΔCq value of sitter. (e) Sitter showed higher expression of *for*-PR4 compared to that of rover (n = 8 for RT-qPCR) (t = 3.92) (df = 14) ($p \leq 0.01$). All ΔΔCqs were calculated relative to mean ΔCq value of sitter.

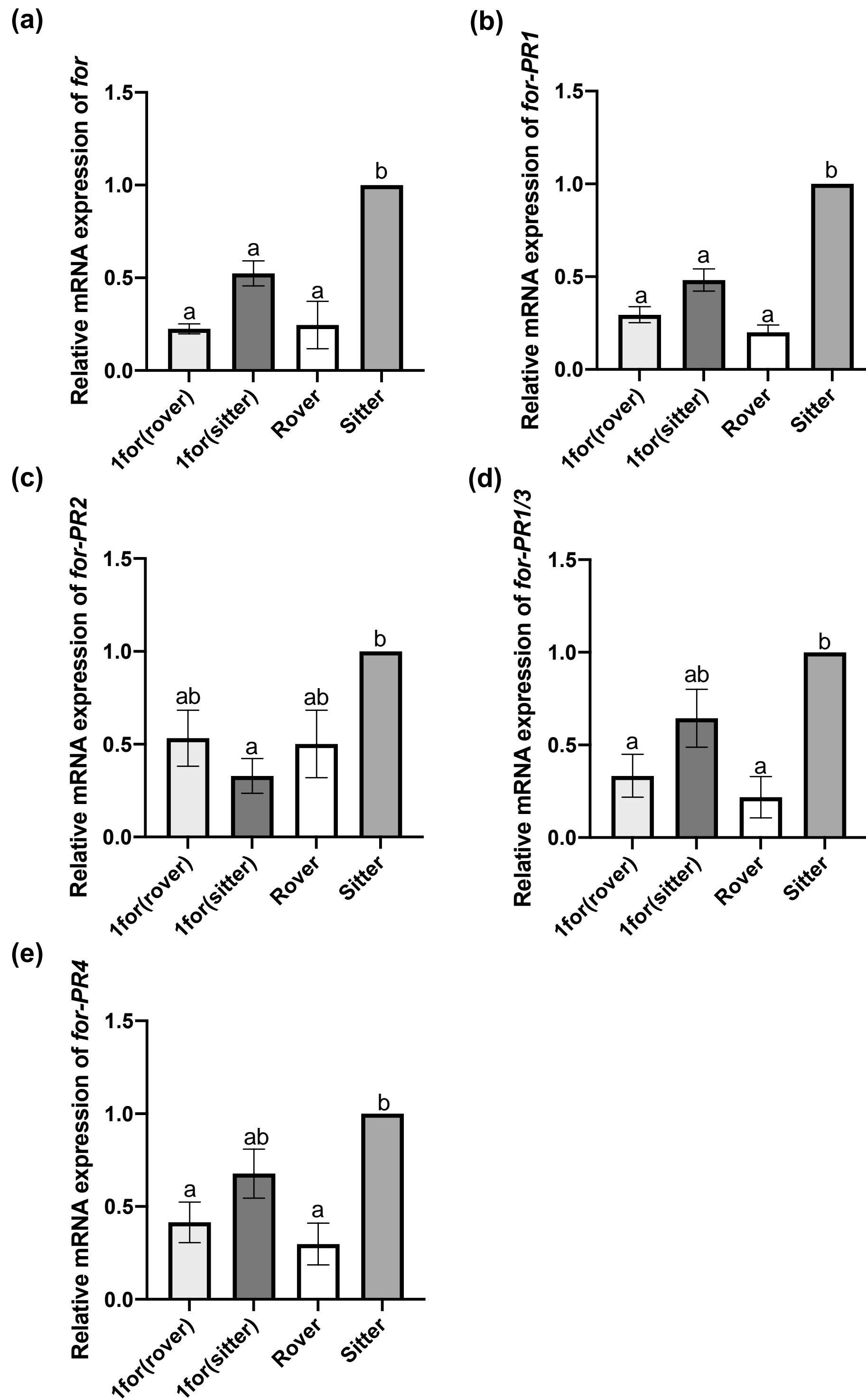


Figure A8. *for* gene expression in whole adult flies of *D. melanogaster* quantified using RT-qPCR. (a) RT-qPCR results *for* products driven by *for*-common promoter differed with sitter flies having significantly higher expression ($F_{(3,8)} = 24.27$) ($p \leq 0.001$) (n = 3 for RT-qPCR). All

ΔΔCqs were calculated relative to mean ΔCq value of sitter. (b) Sitters also have higher *for*-PR1 mRNA expression relative to other groups ($F_{(3,8)} = 72.74$) ($p \leq 0.0001$) (n = 3 for RT-qPCR). All ΔΔCqs were calculated relative to mean ΔCq value of sitter control. (c) *1for*(rover) and *1for*(sitter) strains did not differ in *for*-PR2 expression, neither did rover and sitters ($F_{(3,8)} = 5.11$) ($p \leq 0.05$) (n = 3 for RT-qPCR). All ΔΔCqs were calculated relative to mean ΔCq value of sitter. (d) RT-qPCR results of rover and sitter strains showed significant differences in *for*-PR1/3 expression between rovers and sitters but not between *1for*(rover) and *1for*(sitter) lines ($F_{(3,8)} = 9.76$) ($p \leq 0.01$) (n = 3 for RT-qPCR). All ΔΔCqs were calculated relative to mean ΔCq value of sitter. (e) *1for*(rover) and *1for*(sitter) lines did not differ in *for*-PR4 mRNA expression but significantly differed between rovers and sitters ($F_{(3,8)} = 9.24$) ($p \leq 0.01$) (n = 3 for RT-qPCR). All ΔΔCqs were calculated relative to mean ΔCq value of sitter.

