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Original Article

Evolution of sociability: genome scans and gene validation

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Abstract

Sociability, defined as individuals' tendencies to affiliate with conspecifics, has positive associations with fitness in animals as well as with health, well-being, and longevity in humans. Despite its importance, we still have limited information about natural genetic variation in sociability. As part of a long-term initiative to address this knowledge gap, we quantified changes in allele frequencies in adult fruit flies (*Drosophila melanogaster*) from lineages that we artificially selected to diverge in sociability. Based on our genomic analyses, we generated a short list of 226 single nucleotide polymorphisms (SNPs) representing 169 candidate genes influencing variation in sociability. We also made a shorter list of 41 SNPs from 36 genes that showed the largest average divergence between the low and high sociability lineages. Experiments using knockdowns of 19 of the candidate sociability genes revealed that 18 of them significantly affected sociability, though some effects were sex-specific. Our results provide important insights into a quantitative trait central to the lives of many animals including humans.

Keywords: artificial selection, *Drosophila melanogaster*, population genomics, sociability, social behavior

Introduction

The traditional distinction between social and solitary animals is gradually fading with the growing appreciation among researchers that many animals historically perceived as solitary have rich social lives. For example, although martens and their relatives (family *Mustelidae*) had been assumed to be obligatory solitary, a recent global investigation documented widespread occurrence of individuals in groups (Twining et al., 2024). Similar prevalent social associations have been observed in other historically presumed solitary carnivores including cheetahs (*Acinonyx jubatus*) and pumas (*Puma concolor*) (Caro, 1994; Elbroch & Quigley, 2017; Elbroch et al., 2017; Melzheimer et al., 2020; Wachter et al., 2017). Finally, some researchers have also recognized the limitations of the standard grouping of insects into social and solitary species in their analyses of affiliative behavior in species classified as solitary (Costa, 2006; Prokopy & Roitberg, 2001).

Like many other insects historically categorized as solitary, fruit flies (*Drosophila melanogaster*) show rich social behaviors. Their tendency to aggregate at natural food and egg laying substrates had been known for a long time (Spieth, 1974) and led to the discovery of their aggregation pheromone, cis Vaccenyl Acetate (Bartelt et al., 1985). More recent research has indicated that, given a choice, fruit flies prefer to affiliate with others in both the lab (Bentzur et al., 2021; Durisko & Dukas, 2013; Saltz, 2011; Schneider et al., 2012) and natural settings (Dukas, 2020). Furthermore, fruit flies show a variety of social behaviors including social synchronization of their circadian clocks, social learning, and collective response to danger (Battesti et al., 2012; Ferreira & Moita, 2020; Levine et al., 2002; Ramdya et al., 2015; Sarin & Dukas, 2009).

The rich social life of fruit flies together with the fact that they are a leading model system in evolutionary biology and genetics make them an ideal model system for examining the genetic mechanisms underlying the evolution of social behaviors. While social behavior can be broadly defined as interactions among conspecifics (Ward & Webster, 2016; Wilson, 1975), we focus on a key social trait, sociability, which we define as individuals' tendencies to affiliate with others. Sociability means that individuals either seek each other, tolerate other members of a group, or both, while engaging in activities such as feeding, traveling, resting, and sleeping (Billeter et al., 2024; Scott et al., 2022).

A variety of studies have examined aspects of broadly defined social behavior. Most notably, mouse studies on the genetics and neurobiological basis of social behavior primarily focus on social deficiencies in an attempt to understand autism spectrum disorder (de la Torre-Ubieta et al., 2016; Moy & Nadler, 2008; Silverman et al., 2010). Social hymenopterans have been a major focus of research aiming to

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understand the evolutionary genetics of eusociality (Kocher et al., 2018; Smith et al., 2008; Toth & Rehan, 2017). And genome wide association studies in humans addressed issues of socializing and loneliness (Bralten et al., 2021; Clyde, 2018; Day et al., 2018). Nevertheless, despite the clear importance of sociability for many animals including humans (Bond et al., 2021; Dal Pesco et al., 2022; Gerber et al., 2022; Kajokaite et al., 2022; Snyder-Mackler et al., 2020), we still have limited knowledge about natural genetic variation in sociability.

To examine the genetic basis of natural variation in sociability, we artificially selected replicated lineages of fruit flies that depict either low or high sociability, demonstrating a heritable basis for this trait (Scott et al., 2022). In a previous study, we examined transcriptome-wide variation in gene expression associated with phenotypic divergence in sociability among evolutionary treatments (Torabi-Marashi et al., 2025). In the current study, we report on population genomic analyses aimed to identify alleles that contributed to the response to selection on sociability. We also functionally validated the effects of 19 of the candidate genes on sociability.

Methods

Artificial selection

We previously applied artificial selection on sociability. We derived the artificial selection lineages from a population of ~600 wild *Drosophila melanogaster* females caught in various locations in Southern Ontario, Canada. For each selection treatment, we had four independently evolving lineages. That is, we had four low sociability lineages, four high sociability lineages, and four control lineages. Each generation, we quantified sociability in 12 groups of 16 females, and 12 groups of 16 males, from each of the four low and four high sociability lineages. To quantify sociability, we placed each group of 16 flies inside a sociability arena, which had eight equal sized compartments, each containing a food disc (Scott et al., 2022). Flies could move freely among compartments for 90 min, after which we blocked the passage, and recorded the number of flies in each compartment. From this record, we calculated the sociability score as the variance over mean number of flies in each compartment. Owing to time constraints, we quantified sociability in control lineages every five generations. We then selected four flies from each arena, for a total of 48 males and 48 females per lineage. These 48 pairs generated the next generation of individuals. For the low-sociability lineages, we selected flies from compartments with the lowest numbers of individuals, while for the high-sociability lineages, we selected flies from compartments with the highest number of individuals. For the four control lineages, we randomly selected four flies from each of the 12 groups of 16 same-sex flies per lineage. After 25 generations of selection, the high-sociability lineages showed sociability scores about 50% greater than the low-sociability lineages (Scott et al., 2022).

