THE GENETIC BASIS OF NATURAL VARIATION OF SOCIABILITY IN FRUIT FLIES

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Lay Abstract

Sociability is an individual's tendency to do friendly activities with others. The genetic basis is not clear. To address this gap, we silenced genes that were identified in previous work. We predicted that silencing these genes will alter sociability and used circular arenas to measure it. We found that 10 out of 20 genes affected sociability. Future research includes validating a second set of genes, investigating other social behaviours for verified genes, and to test verified genes in mice.

Abstract

Sociability is defined as the tendency of conspecifics to do non-aggressive activities with each other. In many species, being in a group increases fitness, making it highly relevant to understand. Fruit flies (*Drosophila melanogaster*) are an ideal model system to study sociability because of their complex social lives. A previous artificial selection experiment created evolved lineages of low and high sociability. Extraction, sequencing, and follow up genomic analyses of these lineages allowed us to identify candidate sociability genes. However, a causal link between genes and sociability has yet to be identified. The goal of this thesis was to functionally validate the effect of these genes. We used RNA Interference (RNAi) to knock down genes and measure subsequent changes in sociability between knockdown and control flies. Our predictions were based on the differential expression of each gene: We predicted that genes with lower expression in the low sociability lineages compared to the control lineage would have lower sociability scores than controls, and vice versa. We used circular 3D printed circular arenas like the ones in Scott et al., (2022) to measure sociability. We successfully verified 10 out of the 20 genes we tested. *Sec5*, *CG13197*, *Ir94D*, and *Est-P* altered sociability in the predicted direction. We also found that *thoc5*, *CG8329*, *DJ-1α, Net-A*, *FBgn0033353*, and *ppk28* also affected sociability, but in the opposite than predicted direction. Future work entails validating a second set of candidate genes that were identified based on population genomic work, investigating other social behaviours in some verified genes, and testing orthologs of verified genes in mice to understand sociability from an evolutionary perspective across various species.

Acknowledgements

First, I'd like to thank my supervisor, Dr. Reuven Dukas, who has taught me more about research and science than anyone else. Thank you for being efficient, reachable, and hands-on. I'd also like to thank my co-supervisor, Dr. Ian Dworkin, who helped me with genetics and statistics, all while keeping a positive vibe. I'd like to thank my committee member, Dr. Katrina Choe, for all her support throughout my degree.

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Thank you to my family, there's like 8 of you so I'll just say that you all had your own part in making this journey easier for me. Thanks to my cats (Baggy and Toothless). My "coming home" routine with Toothless at the end of a long day kept me going. Thank you to Bak Feliz, for making me laugh at anything and everything. I could write a whole second thesis about how big of a role you play in my life.

Finally, thank you to my fiancé, Aamir. Thanks for listening to all my presentations before I had to present them, for letting me talk your ear off about fly care, and for still wanting to marry me after all of it.

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LIST OF ALL ABBREVIATIONS AND SYMBOLS

RNAi – RNA Interference

siRNA – small interfering RNA

ASD – autism spectrum disorder

RT – qPCR – Real Time Quantitative Reverse Transcription Polymerase Chain Reaction

GWAS – Genome Wide Association Study

DECLARATION OF ACADEMIC ACHIEVEMENT

I, Dania Daanish, was responsible for the regular maintenance and crosses of the transgenic fruit flies, the majority of the behavioural work, and the academic writing. Dr. Reuven Dukas gave significant input on the experimental design, circular sociability arenas, and academic writing. Dr. Ian Dworkin provided input on the genetic aspect of this experiment, all the statistical analyses, and feedback on the academic writing. Dr Katrina Choe provided feedback with academic writing. Undergraduate students Andrew Roth, Brooke Luo, Sana Abdullah, Caleigh Kloppenburg, Victoria Ricardo, and Vajran Sugunanavalan assisted with the set up and observations for sociability testing.

1 | Introduction

1.1 | Defining sociability

Sociability is defined as the tendency to engage in non-aggressive activities with conspecifics (Scott et al., 2022). Examples include traveling in a group, communal sleeping, and foraging together (Scott et al., 2018). Sociability is ubiquitous, and many animals exhibit it in some form. Honeybees (*Apis mellifera*) show cooperation in nest building and brood rearing (Zayed & Robinson, 2012). We also see aspects of sociability in many species of birds (Emery et al., 2007; Skandrani et al., 2017). Flocks of European starlings *(Sturnus vulgaris)* travel together as they fly in formation before settling down for the night (Feare, 1984). Other commonly studied social animals are baboons (*Papio cynocephalus*) (Silk et al., 2003), naked mole rats (*Heterocephalus glaber*) (Jarvis 1981), and ants (Gordon, 2018)

1.2 | The costs and benefits of sociability

There are many benefits to being social. Being in a social group assists individuals in foraging efficiency (Hoelzel, 1993), protection against predators (Ebensperger & Wallem, 2002), mating success (Parrish, 1995),overall survival (Tung et al., 2023), and lower stress levels (Sharp et al., 2003; Liu et al., 2013; DeVries et al., 2003; Engh et al., 2006). Sociality of wild savannah baboons (Papio cynocephalus) females is associated with infant survival – one aspect of lifetime fitness (Silk et al., 2003). Sociability is critical for humans as well; social isolation due to the lockdowns of the Covid-19 Pandemic increased depression and

anxiety symptoms, stress levels, and other psychological consequences (Matias et al., 2020).

Sociability also has potential costs. For example, killer whales (*Orcinus orca*) seem to benefit from cooperative food searching, but not from cooperative food capture; there is a negative correlation between group size and feeding behaviours (Hoelzel, 1993). Being in a group may increase competition for resources (Koenig, 1999). Overall, sociability in highly relevant for humans and most non-human animals, and therefore vital to understand.

1.2 | Fruit flies as a model system

Fruit flies (*Drosophila melanogaster*) make an ideal model system to study sociability. While they are traditionally thought of as a solitary species, fruit flies show many examples of social behaviours. Both adults and larvae tend to aggregate in both laboratory settings (Saltz & Foley, 2011; Scott et al., 2018, 2021; Bentzur et al., 2021) and in the field (Dukas, 2020; Wertheim et al., 2002); Wertheim et al., 2006) We see evidence of social learning (Durisko & Dukas, 2013; Schneider et al., 2012; Sarin & Dukas, 2009), as well as collective responses to danger (Ferreira & Moita, 2020; Ramdya et al., 2015).