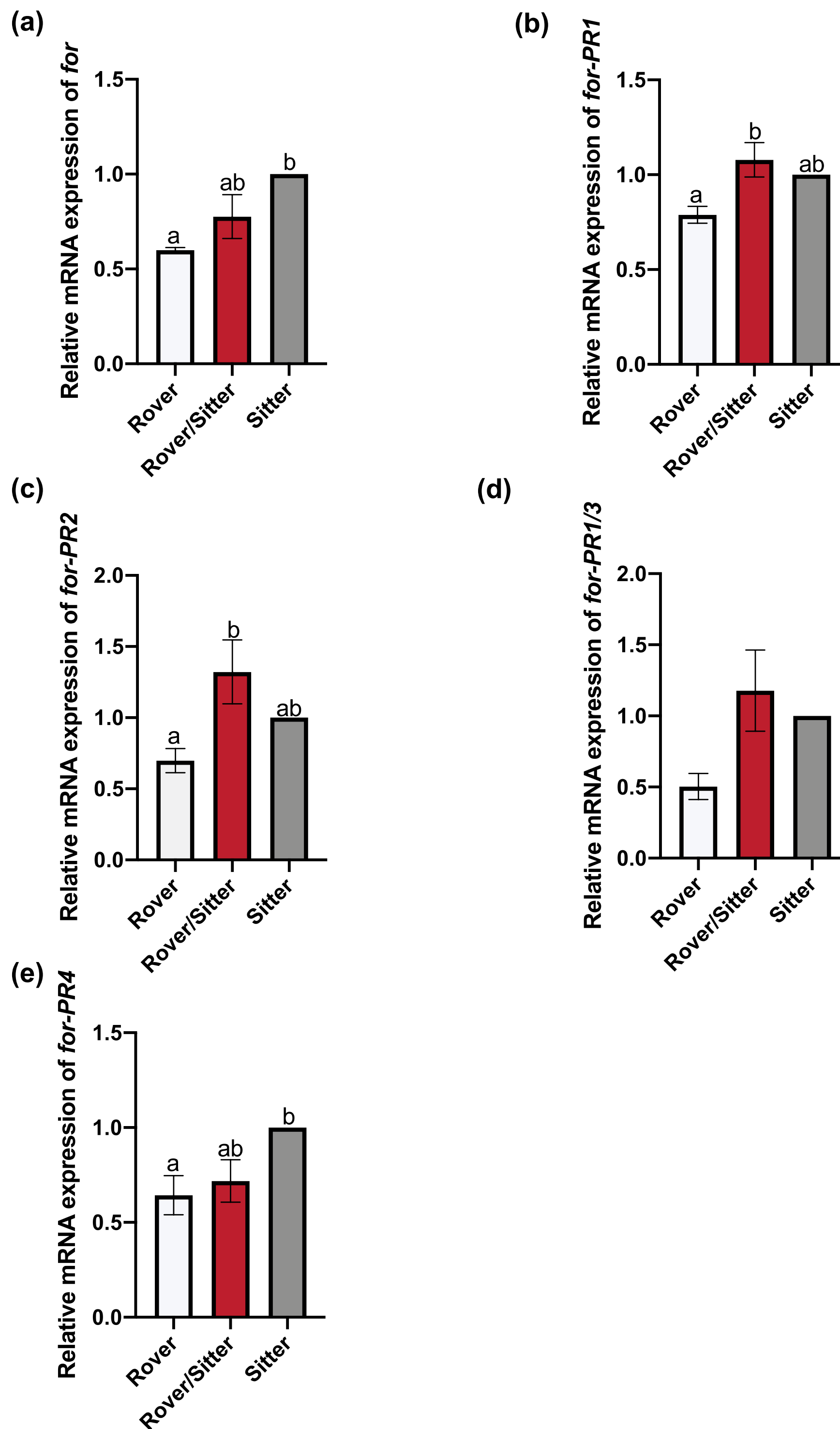


Figure A9. Heterozygotes *for* gene expression in whole adult flies of *D. melanogaster* quantified using RT- qPCR using different primers to drive their respective products. (a) Rover/sitter heterozygote flies showed intermediate expression of *for,* with sitter homozygote controls having

the highest levels and rover homozygote controls having the lowest levels of expression ($F_{(2,11)}$ = 7.38) ($p \leq 0.01$) (n = 5 for RT-qPCR). (b) Homozygote rover flies had the lowest level of expression of *for-PR1* and rover/sitter heterozygote the highest ($F_{(2,12)}$ = 6.61) ($p \leq 0.05$) (n = 5 for RT-qPCR). (c) Expression levels of rover/sitter heterozygote did not differ from homozygote sitter control highest ($F_{(2,9)}$ = 5.08) ($p \leq 0.05$) (n = 4 for RT-qPCR). (d) There were no differences in expression of *for-PR1/3* between the three lines ($F_{(2,12)}$ = 2.60) (p = 0.1156) (n = 5 for RT-qPCR). (e) Rover/sitter heterozygote had intermediate expression values compared to its homozygote controls, with rover having the lowest expression levels and sitter the highest ($F_{(2,14)}$ = 5.13) ($p \leq 0.05$) (n = 6 for RT-qPCR). All ΔΔCqs were calculated relative to mean ΔCq value of sitter. This was analyzed using One-way ANOVA followed by the Tukey-Kramer method as the post-hoc test (*Methods)*. Outliers were removed from dataset. Error bars indicate mean ± SE. Letters indicate statistical significance.