DNA extraction, library preparation, and sequencing

We used a pooled sequencing approach for our genomic analysis, as is commonly used for evolve and resequence studies, in particular from small organisms. This approach is akin to a bulk segregant analysis/"extreme QTL" mapping, but artificial selection generates greater phenotypic differentiation among pools in comparison to extremes from a single population. We sequenced samples from each of the low, control and high sociablity lineages after 25 generations of artificial selection. We also sequenced a sample of the ancestral population collected before initiating artificial selection. Flies were stored in 70% ethanol and kept at −20°C prior to DNA extraction. DNA was extracted by homogenizing adult flies. Each unique sequencing pool consisted of 96 individuals total, but DNA was extracted in groups of 24 individuals (48 males and 48 females) using the Qiagen DNeasy Kit (catalog number 69506). DNA from each extraction was quantified and pooled (within lineage) in an equimolar fashion. Library preparation (PCR library preparation protocol with the NEBNEXT Ultra II kit with IDT dual adapters) and DNA sequencing were performed by Génome Québec. All sequencing was done to a minimum of 200X coverage per pooled sample. Previous research has shown that this level of coverage accurately recovers sample allele frequencies (Schlötterer et al., 2014; Zhu et al., 2012). There were four lineages in each of the four groups (including four distinct replicates of the ancestral population), for a total of 16 unique samples representing a total of 1,536 individuals along with the ancestral population.

Quality checking, filtering, and mapping of reads

Computational analyses were performed using the Digital Research Alliance of Canada. See Table S1 for software versions, scripts and parameters used, and references. We used FASTQC (v0.11.9) and MultiQC (v1.12), to check quality of reads, and ensured that all samples had a mean Phred quality score of >35. Adapters were trimmed using trimmomatic (v0.36), with the parameters of leading and trailing set to "3" and run parameters set to "MAXINFO:20:0.2." After trimming, samples were once again run through FASTQC via MultiQC to confirm adapter removal. We mapped reads with bwa-mem (v0.7.17) to the *D. melanogaster* reference genome (version r6.38), filtered for reads with MAPQ score of >30 using samtools view (v1.15). Using awk and samtools (v1.15), the core genome was extracted (chromosomes 2L, 2R, 3L, 3R, 4, and X) and then duplicate alignments were marked and removed using "samtools fixmate" and "samtools markdup." We used Picard (v2.26.3) to add read groups to samples and GATK (v3.8) to mark and realign around indels. We merged replicate lineages of each selection treatment into a single file with the command "samtools merge," and generated a single mpileup containing all samples using "samtools mpileup."

SNP calling

SNPs were called using PoolSNP (Kapun et al., 2020). For a SNP to be retained, the position had to have a minimum read coverage of 25, a maximum coverage within the 98% percentile of coverage (to account for highly repetitive regions that have increased chances of being mismapped), within a given sample. Across all samples, a minimum cumulative minor allele count of 10 and minimum minor allele frequency of 1% was additionally required. Retained SNPs were output as variant call format (VCF). We then filtered out repetitive regions using RepeatMasker based on a reference genome and list of known transposons. Using a

script from Kapun et al. (2020), we identified indels from the mpileup, and filtered out indels and nearby positions (5 bp on either side of indels). These steps were undertaken to remove the risk of alignment issues that can result in misidentification of SNPs. This may result in the loss of small, but functionally important indel polymorphisms that contribute to variation for sociability (Kapun et al., 2020, 2021), however the positions are likely to still be associated due to linkage disequilibrium. Additional repetitive regions from the ENCODE blacklist (Amemiya et al., 2019) containing potentially unannotated repeats were filtered out using bedtools and scripts from Kapun et al. (2020).

Genetic differentiation among artificially selected treatments

We used "grenedalf sync" to convert unfiltered mpileups into sync files (Czech et al., 2024). Indels and repetitive regions we previously identified were filtered out of the sync file based on positional information from the VCF described above. We used "grenedalf fst" to calculate pairwise F_{ST} for all contrasts of ancestor, low, control, and high using 5KB sliding windows. For a given contrast of interest (low versus control and low versus high) we chose an outlier-based approach of extracting the windows with the top 5% of F_{ST} values within the contrast. We did this separately for windows in the X chromosome and for windows in the autosomes, to account for increased variation on the X chromosome due to sampling (as we sampled three-fourth of the X chromosomes compared to autosomes). Following this, we merged the outliers for the X chromosome and autosomes back together in each contrast. This outlier approach is less stringent than looking for F_{ST} values greater than 3 standard deviations from the mean, and we chose this because we are also comparing these regions to SNPs identified by a Cochran–Mantel–Haenszel (CMH) test (below) and did not want to potentially exclude important SNPs. Given that there was no artificial selection acting upon control lineages, F_{ST} between ancestor and control is a combination of genetic drift and lab domestication (adaptation to lab environment), which we can use to account for lab domestication in our low versus control and control versus high comparisons. To account for this lab domestication, we identified windows with the top 5% of F_{ST} values in the ancestor versus control contrast and filtered those out of the initial list of windows in the low versus control and control versus high lineages.