From a fitness perspective, it is quite advantageous for fruit flies to be social. For example, flies that were kept in social isolation had significant decreases of fibers in the mushroom body (Technau, 2007), a structure implicated in associative learning, olfactory learning, habituation, temperature regulation and sleep (Aso et al., 2014). Social isolation also leads to changes in courtship and courtship memory, chemical communication (Krupp

et al., 2008), and decreases in overall lifespan (Leech et al., 2017). These examples make fruit flies an ideal model to study sociability.

1.3 | Sociability assays

Sociability is measured in various ways, depending on the species. For example, in humans (*Homo sapiens*), sociability is often measured using self-report surveys (Bralten et al., 2021a). In capuchin monkeys (*Cebus capucins*), researchers measured social integration, quantified by observing interaction types such as grooming, support giving/receiving, and foraging (Kajokaite et al., 2022). In fruit flies, common ways of measuring sociability are the nearest neighbour distance (Anderson et al., 2016), triangular social space assays (Yost et al., 2020; Simon et al., 2012), and individuals choosing group preferences at food patches (Scott et al., 2018). Nearest neighbour index essentially refers to the ratio between the mean actual observed distance between two flies and the expected distance by random change (Anderson et al., 2016). The index ranges from 0-2.5, with a smaller score representing higher sociability. The caveat for the nearest neighbour index is the fact that the space between two individuals may not necessarily imply that they are being social. For example, if one fly was simply passing by another fly, and not directly interacting with them, the distance between them may be small, but it may not encapsulate sociability as we define it. In addition, the nearest neighbour index does not include whether the flies are face to face. This is critical, because flies communicate via chemosenstation. Next, triangular social space assays are also commonly used to study social behaviour in fruit flies by measuring social space within a group which uses social space – the distance between two flies (Simon

et al., 2012). The current study uses a 3D printed circular sociability arena (details in section 2.4) that capitalizes fruit flies' tendency to gather at food patches. Since this arena is divided into distinct sections, flies can choose their preferred group freely during the acclimation period.

1.4 | Understanding the evolutionary biology of sociability

Fruit flies have also been well studied from a genetics perspective, linking genes to behavioural phenotypes such as foraging (*for* gene) (Anreiter & Sokolowski, 2019). Scott et al., (2018) documented significant genetic variation in sociability in fruit flies, with a board-sense heritability of 0.24 in males and 0.21 in females. This work led to artificially selecting on low and high sociability to determine costs, benefits, and the genetic correlation of sociability with other relevant traits (Scott et al., 2022). Researchers randomly assigned flies to separate lineages: control, low, and high sociability. They measured sociability using a circular arena and chose the 4 most and 4 least sociable flies to produce the next generation. In this way, Scott et al., (2022) successfully artificially selected for sociability over 25 generations. They found significant differences in sociability between the lineages: the high lineage had 54% and 40% higher sociability than the low lineage in male and females, respectively.

Torabi-Marashi (2023) extracted and sequenced DNA and RNA from these lineages to investigate the genetic architecture of sociability; he wanted to identify genes that were correlated with the differences in sociability between each lineage, and therefore contributing to the phenotypic variation of sociability. He was able to identify a set of genes

that were differentially expressed between the low and high sociability lineages using RNA sequencing. He also found a second set of genes that differed in their allelic frequencies between the low and high sociability lineages using DNA sequencing and a population genomics approach. Overall, Torabi-Marashi (2023) was able to identify two sets of genes, with either differences in expression or allelic frequency between the lineages.

1.5 | Functionally validating candidate sociability genes

These candidate genes were based on computational analysis and are correlated with sociability. However, a causal link is yet to be identified between the candidate genes and sociability. Our goal was to functionally validate the candidate genes that significantly differed in their expression between the lineages. Behaviour can be shaped by genetic variation, genetic drift, and selection (Niepoth & Bendesky, 2020) – using a controlled laboratory environment, robust genetic approaches, and fruit flies as a model system, can help us create this causal link between genes and sociability

We predicted that knocking down the candidate genes will alter sociability. The specific prediction for each candidate gene is dependent on its respective differential expression (Fig 1). We predicted that candidate genes with higher expression in the low sociability lineage, compared to controls and high- lineages, will have higher sociability scores when knocked down. Similarly, we predicted that candidate genes with lower expression in the low lineage, compared to controls and high- lineages, will have lower sociability scores when knocked down.