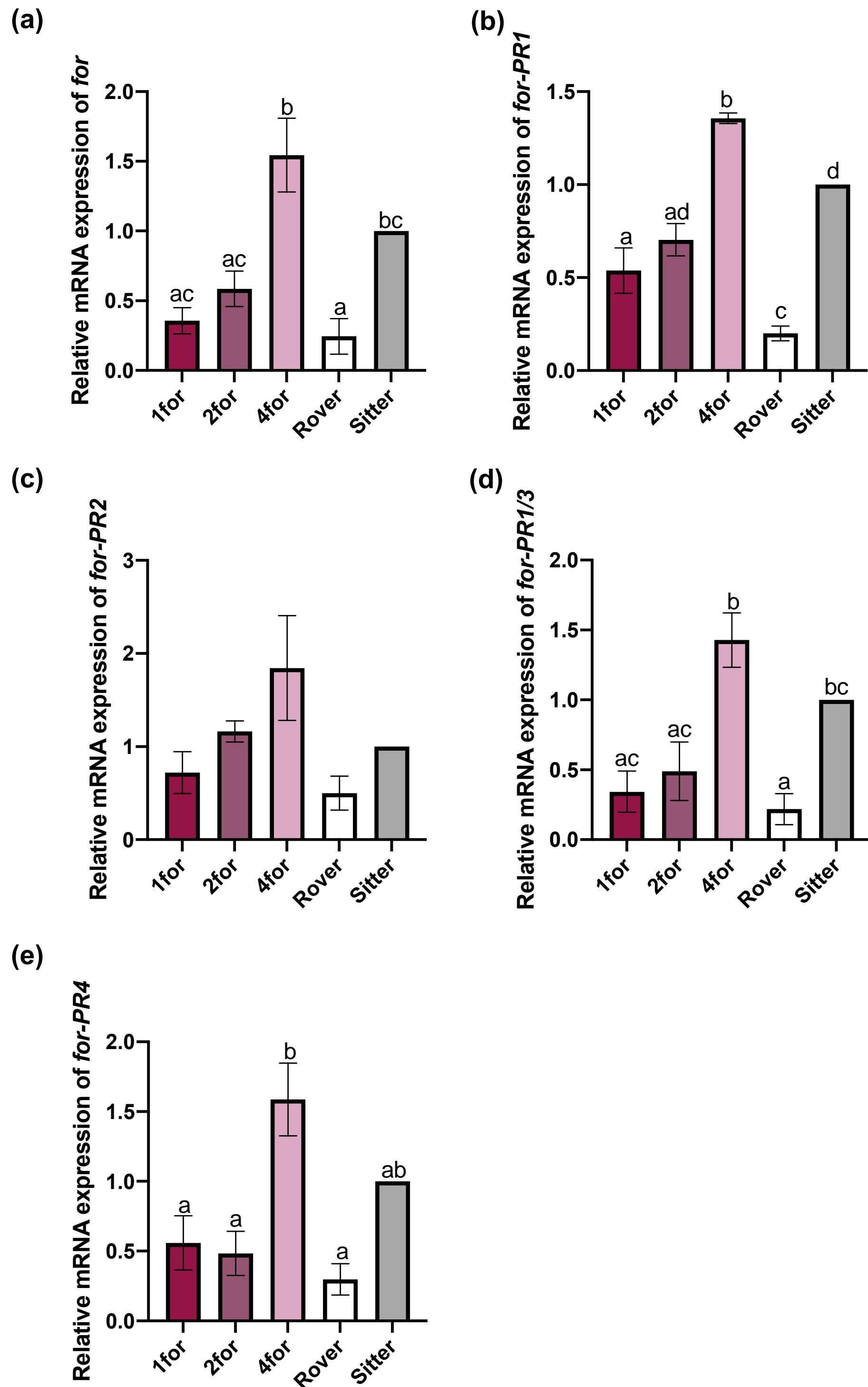


Figure A10. Dosage *for* gene expression in whole adult flies of *D. melanogaster* quantified using RT- qPCR using different primers to drive their respective products. (a) Dosage effect of *for* is reflected in expression levels of *for,* with *4for* line having the highest levels of *for*