CMH test, adapted for pooled resequencing

To identify positions that are potentially under selection, we utilized a modified CMH test. The CMH test is an extension of the χ^2 test (Cochran, 1954; Mantel & Haenszel, 1959). We used a modified CMH test that accounts for the effects of genetic drift and pooled sequencing in the R package ACER (Spitzer et al., 2020). Rather than using the sync file obtained previously from the merged replicates, we needed to generate a new sync file where replicates are not merged. To do so, we went through our pipeline as usual but omitted the merging step. For a given contrast, we first split our dataset into X chromosome and autosomes. We used ACER to identify positions in the genome that show evidence of genetic differentiation between low versus control, control versus high, and low versus high sociability contrasts. Each of the contrasts were run separately and the output was *p*-values associated with positions along the genome. We then applied a *p*-value adjustment, using the R function "p.adjust()" with "method = "BY" ," referring to the Benjamini and Yekutieli method of controlling false discovery rate (Benjamini & Yekutieli, 2001). We filtered the list for the lowest 1% of adjusted *p*-values (a maximum FDR of 4.9e-13 amongst the top 1% of sites), which left us with positions of the genome showing strong genetic differentiation, after having accounted for the influence of drift and random sampling during sequencing. We merged results for X and autosomal chromosomes back together. We chose the lowest 1% of adjusted *p*-values as this provided us with a large list (>20,000) of outlier positions that are potentially under selection that we could compare back to our windows with the top 5% of F_{ST} values.

SNP annotation and extraction

We chose to compare the list of top 5% F_{ST} values with the positions from the CMH test that also corresponded to the lowest 1% of adjusted *p*-values. In both cases, we chose an outlier-based approach to identify regions/positions, which, if used exclusively, may not be the most sensible approach as there is a chance that it introduces noise in the list by solely choosing the highest (or lowest) values. Instead, we chose to see what positions are identified by both analyses (F_{ST} and CMH) as the intersection between the two methods. We created a list of SNPs that overlapped between the two lists of high F_{ST} and statistically significant CMH. To do so, we took the previously generated lists of F_{ST} and CMH and manually converted them into bed files. Then, using the command "bedtools intersect" (Quinlan & Hall, 2010), we generated a bed file that included only the regions of the genome where the F_{ST} window and CMH position overlapped. We then annotated this list using SnpEff (Cingolani et al., 2012). SnpEff, required a VCF file, so similarly to subsetting our sync file from a VCF, we created a script that subsetted our initial VCF with only positions from our bed file. Another benefit of performing this subsetting is that it filters out positions identified in the CMH test that do not appear in the merged replicate VCF, as the positions in the CMH list come from the unmerged VCF. SnpEff provides preliminary predictions of impacts of coding variants identified, which are low (synonymous), moderate (missense and inframe mutations), and high impact (frameshift and nonsense mutations). Noncoding, splice, and other types of variants are described as modifiers. We extracted SNPs that had high and moderate impact variants along with those described as "modifers." If a given contrast had no SNPs labeled as high effect variants, we used the list of moderate and modifiers and vice versa. We followed this up by manually checking estimates based on visualization of allele frequencies by lineage (Supplementary file 1), removing sites that were "significant" but likely due to (potentially spurious) effects of single lineages within an evolutionary treatment, to focus on candidate variants with parallel responses (among replicate lineages) to selection. Specifically, to generate the short list of 226 variants, the frequencies of at least three high lineages had to be lower or higher than those of all low lineages. To generate the shorter list of 41 alleles, the frequencies of all four high lineages had to be lower or higher than those of all low lineages.

We then created a list of the genes associated with the overlapping SNPs in a given contrast and compared that list to the list of differentially used transcripts or expressed

genes of the same contrast to see if any genes overlapped between the two analyses. We also created a list of genes associated with the SNPs with predicted high and moderate impact variants for a given contrast and compared that list to the list of differentially used transcripts or expressed genes of the same contrast.

Gene ontology overenrichment analysis

We performed an overenrichment analysis on the 169 identified candidate genes based on gene ontology. We contrasted our list of 169 genes to the reference list of 11,379 *D. melanogaster* genes with gene ontology information (Biological Process database). We included only categories with a minimum of five genes and adjusted for multiple comparisons using the Benjamini–Hochberg FDR (FDR < 0.05). We performed the analyses (April 23, 2025) using the Web-based Gene set analysis toolkit 2024 (Elizarraras et al., 2024), available at https://www.webgestalt.org/.

Comparison to other relevant studies

We conducted a few comparisons. First, we previously examined gene expression in the adult heads of flies from generation 25 of the same artificial selection study used for the population genomics analyses reported here (Torabi-Marashi et al., 2025). In that study, we recorded 328 differentially expressed genes and 508 genes that showed evidence of differential transcript usage in contrasts among the low, high, and control sociability lineages. Second, Bralten et al. (2021) performed a GWAS on people using the UK Biobank and identified 56 genes associated with sociability. Finally, Shpigler et al. (2017) compared sociability genes they identified in honey bees (*Apis mellifera*) to genes linked to autism spectrum disorder in humans. They concluded that there are conserved molecular mechanisms for social behaviors in invertebrates and vertebrates. Specifically, they performed differential gene expression analysis on RNA obtained from the mushroom body of the brain of honey bees that only responded to a territorial threat, bees that only showed nursing behavior toward a queen larva, and bees that responded to neither. They identified 1,057 differentially expressed genes between all three groups of social responsiveness (Shpigler et al., 2017).