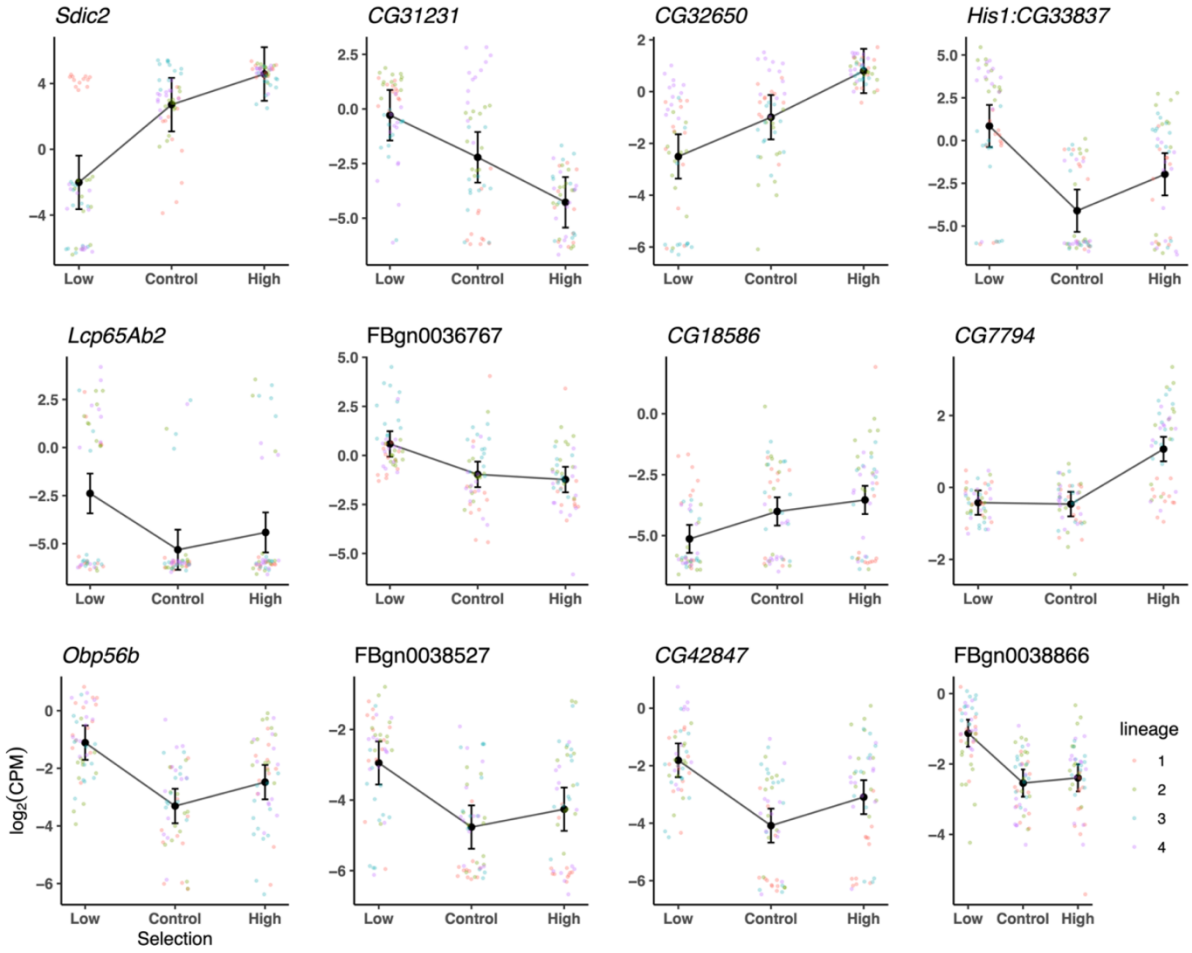

Figure 1. Differential expression graphs of top candidate genes. Each plot shows fitted gene expression in log2(cpm) as obtained by emmeans with their 95% confidence interval. The individual points indicate the log2(cpm) of each sample, where the 4 colours are the 4 lineages of each treatment. Figures created by Arteen Torabi-Marashi for Torabi-Marashi (2023).

2 | Methods

2.1 | Drosophila melanogaster lines and maintenance

We chose 20 candidate genes to test for sociability. We picked genes using two criteria: 1) highest differential expression between low-, high- and control sociability lineages (Torabi-Morashi, 2023), and 2) availability of RNAi lines. We ordered all lines from either Bloomington Drosophila Stock Center (BDSC) or Vienna Drosophila Resource Center (VDRC) (Tables 1.1 and 1.2). RNAi lines from BDSC were from the Transgenic RNAi Project (TRiP) collection and RNAi lines from VDRC were from the KK library, except for VDRC # 25725 which was from the GD library. Some of the lines contained a balancer in which case we only used focal flies without markers (e.g. CyO, Sb). All lines were maintained at 25^{o} C and ~60% RH on standard food (1 L standard food = 900ml water, 90g sucrose, 75g cornmeal, 10 g agar, 32 g yeast, and 2 g methyl paraben dissolved in 20 mL ethanol).

2.2 | The GAL4-UAS system

We silenced the expression of the candidate genes to test their effect on sociability using the GAL4/UAS System and RNAi constructs of each Gene-of Interest (GOI). The GAL4 is a protein that can be put under many types of promoters allowing expression anywhere in the fly (Duffy, 2002). We used a general nervous system GAL4 or elav-GAL4 which is expressed in the brain. An Upstream Activator Sequence (UAS) is a construct whereby a GOI can be placed downstream of it. When one fly with the GAL4 protein is crossed to another fly carrying an Upstream Activator Sequence (UAS) construct, the progeny will

express whatever GOI was placed downstream of the UAS. We used UAS-RNAi lines, where an RNAi construct was placed downstream of a UAS (Del Valle Rodríguez et al., 2012; Duffy, 2002); in this way, we were able to reduce the expression of candidate genes in progeny.

We obtained two elav-GAL4 lines. The first one is the elav-GAL4 with a UAS-dcr2 (BDSC# 25750). This line contains Dicer2 which enhances the effectiveness of RNAi because Dicer can generate siRNAs (small interfering RNA) from the GAL4 mRNA (Bloomington Drosophila Stock Center, 2021). In this way, Dicer2 can increase the efficiency at which mRNA is translated. This stability and efficiency of the GAL4 mRNA results in more production of the GAL4 protein which can then bind to the protomer of the GOI and activate transcription with greater magnitude. Therefore, crossing an RNAi GOI to an elav-GAL4 with a UAS-dcr2 results in a strong silencing effect of the target gene (Bloomington Drosophila Stock Center, 2021). Dicer2 is on the X chromosome, so only females can be used. In some cases, Dicer2 can lead to off-target effects where degradation of unintended mRNA occurs, which may cause deleterious effects, like offspring mortality. If this happened, we used the elav-GAL4/CyO line (BDSC# 8765). This line contains CyO balancer, which carries a visible genetic marker, CyO (curly wings) (Bloomington Drosophila Stock Center, 2021). Offspring of this line will either have the GAL4 or the CyO mutation, and so we only tested focal flies without curly wings. The effect of the GAL4 in this line may be weaker due to the lack of Dicer2. We verified the expression of elav-GAL4 in both GAL4 lines by crossing them to a Green Fluorescent Protein (GFP) strain, which allowed us to visualize the GAL4 in the fly brain.

163 2.3 | Making the crosses

164 For each candidate gene, we made two crosses: elavGAL4/GOI-RNAi (Knockdown), elav-
165 GAL4/TRiP-Control (Control). We sexed 15 virgin females and 15 virgin males under light
166 CO2 anesthesia within 7 hours of eclosion. We placed flies in same-sex vials with 5ml of
167 standard food. We made two crosses per gene once the flies were 2-3 days old. Elav-GAL4
168 females were crossed with either GOI-RNAi males (Knockdown group) or TRiP-Control
169 males (Control group). We transferred flies into new food vials with live yeast every day
170 for 5 days. Offspring of crosses eclosed ~10 days after egg laying. The Knockdown group
171 consisted of offspring with the expression of the GOI silenced, whereas the Control group
172 consisted of the offspring without the GOI's expression reduced.

173 After crossing BDSC# 50556 males with the stronger elav-GAL4, the offspring did
174 not survive. We also tried to cross them with the weaker elav-GAL4, however the wings of
175 the offspring did not develop. To avoid any confounds involving healthy wing development
176 and sociability, we did not test this gene. We also found the same issue with VDRC#
177 101616, and VDRC# 100094, where offspring either died or had uninflated wings, and
178 therefore did not test these lines either. In total, we tested the effect of 17 genes on
179 sociability. All genes were tested using the GAL4 + Dicer2, except thoc5, which was tested
180 using GAL4/CyO.