expression ($F_{(4,10)} = 12.74$) ($p \leq 0.001$) (n=3 for RT-qPCR). (b) Expression levels of *for-PR1* product is the highest in *4for*, dosage effect of the lines is also observed here ($F_{(4,10)} = 39.23$) ($p \leq 0.0001$) (n=3 for RT-qPCR). (c) Expression levels of *2for* line was intermediate between *1for* and *4for* but no significant differences were found across groups ($F_{(4,10)} = 3.19$) ($p = 0.0623$) (n=3 for RT-qPCR). (d) Expression levels of *for-PR1/3* was highest in *4for* line ($F_{(4,10)} = 11.09$) ($p \leq 0.01$) (n=3 for RT-qPCR). (e) Expression levels of *for-PR4* for the *4for* were the highest ($F_{(4,10)} = 9.28$) ($p \leq 0.01$) (n=3 for RT-qPCR). All ΔΔCqs were calculated relative to mean ΔCq value of sitter. This was analyzed using One-way ANOVA followed by the Tukey-Kramer method as the post-hoc test (*Methods)*. Outliers were removed from dataset. Error bars indicate mean ± SE. Letters indicate statistical significance.

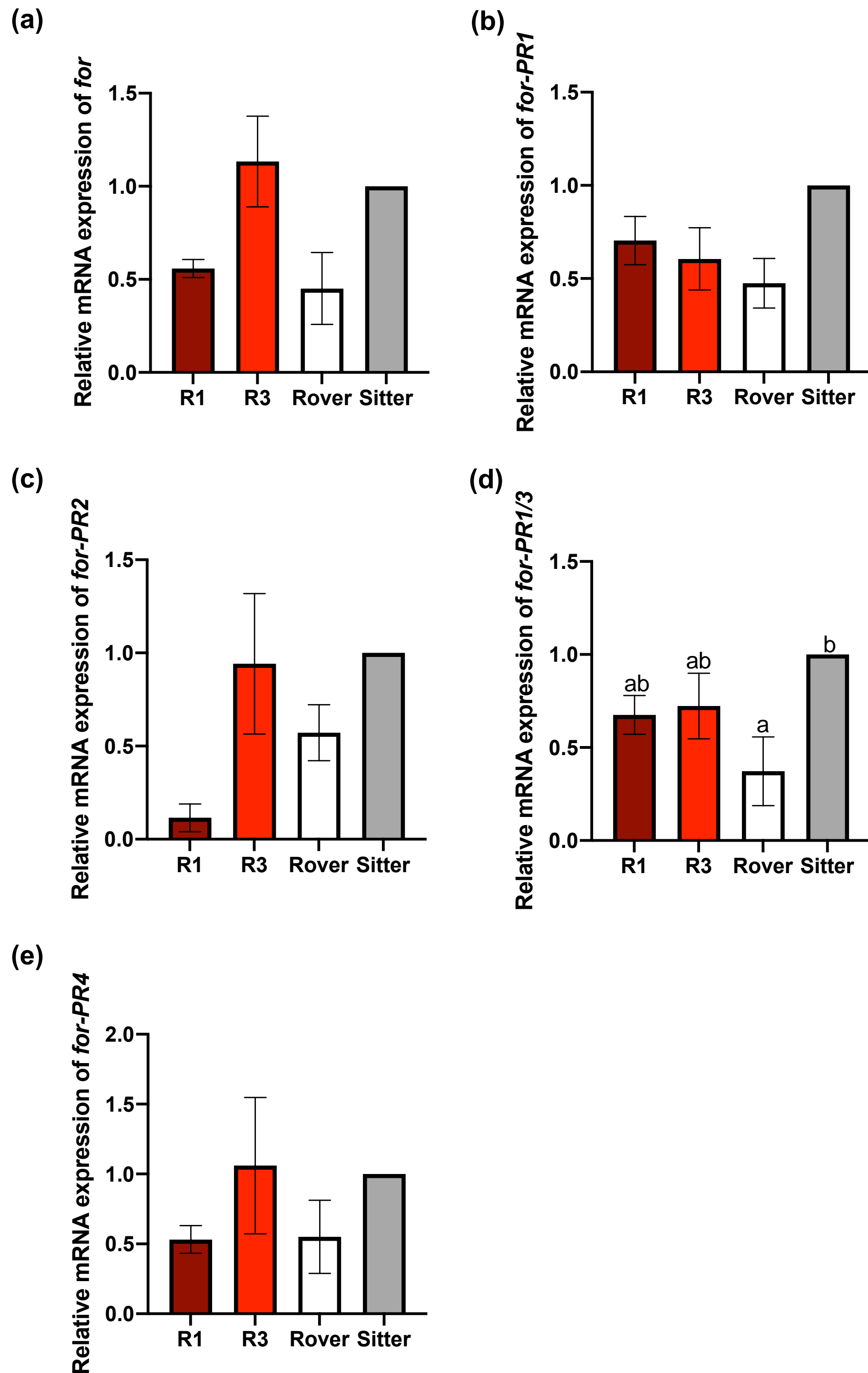