To evaluate whether there was more overlap than expected by chance between genes in our current list and each of the three studies mentioned above, we used a simulation approach. We generated a random set of genes from each organism, with the number of genes remaining fixed based on the number of significant hits observed from the relevant study. From this we identified the number of overlapping genes in this random set. We evaluated our observed number of overlapping genes, relative to distribution based on overlaps for random sets of genes and adjusting for proportion of orthologous genes (genome-wide) for the two studies of consideration. We performed 100,000 iterations of the simulation for each study (in comparison to the current study).

Our final comparison involved the gene *degrees of kevin bacon* (*dokb*), which affects fruit flies' betweenness centrality, a measure that indicates how important individuals are within their social network (Rooke et al., 2024). Our initial filtering of candidate variants did not identify variants within *dokb* as showing extreme patterns of differentiation associated with artificial selection for sociability. However, we examined all SNPs in this gene to see if there was evidence of any consistent changes in allele frequencies that we may have removed as a result of overly stringent filtering of variants.

Candidate gene validation

We chose 20 candidate genes that showed large and consistent differences in allele frequencies between low and high sociability lineages (including candidate variants that were noncoding), subject to the availability of TRiP RNAi strains (Dietzl et al., 2007; Zirin et al., 2020). We used TRiP-control strains that are coisogenic with the TRiP RNAi knockdown strains for control crosses (Table S2). We crossed males from each RNAi strain with females from a pan-neuronal nervous system Gal4 strain (*elav*-Gal4) to knockdown gene expression of each chosen candidate gene in neurons throughout the whole lives of focal flies. Our default Gal4 line was BDSC 25750. This strain contains the $elav^{c155}$-Gal4 enhancer trap insertion that accurately reflects endogenous expression of the *elav* gene, expressed in all postmitotic neurons across all developmental stages (Ogienko et al., 2020; Robinow & White, 1988; Yao & White, 1994). This strain also has a UAS-dicer2 construct to increase efficiency of RNA interference. We had high offspring mortality with the UAS-*rg*.RNAi strain when using the default Gal4 strain. We thus replicated the experiment for this line with a weaker Gal4 line (BDSC 8765). This is also an *elav*-Gal4 line, generated as a reporter construct using a 3.5-kb genomic fragment upstream of *elav*, including the core promoter (Yao & White, 1994). This has a somewhat reduced range of expression in comparison to our default Gal4 (Ogienko et al., 2020). Data from both crosses were similar and we included both data sets in the analyses. We verified expression of *elav*-Gal4 in both Gal4 strains by crossing them to a UAS-GFP.NLS strain, allowing us to confirm patterns of Gal4 expression in the larval *Drosophila* brain (Ogienko et al., 2020). We used the information at https://fgr.hms.harvard.edu/trip-in-vivo-fly-rnai to determine if there were any known off-target effects for the TRiP strains we used (none were found).

Each experimental block had an equal number of assays for the control and experimental genotypes, in which we crossed males from the coisogenic TRiP-control strains (BDSC stock 36303 or 36304 depending on the location of the transgene insertion) to *elav*-Gal4 females. We maintained all strains at 25°C and 60% RH on media in which each 1 L contained 90 g sucrose, 75 g cornmeal, 10 g agar, 32 g yeast, 2 g methyl paraben dissolved in 20 ml ethanol, and water. We failed to produce offspring in 1 strain (BDSC# 40945), leaving us with 19 candidate genes (Table S2).

We quantified sociability using a protocol similar to that of Torabi-Marashi et al. (2025). For each candidate gene, we sexed groups of eight newly eclosed, unmated offspring from the knockdown group and control group and placed each group into a same-sex and same-treatment food vial with 5 ml of standard food. We sexed 12 sets of focal flies per sex, treatment and day, for a total of 384 flies per day, and quantified sociability once individuals were 3 days old. We used 3D-printed circular arenas 50 mm wide and 6 mm high divided into eight compartments by thin walls with openings 6 mm wide and 3.5 mm high (Figure S1). Flies readily trav-

eled among the eight sections. The top of the arena consisted of a plexiglass sheet with a 3D-printed circular edge and a 3-mm hole. We placed a 7.5 mm wide and 2 mm high circular patch made of regular medium in each compartment, and covered it with 5 µl of juice solution made of 2 g live yeast dissolved in 10 ml orange juice. Every morning at 10 a.m., we placed fresh food patches in each arena, attached the covers, and gently aspirated groups of eight same-sex flies into each arena through the hole in the top. We then covered the hole with a small piece of transparent, sticky tape. We prepared 12 arenas per-sex, per-treatment, and 48 arenas in total per day, and placed the arenas inside a humidified container maintained at 25°C and 50% RH. We completed setting up the arenas around 11:30 a.m. and then left the experimental room to allow flies to settle undisturbed in their new settings until 2 p.m.

At 2 p.m., an observer blind to treatment counted the number of flies in each compartment within each arena every 15 min for 1 h. We calculated the five sociability scores for each arena as the variance over the average number of flies in each arena (Durisko et al., 2014; Scott et al., 2018). Using this measure, the minimum sociability score of 0 indicates 1 individual within each of the eight sections of the arena and hence social avoidance. The maximum sociability score of eight means that all eight flies form a single group in one section of the arena, indicating high sociability. Scores significantly greater than 1 indicate higher social grouping than expected at random (Figure S1). After scoring the arenas, we discarded flies, washed the arenas with detergent and water and let them dry overnight. We conducted 3 test days for a total of 144 arenas for each of the 19 candidate genes, but had fewer than 144 arenas for a few genes owing to insufficient numbers of flies.