181

182 2.4 | Testing sociability

183 We sexed groups of 8 virgin offspring from the Knockdown group and Control group and
184 placed them into same-sex and same-treatment food vials with 5 ml of standard food. We

sexed 12 sets of focal flies per sex per treatment per day, with a total of 384 flies per day. We tested sociability in focal flies once they were 3 days old.

We used 3D-printed circular arenas 50 mm wide and 6 mm high divided by thin walls into 8 compartments. Each arena consisted of a circular dish (53mm wide x 6mm high) that had 8 equally sized sections divided by thin walls with openings (6mm wide x 3.5 mm high) to allow flies to move between sections (Fig 2). The top of the arena consisted of plexiglass with a 3D printed circular edge and a 3 mm hole. We aspirated flies into the arena through the hole and then covered it with a small piece of tape. We placed a circular (7.5mm wide and ~2mm high) food patch in each compartment. We pipetted 50ul of juice solution made of 2g live yeast dissolved in 10 ml orange juice. At 8am, we placed food patches in each arena, attached the covers, and gently aspirated groups of 8 same-sex flies into each arena. We then placed the arenas inside a humidified container maintained at 25^{o} C and 50% RH. We had 12 arenas per sex per treatment, and 48 arenas in total per day. We allowed flies to settle until 2pm. Then, an observer blind to treatment scored each arena every 15 minutes by counting the number of flies in each compartment. Sociability scores were calculated using the following formula: $\frac{variance}{mean\ number\ of\ flies\ in\ each\ compartment}$ (Scott et al. 2018) for each arena. The sociability scores range from 0 (one fly per compartment) to 8 (all flies in one compartment). A lower score represents lower sociability, and a higher score represents higher sociality. We compared compare sociability scores between the knockdown group and the control group.

Observers were blind to treatment. All observers also wore white coloured clothing and/or a white lab coat to mimic the white walls of the observation rooms. Observers were

also required to wear no fragrances, avoid any sudden movements, and speak at a low volume. We completed 5 observations per arena per day, and a total of 3 test days per candidate gene. After all observations were completed, we discarded the flies, thoroughly cleaned the arenas with soap and water to remove the food patches. We also cleaned arenas with 70% ethanol and let them air dry overnight. A timeline of the entire experiment (from virgin parents, to making crosses, to testing focal offspring) is illustrated in Table 3.

2.5 | RNA extraction and RT-qPCR

While sexing offspring from each cross, we made sure to sex 2 extra sets of flies (16 total) from each treatment and sex. We flash froze these flies with liquid nitrogen which effectively broke apart the extremities of the fly, including the head, which we separated and preserved in RNA*later* Stabilizing Solution (Invitrogen, Carlsbad, CA) as per manufacturer's instructions. To extract RNA from the head samples, we first homogenized heads in 1.5 mL Eppendorf tubes using 0.5mm metal beads and the NextAdvance Bullet Blender (NextAdvance, Troy, NY, USA). We extracted and purified total RNA using a Qiagen RNeasy Mini Kit (Qiagen Science, Gemantown, MD) per the manufacturer's protocol. We checked the RNA concertation in each sample using a NanoDrop Spectrophotometer (ND 1000, Thermo Fisher). We used the iScript cDNA Synthesis Kit (Bio-Rad) to synthesize cDNA, with two biological replicates and two technical replicates/samples.

We will be using RT-qPCR analysis employing the SYBR Green method to confirm that the candidate genes were successfully reduced in expression in the knockdown group

compared to the control group. We will use Rap2l, Appl, and elF-1A as reference genes (Ling & Salvaterra, 2011).

2.6 | Statistical Analysis

For each gene, we analysed the sociability data by fitting a generalized linear mixed effects model in R v4.3.3 (R-Core-Team, 2023) with the glmmTMB package v 1.1.8 (Bolker et al., 2017). We modeled the effect of Treatment, Sex, and their interaction as fixed effect (categorical) predictors. As we conducted exploratory data analysis (EDA) plotting, we noticed that in some cases, sociability scores were affected by Time (we conducted observations 5 times per day). Therefore, we included Time as a continuous predictor in our model. The effects of (trial) Day were allowed to vary as a random effect (random intercept). Additionally, to account for among arena (subject) level variation for time, we allowed a random slope for time across Arenas (themselves nested within day). Sociability score was the response variable. Since our sociability scale is from 0-8, with no negative values, we could have issues with the mean-variance relationship as knockdown flies with lower sociability scores get pushed towards zero, thereby reducing variance. We used the Tweedie distribution (Dunn, 2022) with log link function to account for the non-normal distribution. We considered modeling the data using the Gamma distribution, given that our sociability index is positive valued and continuous. However, our sociability index scores include values of 0. Thus, a Tweedie distribution, with the power constrained ($1 < p < 2$) results in a compound Gamma-Poisson distribution allows us to use positively valued continuous data with zeros. We did use the Gamma distribution in one data set, BDSC

61998, because this data set did not contain any values of 0. All other models were fitted using the Tweedie distribution. We used the emmeans function from the emmeans R package version 1.10.0 (Lenth, 2024) to estimate contrasts of treatment effects, including sex specific treatment differences, both for treatment averaged over sex, as well as the effect of sex averaged over treatment. We reported the estimated marginal means, standard error, confidence intervals, and p-values for these comparisons. Lastly, we used the ggplot2 package (Wickham, 2016) to create all plots.

Gene	Stock Center ID	Genotype
CG13197	BDSC 39052	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01972}attP2/TM3, Sb[1]
FBgn0032436/CG5418	BDSC 58274	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMJ22357}attP40
Est-P	BDSC 55928	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMC04216}attP40
Fbgn0033353/BaraB	BDSC 61998	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMJ23624}attP40/CyO
ppk28 (Chr 3)	BDSC 31878	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02153}attP2
thoc5	BDSC 55206	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMC03921}attP40
Sec 5	BDSC 27526	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02676}attP2
CG31231	VDRC 25725	w1118; P{GD10198}v25725/TM3
CG32650	VDRC 109827	P{KK109777}VIE-260B
FBgn0038866	VDRC 105450	P{KK103323}VIE-260B
Ir94D	VDRC 330479	P{VSH330479}attP40
Dgat2	VDRC 107788	P{KK109140}VIE-260B
CG8329	VDRC 101603	P{KK104128}VIE-260B
Nmdmc	VDRC 110198	P{KK102478}VIE-260B
NetA	VDRC 108577	P{KK101369}VIE-260B
DJ-1α	VDRC 104329	P{KK107549}VIE-260B
Ir68a	VDRC 106708	P{KK103530}VIE-260B

Table 1. List of candidate genes tested, stock center IDs, and genotypes. BDSC refers to Bloomington Drosophila Stock Center. VDRC refers to Vienna Drosopshila Resource Center.