Figure A11. *for* gene expression of rover-sitter allelic variants in whole adult flies of *D. melanogaster* quantified using RT- qPCR using different primers to drive their respective products. (a) No significant differences in *for*-common mRNA expression were found between

the four groups tested ($F_{(3,8)} = 4.42$) ($p = 0.0547$) (n=3 for RT-qPCR). (b) Expression levels of *for-PR1* product is the higher in sitter flies, but these differences are not significant ($F_{(3,12)} = 3.19$) ($p = 0.0628$) (n=4 for RT-qPCR). (c) Expression levels of *for*-PR2 was lowest in R1 line, but no significant differences were found across groups ($F_{(3,11)} = 3.081$) ($p = 0.0723$) (n = 4 for RT-qPCR). (d) Expression levels of *for-PR1/3* was highest in sitter line ($F_{(3,12)} = 3.49$) ($p \leq 0.05$) (n=4 for RT-qPCR). (e) Expression levels of *for-PR4* did not differ across lines tested ($F_{(3,12)} = 1.02$) ($p = 0.4169$) (n=4 for RT-qPCR). All $\Delta\Delta$Cqs were calculated relative to mean ΔCq value of sitter. This was analyzed using One-way ANOVA followed by the Tukey-Kramer method as the post-hoc test (*Methods)*. Outliers were removed from dataset. Error bars indicate mean ± SE. Letters indicate statistical significance.