The response variable from sociability scores for each arena are semicontinuous, positively valued, with rare 0s. We analysed sociability data for each gene by fitting a generalized linear-mixed effects model with the glmmTMB package (v 1.1.8, Brooks et al., 2017) using a Tweedie distribution with a log link function, in R v4.3.3 (R Core Team, 2023). In this implementation, the Tweedie power-parameter is constrained between 1 (Poisson) and 2 (Gamma). We modeled treatment, sex, and their interaction as fixed effects, while time from onset of scoring was a continuous predictor. We fit a random effect, allowing the intercept to vary for day of experiment, an independent random effect, allowing intercept, and a random slope for time within experiment to vary according to individual arena (unit of sampling). We also included a final random effect for experimental block. For the model fit, we had a singular convergence warning. As such, we confirmed the stability of fixed effect estimates with a model, where we removed the random slope associated with time for the random effect of individual arenas, but otherwise identical to the model described above. Both models produced virtually identical estimates and confidence intervals for fixed effects, which are the focus of this study. We used emmeans and contrast functions from the emmeans package (v1.10.0, Lenth, 2022) to estimate custom contrasts of treatment effects, averaged over sex, as well as the interaction contrasts for treatment and sex effects (to assess sex specific effects of RNAi-mediated gene knockdowns). These were adjusted for multiple comparisons using the Dunnettx approach, adjusting for 19 comparisons.

qPCR assessment of RNAi

We verified efficacy of RNAi examining RNA expression in knockdown compared to control flies using qPCR (quantitative polymerase chain reaction). We selected eight genes for qPCR analysis based on the results from candidate gene validation. For each gene, we extracted RNA from adult heads (reared as described above) from vials consisting of ten flies each. We had two vials per treatment and sex, resulting in eight unique biological samples per qPCR assay. Flies were stored in RNA*later*™ Solution (Invitrogen) and kept at −80°C prior to RNA extraction. Total RNA was extracted from groups of 10 flies by homogenizing adult fly heads submerged in Buffer RLT from the Qiagen RNeasy Mini Kit (catalog number 74106) using The Bullet Blender® Homogenizer (Next Advance) and Froggabio Zirconium Oxide Beads 0.5 mm RNase Free (Item Code: ZROB05-RNA). We aspirated samples through 25G needles and followed the protocol for the Qiagen RNeasy Mini Kit (catalog number 74106) and RNAse-Free DNase Set (catalog number 79254). To quantify RNA extracted, we used the DeNovix QFX Fluorometer with the Qubit™ RNA HS Assay Kit. To synthesize cDNA, we used the PCR Biosystems UltraScript® cDNA Synthesis Kit (Item Code: PB30.11–10) for the *Cpsf100* and *Sec5* assays and the Bio-Rad iScript™ Reverse Transcription Supermix for RT-qPCR (catalog number 1708840) for the other six assays. qPCR reactions were performed on an Applied Biosystems™ StepOne™ Real-Time PCR System using custom primers (Table S3), PCR Biosystems SyGreen® Blue Mix Hi-ROX (Item Code: PB20.16–05) for the *Cpsf100* and *Sec5* assays and Applied Biosystems™ PowerTrack™ SYBR Green Master Mix for qPCR (catalog number A46012) for the other six assays. *elf1A* and *Rap2* were used as reference genes for each assay. For each biological replicate, we used three technical replicates for our gene of interest and reference genes, totaling in nine wells on our qPCR plate per biological replicate. We used the StepOne™ Software v2.3 automatic threshold function for analysis. We omitted samples that failed to amplify or had multiple T_m peaks.

To calculate ΔC_T, we subtracted each C_T value from the average C_T value of the corresponding reference genes. We analysed qPCR data for each gene by fitting a linear model in base R v4.3.3 (R Core Team, 2023). We used emmeans and contrast functions from the emmeans package (v1.10.0; Lenth, 2022) to estimate $\Delta\Delta C_T$, averaged over sex, as well as the interaction contrasts for treatment and sex effects. For one gene we wished to evaluate, *Wnt2*, we always generated multiple amplicons and could not get high quality qPCR data. We thus tested this line phenotypically by crossing the strain to a nubbin-Gal4 driver expressed during wing development and demonstrated that it had the known loss of function phenotype of held out wings in the F1 (Kozopas et al., 1998).

Results

Candidate sociability genes

Our genome scans of lineages diverged for sociability were consistent with a highly polygenic response, with variants spread across the genome, as indicated by the distribution of genomic regions showing genetic differentiation (Figure S2). Using a Holm's adjustment for multiple comparisons, there

Table 1. The shorter list, sorted in alphabetic order, of 41 alleles. Each allele on this list had lower or higher frequencies in all four high sociability lineages than those in all four low sociability lineages. The right column indicates whether allele frequencies were lower or higher in the high sociability lineages.