Gene	Stock Center and ID	Genotype
elav-GAL-4	BDSC 8765	P{w[+mC]=GAL4-elav.L}2/CyO
elav-GAL-4, UAS-Dicer2	BDSC 25750	P{w[+mW.hs]=GawB}elav[C155] w[1118]; P{w[+mC]=UAS-Dcr-2.D}2
TRiP Control (Chr 3-attP2)	BDSC 36303	y[1] v[1]; P{y[+t7.7]=CaryP}attP2
TRiP Control (Chr 2-attP40)	BDSC 36304	y[1] v[1]; P{y[+t7.7]=CaryP}attP40

Table 2. GAL4 and TRiP control lines used for crosses, including gene names, stock center IDs, and genotypes. BDSC 36304 was used for genes on the 2nd chromosome and BDSC 36303 was used for genes on the 3rd chromosome. BDSC refers to Bloomington Drosophila Stock Center. VDRC refers to Vienna Drosophila Resource Center.

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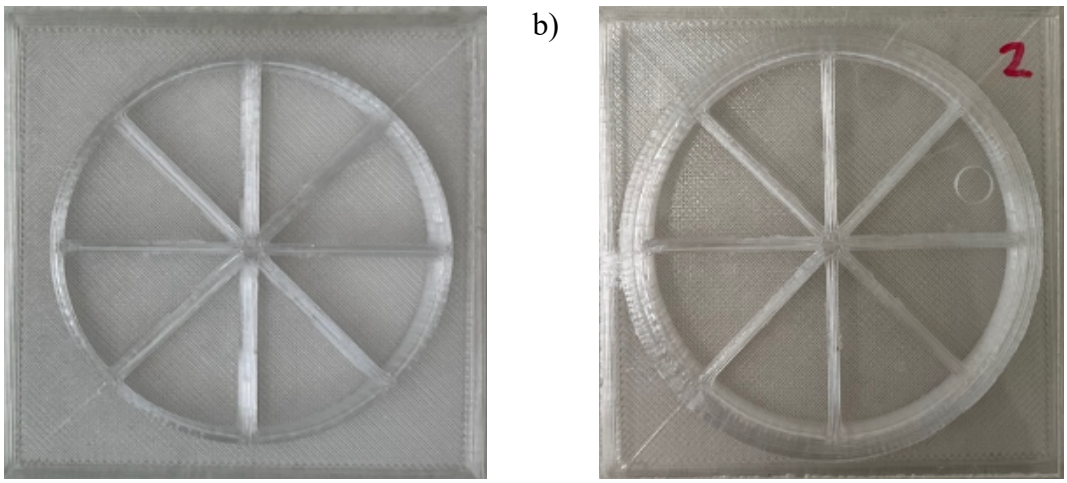
a) b)



300 **Figure 2. Sociability Arenas.** (a) Bottom half of the sociability arena. It contains 8
301 sections, each of which is divided by a thin wall with for flies to move through. (b)
302 Sociability arena with the lid, which contains a small hole from which flies are gently
303 aspirated into the arena.

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Egg laying for Parents	Wait Period	Sex Parents	Wait Period	Make Crosses + Egg laying	Egg laying	Wait Period	Sex Offspring	Test Sociability
3 days	~10 Days	1 day	2-3 days	1 day	4 days	~10 Days	3 days	3 days

Table 2. Timeline of each experiment.

3 | Results

10 of the 17 candidate genes showed significant differences in sociability (Table 4). Four of these 10 genes showed differences in sociability in the predicted direction: *Sec5*, *CG13197*, and *Ir94D* showed lower sociability scores and *Est-P* showed higher sociability scores when knocked down compared to controls (Fig. 3). *Ppk28*, *thoc5*, *FBgn0033353*, *CG8329*, *NetA*, and *DJ-1α* also showed significant differences in sociability, however, the effect of these genes occurred in the opposite direction than predicted (Table 4, Fig. 4). Some of the candidate genes also had sex specific differences. Knockdown of *ppk28*, *thoc5*, and *Nmdmc* only affected males, whereas knockdown of *CG13197*, *Est-P*, and *CG8329* only affected females (Table 4).

Gene	Stock #	Predicted effect of knockdown on sociability	Actual effect of knockdown on sociability	Estimate (Control – RNAi) ± Standard Error	p-value	Sex effect
Sec5	B 27526	Lower	Lower	0.47 ±0.08	<.0001	No
CG13197	B 39052	Lower	Lower	0.17±0.06	0.005	Yes
Ir94D	V 330479	Lower	Lower	0.16± 0.07	0.019	No
Est-P	B 55928	Higher	Higher	-0.14±0.06	0.025	Yes
thoc5	B 55206	Lower	Higher	-0.15±0.07	0.024	Yes
CG8329	V 101603	Lower	Higher	-0.14±0.07	0.032	Yes
DJ-1α	V 104329	Lower	Higher	-0.12±0.06	0.039	Yes
NetA	V 108577	Higher	Lower	0.28±0.07	0.0002	No
FBgn0033353	B 61998	Higher	Lower	0.125±0.06	0.023	Yes
ppk28	B 31878	Higher	Lower	0.19±0.78	0.017	Yes
CG32650	V 109827	Lower	Higher	-0.05±0.06	0.454	No
Ir68a	V 106708	Lower	Higher	-0.04±0.07	0.536	No
CG31231	V 25725	Higher	Lower	0.06±0.06	0.294	Yes
FBgn0038866	V 105450	Higher	Lower	0.06±0.07	0.368	No
Dgat2	V 107788	Higher	Higher	-0.10±0.07	0.1578	No
Nmdmc	V 110198	Higher	Higher	-0.15±0.08	0.055	Yes
FBgn0032436	B 58274	Higher	Higher	-0.01±0.07	0.872	No

Table 4. Effect of knockdown of GOIs on sociability scores compared to controls.

The candidate genes tested and results. In the "Stock #" column, "B" refers to Bloomington Drosophila Stock Center (BDSC), "V" refers to Vienna Drosphila Stock Center (VDRC). The Table is divided into 3 coloured categories: Knockdowns that significantly affected sociability in the predicted direction on the top (dark blue), followed by knockdowns that significantly affected sociability in the opposite than predicted direction (blue), and knockdowns with no significant effect on sociability at the bottom (light blue). The Sex effect column refers to where or not there was a sex specific effect on sociability, whereby only one sex's sociability scores were affected by knockdown: "Yes" indicates that there was a sex-effect, "No" indicates that there was not a sex-effect.