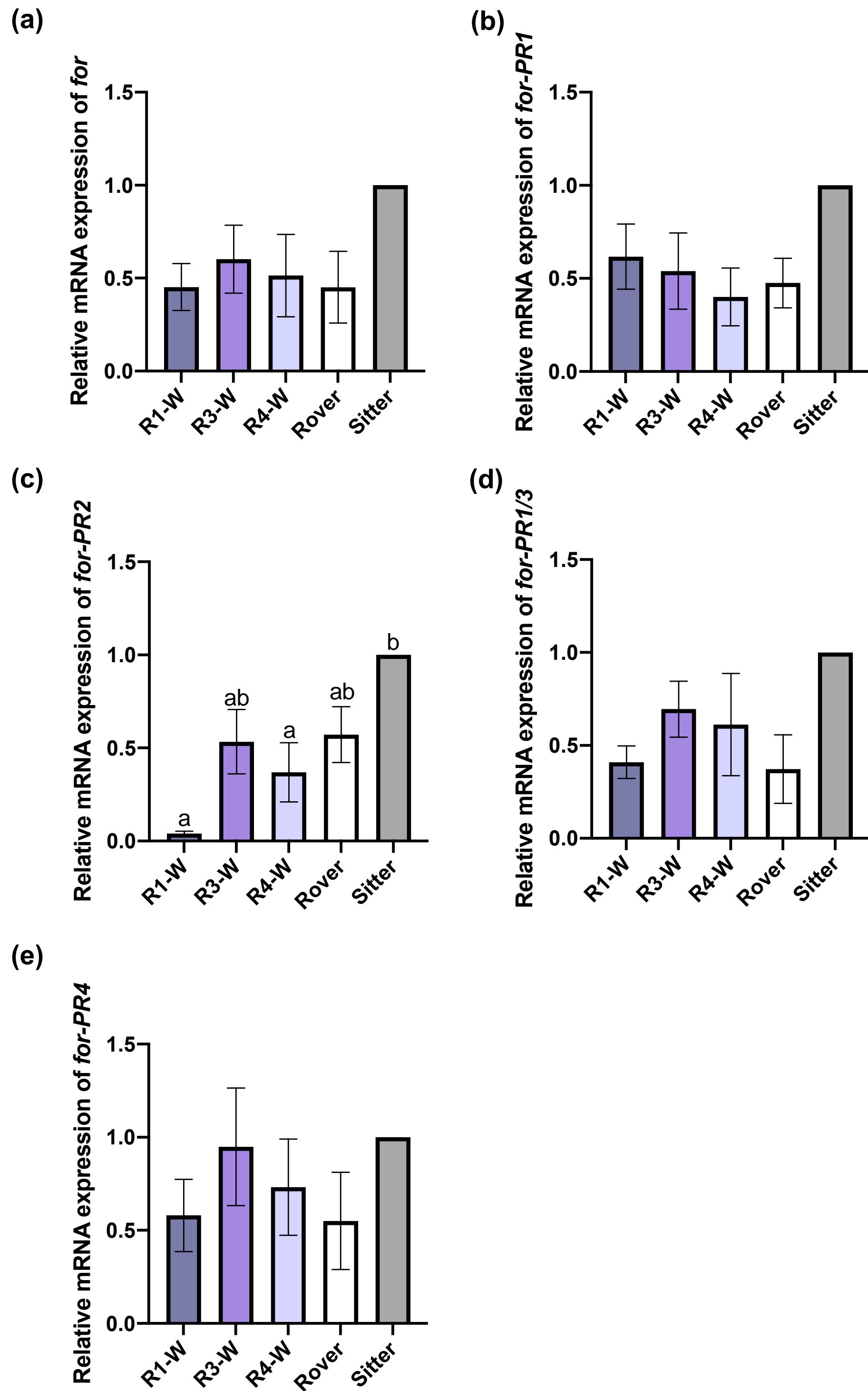


Figure A12. *for* gene expression of rover-sitter allelic variants (white mutant) in whole adult flies of *D. melanogaster* quantified using RT- qPCR using different primers to drive their respective products. (a) No significant differences in *for*-common mRNA expression were found

between the five groups tested ($F_{(4,10)} = 1.95$) ($p = 0.1785$) (n = 3 for RT-qPCR). (b) No significant differences in *for*-common mRNA expression were found between the five groups tested ($F_{(4,15)} = 2.40$) ($p = 0.0960$) (n = 4 for RT-qPCR). (c) Expression levels of *for*-PR2 was highest in sitter flies and lowest in R1-W flies ($F_{(4,14)} = 6.43$) ($p \leq 0.01$) (n = 4 for RT-qPCR). (d) Expression levels of *for-PR1/3* was higher in sitter flies but no significant differences were found across groups ($F_{(4,10)} = 11.09$) ($p \leq 0.01$) (n = 3 for RT-qPCR). (e) Expression levels of *for-PR4* did not differ across groups ($F_{(4,15)} = 0.78$) ($p = 0.5560$) (n = 4 for RT-qPCR). All ΔΔCqs were calculated relative to mean ΔCq value of sitter. This was analyzed using One-way ANOVA followed by the Tukey-Kramer method as the post-hoc test (*Methods)*. Outliers were removed from dataset. Error bars indicate mean ± SE. Letters indicate statistical significance.

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