SNP	Type	Gene	Gene_ID	Frequency in high lineages
X_10290357	Intron_variant	*alpha-Man-Ia*	FBgn0259170	Higher
3L_4676265	Intron_variant	*axo*	FBgn0262870	Higher
3R_26680488	Intron_variant	*beat-VII*	FBgn0250908	Higher
3R_22081571	Intron_variant	*CG13408*	FBgn0038929	Higher
4_234884	Synonymous_variant	*CG1674*	FBgn0039897	Lower
3R_23643976	Intron_variant	*CG31145*	FBgn0051145	Lower
3L_3741911	Intron_variant	*CG32264*	FBgn0052264	Higher
3L_1282416	Intron_variant	*CG32333*	FBgn0052333	Lower
3L_1291581	Synonymous_variant	*CG32333*	FBgn0052333	Lower
2L_10967132	Intron_variant	*CG33129*	FBgn0053129	Lower
X_17495415	Intron_variant	*CG43658*	FBgn0263706	Higher
3R_30203409	Intergenic_region	*CG9743-RpS7*	FBgn0039756–FBgn0039757	Higher
3R_14098601	Intergenic_region	*CG9920-PK1-R*	FBgn0038200–FBgn0038201	Higher
3R_23198088	Intron_variant	*cnc*	FBgn0262975	Lower
3R_29036818	Missense_variant	*Cpsf100*	FBgn0027873	Higher
3L_8841946	Intron_variant	*dally*	FBgn0263930	Lower
X_14347162	Intron_variant	*dpr8*	FBgn0052600	Lower
2L_8403579	5_prime_UTR_variant	*emb*	FBgn0020497	Higher
3L_14310395	Intron_variant	*fz*	FBgn0001085	Higher
3L_14310403	Intron_variant	*fz*	FBgn0001085	Higher
3L_14310430	Intron_variant	*fz*	FBgn0001085	Higher
2L_914378	Synonymous_variant	*GluRIIC*	FBgn0046113	Lower
3L_5358430	Synonymous_variant	*Klp64D*	FBgn0004380	Lower
3R_20880956	Intergenic_region	*lncRNA:CR44048-tRNA:Thr-CGT-1–1*	FBgn0264840–FBgn0051480	Higher
3R_28976154	Intergenic_region	*lncRNA:CR45669-wat*	FBgn0267229–FBgn0039620	Lower
3R_28976368	Intergenic_region	*lncRNA:CR45669-wat*	FBgn0267229–FBgn0039620	Lower
2L_18962002	3_prime_UTR_variant	*Nak*	FBgn0015772	Higher
3L_2966024	Intergenic_region	*Or63a-FBti0020025*	FBgn0035382–FBti0020025	Higher
3L_3811904	Intron_variant	*PIG-B*	FBgn0035464	Higher
2R_24421599	Intron_variant	*prom*	FBgn0259210	Higher
3R_29446019	Intron_variant	*Ptp99A*	FBgn0004369	Lower
X_18800322	Intron_variant	*S6KL*	FBgn0283473	Lower
3L_6499688	Intron_variant	*sfl*	FBgn0020251	Higher
2R_19597518	Intron_variant	*sm*	FBgn0003435	Lower
3L_4615602	Intron_variant	*Src64B*	FBgn0262733	Lower
3L_13482839	3_prime_UTR_variant	*stv*	FBgn0086708	Lower
3L_13501425	Synonymous_variant	*Tgi*	FBgn0036373	Lower
3L_13501499	Missense_variant	*Tgi*	FBgn0036373	Lower
3R_13181185	Intergenic_region	*timeout-CG8138*	FBgn0038118–FBgn0038122	Lower
3R_19219857	Intron_variant	*vib*	FBgn0267975	Higher
2R_9499163	Intron_variant	*Wnt2*	FBgn0004360	Lower

were 132,524 polymorphic sites that exceeded an adjusted alpha of 0.05 from the CMH test. Many of these associations likely reflect linkage disequilibrium with actual causal sites. To narrow this to a far more manageable set of candidate genes, we used substantially more stringent filtering (see the section "Methods") and identified 226 SNPs representing 169 genes (Supplementary file 2) with evidence of consistent genetic differentiation based upon both F_{ST} and the CMH test. Overenrichment analysis was consistent with biological processes such as generation of neurons (GO:0048699, ratio = 3.12, FDR = 3.5×10^{-6}), and nested processes like nervous system development (GO:0007399, ratio = 2.71, FDR = 6.4×10^{-6}) among GO categories showing overenrichment (Supplementary file 3). Using SnpEff, 18 of the polymorphisms were predicted as low priority and five as moderate priority (missense mutations). The missense mutations were in the genes: *stv, Tgi*, *CG5013*, *Cpsf100*, and *Mec2*. The remainder of the sites were in introns or untranslated regions of a gene, or were intergenic, and as such SnpEff does not prioritize those. We manually curated based on the additional criteria of large, average magnitude of differences in allele frequencies, and parallel responses among multiple independently evolved lineages. This subset included 41 SNPs, representing 36 genes (Table 1), that, based on our objective evaluation, are top candidates for functional evaluation. We also added to this list three genes (*kek6*, *nlg1*, and *rg*) from the longer list of 169 genes owing to their known neurobiological function. We provide further information about the 39 candidate sociability genes in Supplementary file 4. Figure 1 depicts allele frequencies in