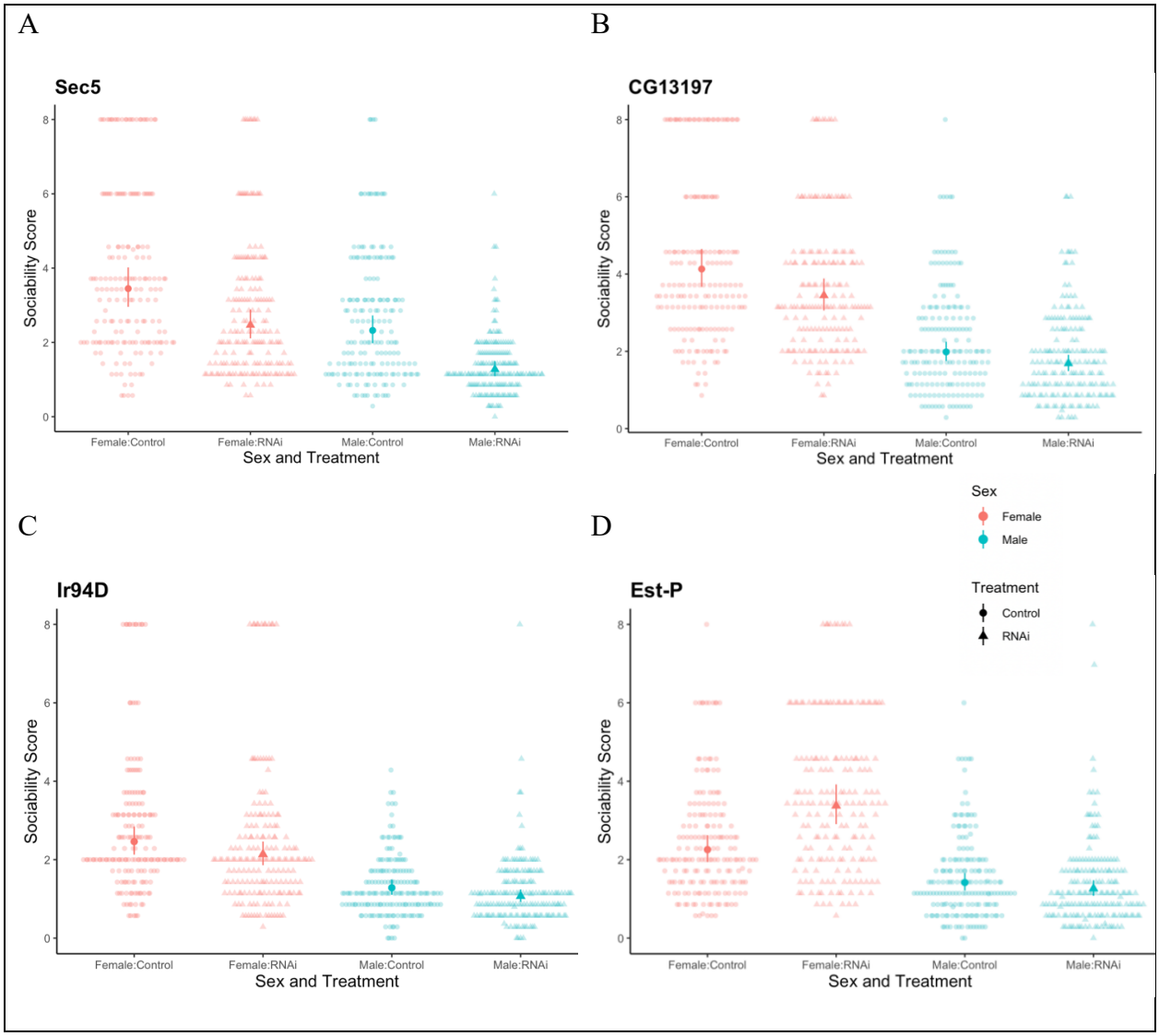

Figure 3. Effect of knockdown on sociability scores in the predicted directions. Knockdown genes that showed the predicted effect on sociability compared to controls. Plotted values represent sociability scores for each arena (n=144 arenas per gene), estimated means and 95% confidence intervals.

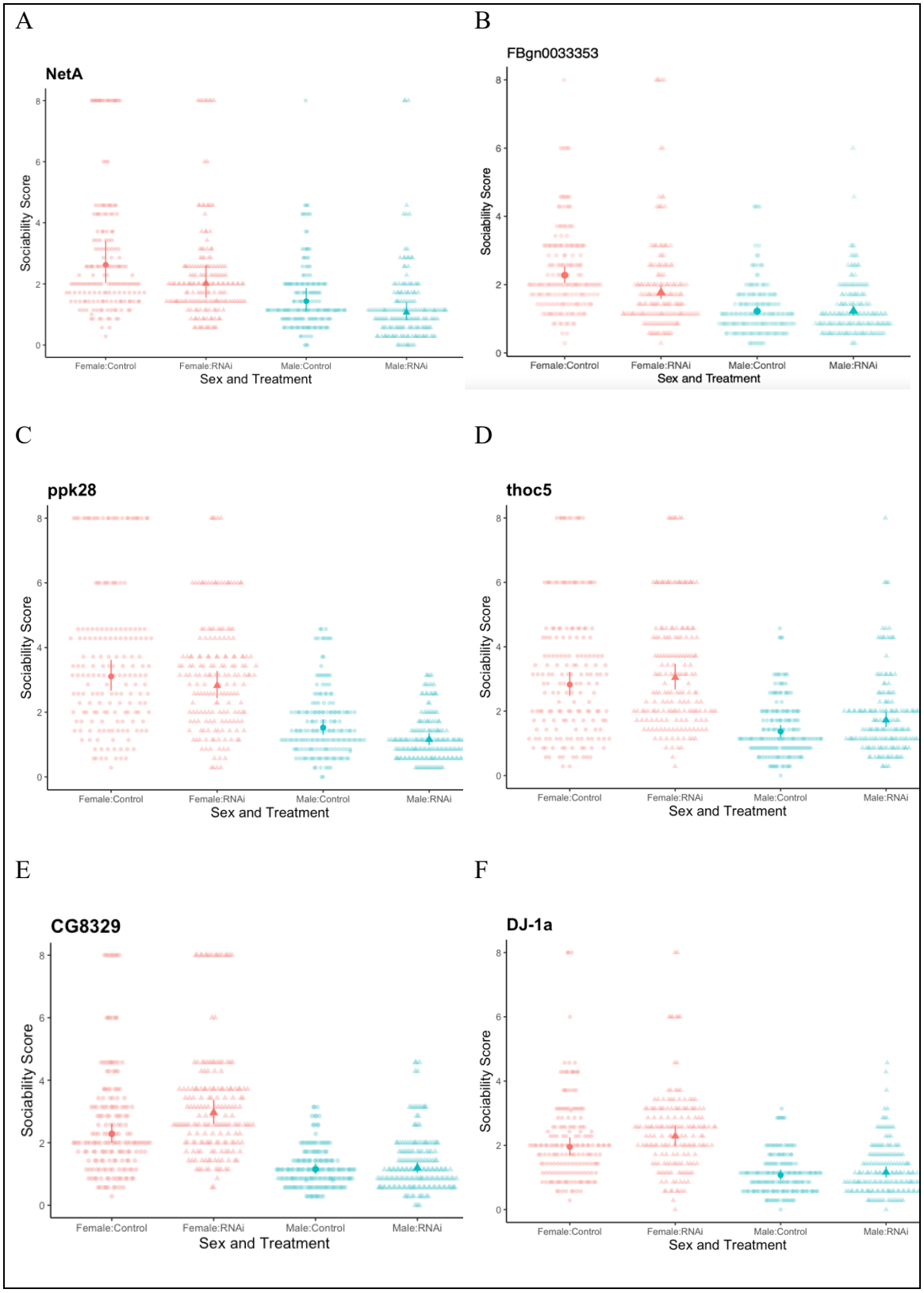
A
NetA
B
FBgn0033353
C
ppk28
D
thoc5
E
CG8329
F
DJ-1a
Sociability Score
Female:Control
Female:RNAi
Male:Control
Male:RNAi
Sex and Treatment