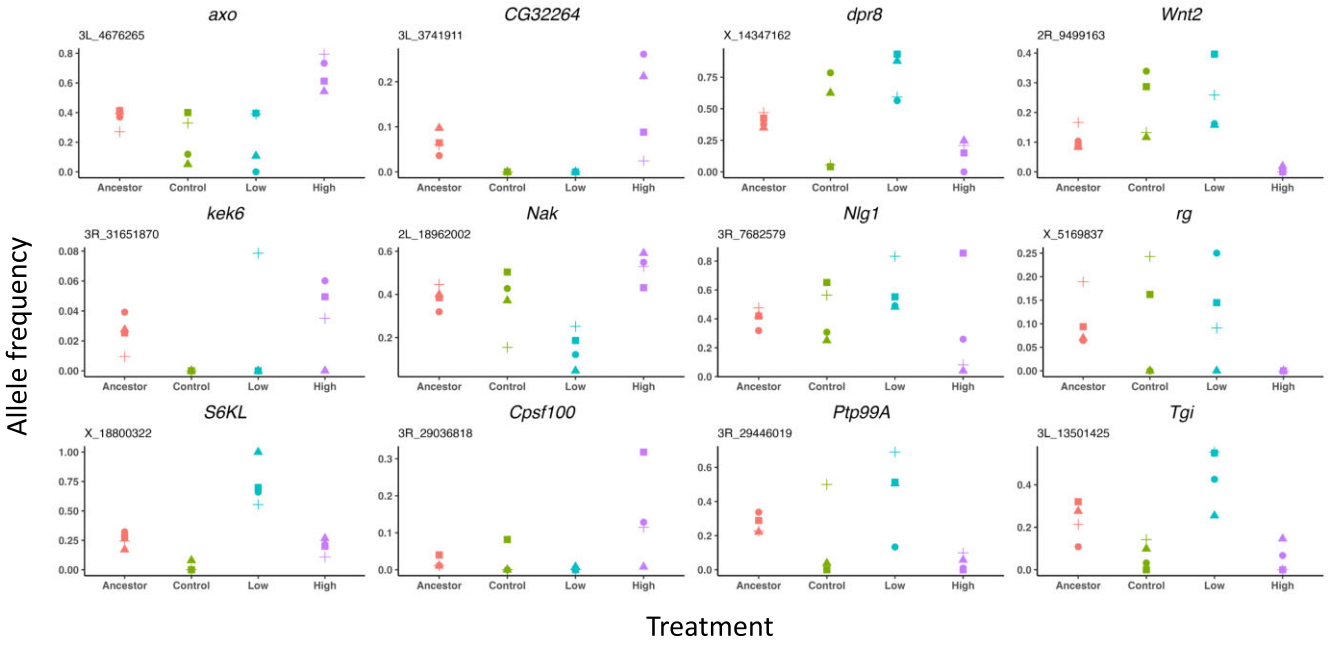

Figure 1. 12 candidate genes with large differences in allele frequencies. Depicted in each panel are the mean ± SE for the ancestral population, and values for each of the four control, four low, and four high sociability lineages. Symbols identify the individual lineages. SNP identity is indicated at the top left of each panel. See Supplementary file 2 for the full gene list.

the ancestral population and the evolved lineages for 12 top candidate genes.

Out of the high priority list of 41 SNPs (Table 1), six SNPs across four genes influence WNT signaling. These are one of the ligands, *Wnt2* (wingless-type MMTV integration site oncogene analog 2), a WNT receptor, *fz* (*frizzled*), and two genes, *sfl* (*sulfateless*) and *dally* (*division abnormally delayed*), which influence ligand mobility. Furthermore, three SNPs across two additional genes that influence WNT signaling appear in our short list of 226 SNPs (Supplementary file 2). These are *Hs6st* (*Heparan sulfate 6-O-sulfotransferase*) and a regulator of *fz*, *grh* (grainy head).

Comparison to other relevant studies

In the comparison to our previous list of 328 genes identified by differential gene expression in adult heads across the artificial selection treatment (Torabi-Marashi et al., 2025), we found no genes that overlap. Our simulations under the assumption of no expected overlap had a median of three shared genes and 99% upper limit of eight shared genes. In the comparison to the candidate genes identified in the GWAS for human sociability (Bralten et al., 2021), we also observed no overlap. Our simulations under the null suggest a median of zero genes with a 99% upper limit of two shared genes (and only 20.4% of simulations having any shared genes). In the comparison with genes identified as being associated with variation in social behaviors in honeybees (Shpigler et al., 2017), we observed an overlap of 10 genes. Simulations under the null expected a median of 10 genes overlapping, with the 99% upper limit being 18 shared genes.

We identified 42 SNPs across ~1.45 kb region of the *degrees of kevin bacon* (*dokb*) gene identified by Rooke et al. (2024). Amongst these, several sites exceeded the nominal FDR adjusted threshold. The C/A SNP at position (3L:13,441,958) encoding the alanine/glutamine nonsynonymous polymorphism described by Rooke et al. (2024) segregated in the ancestral founding population for our lineages (minor allele frequency = 0.15). This SNP did not show evidence for consistent divergence between low and high sociability treatments in our study (Figure S3A). Yet, several other SNPs, not examined in Rooke et al. (2024), showed consistent allele frequency changes associated with the treatments. This included an A/C polymorphism also encoding a glutamine/alanine nonsynonymous polymorphism 30 bp (3L:13,441,928) from the SNP evaluated in Rooke et al. (2024) (Figure S3B).

Candidate gene validation

18 of the 19 candidate genes we functionally tested showed significant effects on sociability in at least one sex. The sole exception was the *fz* knockdown, which showed no effect in either sex (Figure 2; Figures S4 and S5). Two of the most obvious results were that most genes had a sex-specific effect, and that females had sociability scores twice as high as those of males. Seven gene knockdowns had lower sociability only in females, while seven gene knockdowns had higher sociability only in males. Only one of these gene knockdowns, *kek6*, had opposing effects, decreasing sociability in females and increasing it in males. Three additional gene knockdowns decreased sociability in both females and males, and two additional gene knockdowns increased sociability only in females. Notable genes with large effects in both sexes included *Nlg1* and *Cpsf100*, in which knockdown flies had between 25% and 50% lower sociability. In females, knocking down *rg* and *Ptp99A* led to the largest reduction in sociability. In males, on the other hand, the two largest effects of knocking down genes were associated with an increase in sociability in knockdowns of *dpr8* and *sfl* (Figure 2; Figures S4 and S5). In a subset of the strains, we confirmed that the