Figure 4. Effect of knockdown on sociability scores in the opposite direction of predictions. Knockdown genes that showed the opposite effect to what we predicted on sociability compared to controls. Plotted values represent sociability scores for each arena (n=144 arenas per gene) estimated means and 95% confidence intervals.

4 | Discussion

4.1 | Main Findings:

Overall, the results of our experiments partially supported our predictions; some candidate genes did indeed alter sociability in fruit flies. We were able to validate 10 of the 17 chosen candidate genes using RNAi knockdowns (Table 4). Four out of the 10 genes affected sociability in the predicted direction (Fig 3), and 6 genes affected sociability in the opposite than predicted direction (Fig 4).

Sec5, *CG13197*, *Ir94D*, and *Est-P*, altered sociability in the predicted direction. *Sec5* is a member of the exocyst complex, where it promotes vesicle trafficking; it is involved in binding secretory vesicles to the plasma membrane (Heider & Munson, 2012). Until now, no clear link has been made linking *Sec5* to behaviour in drosophila. The human ortholog however, *EXOC2*, has been linked previously to severe defects in human brain development, such as severe developmental delay, variability associated with epilepsy and poor motor skills (Van Bergen et al., 2020). *EXOC2* is a part of the exocyst complex which contain many other *EXOC* genes (Halim et al., 2023), some of which have previously been linked to autism spectrum disorder (ASD). *EXOC4* was silenced in utero electroporation (IUE) in mice which resulted in neuronal and dendritic abnormalities that have been linked to ASD (Sung-Oh et al., 2023). In addition, a human patient with a disruption of *EXOC6B* exhibited intellectual disability, epilepsy, and behavioural features resembling ASD (Frühmesser et al., 2013). One of many roles that *EXOC2* has is mediating a protein called RALA, which has previously been implicated in ASD (Halim et al., 2023). Next, *CG13197* is linked to polynucleotide 5'-phosphatase and is orthologous to *DUSP11* (Dual-specificity

phosphatase 11) in humans. Many *DUSP* genes have been previously linked to cancer and autoimmune disorders (Bermudez et al., 2010; Lang et al., 2006). More recently, *DUSP* genes have also been linked to mental health disorders such as depression, as well as neurological disorders such as Alzheimer's disease (An et al., 2021). *Ir94D* (Ionotropic receptor) is predicted to be involved with ligand-gated monatomic ion channel activity. In drosophila larvae, most of the *IR* genes are implicated in taste perception, while others are expressed in neurons that seem to be involved in chemo sensation (Stewart et al., 2015). Specifically, *Ird94D* was expressed in larval neurons that may have a chemosensory role in mediating escape behaviour as a response to hypoxia or hyperoxia (Stewart et al., 2015).Est-P (Esterase P) enables carboxylesterase activity. One of the human orthologs, *CES1* (Carboxylesterase) is an enzyme involved with metabolizing drugs such as pesticides, xenobiotics (such as cocaine, and heroine), and environmental pollutants (Her & Zhu, 2020).

4.2 | Potential reasons for no effect on sociability:

We successfully validated 10 out of 17 genes. However, 6 of these genes affected sociability in the opposite than predicted direction, and 7 of these genes had no effect on sociability. There are some possible reasons for why we saw these unexpected results. First, we knocked down a single gene at a time using RNAi. However, most traits, physiological and behavioural, are polygenic. Sociability is an extremely complex trait which might have hundreds of genes involved in shaping such a complicated behaviour. One potential reason for these unexpected results may be that we are missing potential gene-gene interactions.

It is possible that silencing only one gene may not be capturing the interactions with other nearby genes (Epistasis) and therefore producing no effect on sociability. Second, we must consider the effect of genetic backgrounds. Phenotypes of mutated genes are often expressed differently based on their distinct wild-type genetic background (Chandler et al., 2013). For example, the *Indy* gene of Drosophila was linked to increased life span in Canton-S wild-type background. However, these effects disappeared once the mutations were outcrossed into a different wild-type strain (Chandler et al., 2013). The artificial selection study (Scott et al., 2021) was conducted on wild-type flies native to Hamilton, ON. The current experiment used TRiP-RNAi lines containing specific backgrounds with docking sites on the 2nd and 3rd chromosomes. It is possible that the effect of the candidate genes may be more prevalent in the specific Hamilton wild-type strain used in the artificial selection study. Lastly, we must also consider off-target effects of RNAi, where small interfering RNA (siRNA) may not target only one specific gene; transcripts with partial identity to the siRNA sequence may silence additional transcripts separate from the target gene (Jackson et al., 2003). *Nmdmc*, *FBgn0032436*, and *CG31231* all have at least one other matching transcript. All three of these candidate genes did not show differences in sociability compared to our control flies (Table 4). *CG8329* is also another candidate gene with a potential off-target effect. Knocking down this gene did have an effect on sociability, however, it was in the opposite than predicted direction (Table 4).

4.3 | Sex-specific effects:

We found sex-specific effects for some of our candidate genes, where the effect of knockdown only caused changes in sociability in one sex and not the other. We did not expect these differences, as none of our candidate genes had sex-specific differential expression patterns. All our genes were either on the 2nd or 3rd chromosome, none of them were sex-linked. Such differences have been seen in previous literature. Nlg3, an autism candidate gene, also had sex-specific effects such that males, but not females, showed decreased social space after recovery from isolation compared to controls (Yost et al., in press). In addition, they investigated the role of dopamine in social space; They found that dopamine was required as a response to social isolation and recovery from social isolation in males but not females. Scott et al., (2021) found that females in the high-sociability treatment had approximately 40% higher sociability compared to the low treatment, but males from the high treatment had 54% higher sociability compared to the low treatment. They also found that males had higher sociability scores overall compared to females. It is unclear why we see these sex-specific differences in sociability in fruit flies, but not entirely surprising. Variation in sociability is bound to occur given that these fruit fly samples across studies were all from very different populations, with entirely different genetic material.

4.4 | Genetics of sociability across species:

Sociability is ubiquitous, and so our long-term goal is to understand sociability across many different animals. Specifically, we want to understand how gene expression is associated with sociability in other species. The genetic underpinnings of sociability have become a popular topic in recent literature. Mice are a common model system, in which the two

482 neuropeptides, oxytocin and vasopressin, have been repeatedly implicated with the
483 neuronal makeup of sociability (Caldwell, 2017) and social familiarity (Ferguson et al.,
484 2000). In male zebra finches (*Taeniopygia guttata*), *egr1* is expressed in the auditory
485 forebrain as a response to hearing the song of a male conspecific (Mello et al., 1992). This
486 expression is not evident when white noise or other tones are played, suggesting that *erg1*
487 expression is associated with a social signal. In humans, 18 independent loci and 56 genes
488 have been identified using a large-scale Genome Wide Association Study (GWAS) and a
489 self-report sociability survey (Bralten et al., 2021b). Honeybees are another common model
490 for studying social behaviours. Shpigler et al., (2017) found genes in honeybees (Apis
491 mellifera) that were associated with human social biology. They used RNA sequencing and
492 differential expression graphs to identify overlapping genes between bees that were
493 unresponsive to social stimuli and human genes related to ASD. While sociability is
494 ubiquitous, it is complicated to understand across species. Understanding the genetic
495 underpinnings using robust model systems may provide insight on these similarities and
496 differences
497
498 4.5 | Limitations:
499 These experiments do have some caveats. For one, the sociability arenas were only 6 mm
500 in height. While the flies were able to move through the spaces between the arenas, they
501 were not able to fly sufficiently. Future studies could create arenas with sufficient height to
502 allow flying, which could potentially make a more ecologically valid assay. Second, the
503 flies are aspirated into the arenas at ~8am and tested for sociability at 2pm; this means that

the flies are not able to fly for a sufficient portion of the day. A reduction in the time interval from aspirating flies to testing sociability may resolve this issue. Another potential caveat is the fact that our observations are conducted live. The sociability arenas are placed in an observation room that is closed. However, the door opens for the observers to enter the room during testing. While observers are trained to walk extremely slowly, quietly, and cautiously, the door opening and closing may be noticed by the focal flies. Also, due to the nature of our set up, one observer counts the number of flies in each section out loud, while another records them. There is no clear evidence that flies can hear a human voice, but there is also no evidence that they cannot. This information is relevant because our data show that sociability scores are lower at the beginning of testing (Time 1) versus the end (Time 5). Anecdotally, it also seems that the flies are more settled (moving around less) towards the end of our observation period compared to the beginning. It is possible that the flies re-acclimatize into their preferred groups towards Time 5, after researchers have been in the room for some time. We have statistically accounted for this in our model by including Time as a continuous predictor. One solution to this issue is video recording, which eliminates the entering and leaving of the observation room. Lastly, the knockdown of each candidate gene is yet to be validated via RT qPCR. A confirmation of knockdown will confirm the effect of the candidate genes on sociability.

4.6 | Future work:

A second set of candidate genes have been identified using a population genomics approach looking at genes with distinct changes in allele frequency between sociability lineages

(Torabi-Marashi, 2023). These DNA data can help answer questions regarding evolutionary changes related to differences in sociability as a result of changes in allele frequency. These candidate genes are currently being tested for sociability using the same methods as the current experiments.

The verified genes create avenues for future research to investigate other social behaviours. *Sec5* makes for a good candidate for follow up behavioural experiments. Recently, the Dukas lab has investigated the dynamics of social interactions. In the current experiment, we were able to measure the sociability scores after the settlement period, but we may be missing critical information about how the groups were formed. We wanted to look at how flies interact from the introduction to the arenas until the settlement period where they form their preferred groups. This work has so far had some promising results, one of which includes replicating the *Sec5* sociability result from the current experiment.

Sec5 would also make a good candidate to investigate egg laying in females. There has been relevant literature regarding social effects on egg laying. Sarin & Dukas (2009) showed social learning in female fruit flies with respect to egg-laying. They showed that focal naïve females developed a stronger preference to lay eggs on food that was experienced with mated females who had also laid eggs on that food. Bailly et al., (2023) show that female flies produce eggs faster when their group size increases. Future research could include looking at the number of eggs laid on food patches by *Sec5* knockdown female flies compared to control females.

Lastly, future work also includes testing *EXOC2* in mice. As mentioned, *EXOC2* is the ortholog for *Sec5*, and some *EXOC* genes have been previously linked to ASD. These plans can help us understand the genetic basis of sociability across animals.

5 | Conclusions and Final Remarks

Overall, it is clear that sociability is a complex trait. This work provides further understanding about sociability and its genetic architecture. It answers some mechanistic questions regarding causal links between genes and behaviour. We have functionally validated the effect of genes on sociability in fruit flies. There are several future directions, some of which are already in motion. These data showcase the robustness and resourcefulness of fruit flies as a model system for both social behaviours and genetic work. Using fruit flies as a tool allows us to understand our long-term goal, which is to investigate the genetic basis of sociability and other social behaviours across various species.

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