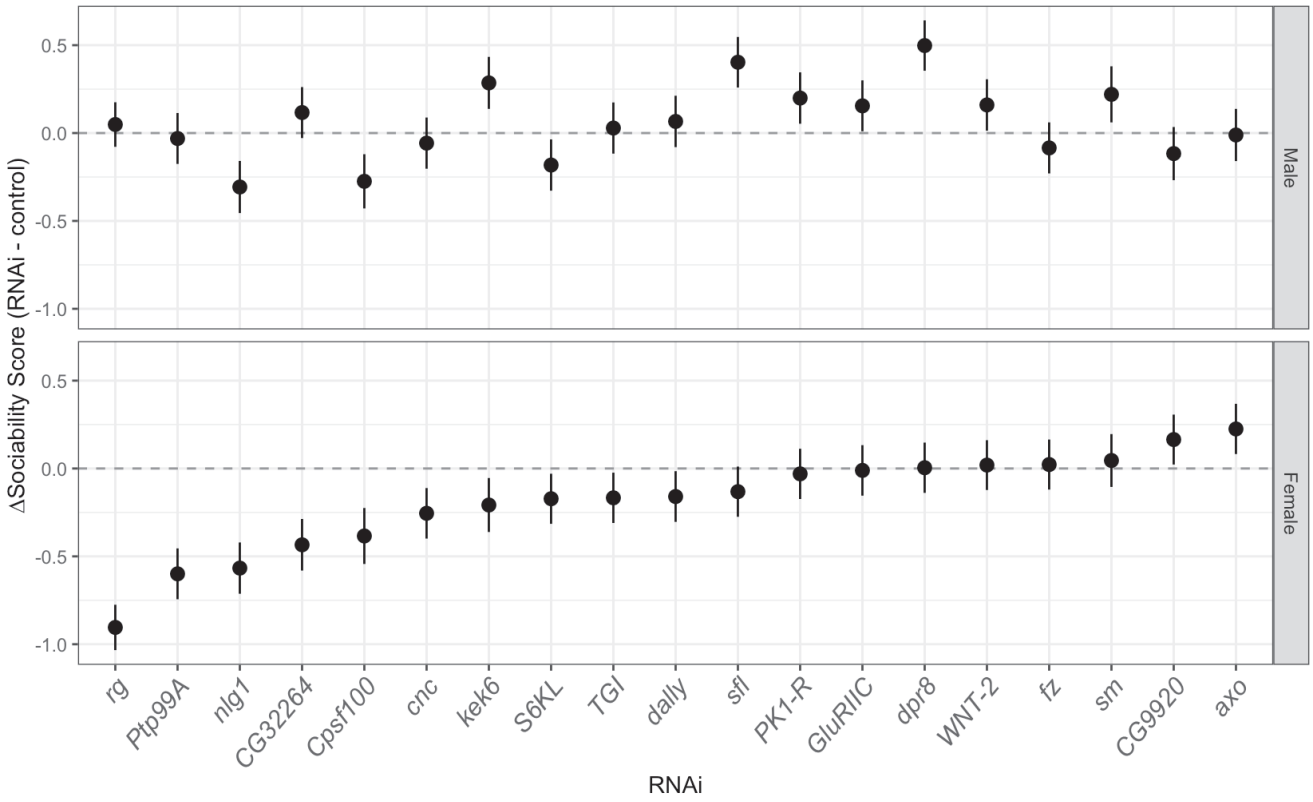

Figure 2. Treatment contrasts for sociability between RNAi knockdown and their respective controls in females and males. Contrasts are on a natural log scale. 95% confidence intervals for contrast estimates are adjusted for 19 comparisons to the control treatment (Dunnett adjustment). Values below zero indicate lower sociability in knockdowns.

crosses with the RNAi constructs reduced RNA expression (Figure S6). We observed the expected reduction in mRNA in most genes, however two genes (*Cpsf100* and *dpr8*) showed evidence of increased mRNA in one sex, for unknown reasons.

Discussion

Population genomics

Sociability is a prominent trait with major effects on fitness and well being in many animals including humans. It is thus essential that we enhance our knowledge about the genetic basis of natural variation in sociability. To this end, we conducted genome scans based on changes in allele frequencies from lineages selected for low and high sociability (Scott et al., 2022) in order to identify candidate sociability genes, and to functionally validate a set of these genes. Based on the genomic analyses, we generated a short list of 226 SNPs representing 169 candidate genes influencing sociability (Supplementary file 2). We also prepared a shorter list of 41 SNPs representing 36 genes (Table 1) that showed the largest average divergence between the low and high sociability lineages. Some of the top candidate sociability genes have been previously linked to social behavior. These include *axo*, *Nlg1*, and *rg* (Figure 1). The human ortholog of *axo*, *CNTNAP2* is involved in social behavior. Furthermore, a mouse *Cntnap2* knockout is a major model for research on the neurobiological mechanisms of autism spectrum disorder (Choe et al., 2022; Peñagarikano et al., 2011). The *Nlg* (*Neuroligin*) gene family plays important roles in synaptic formation and function. *Neuroligin* genes have been implicated in the social behavior of fruit flies (Corthals et al., 2017; Hahn et al., 2013; Yost et al., 2024) and linked to autism spectrum disorder in humans (Südhof, 2008; Uchigashima et al., 2021). Finally, *rg* (*rugose*) encodes a protein that affects synaptic architecture and brain morphology. Fruit flies with *rg* loss of function mutations show impaired associative learning and social behavior (Volders et al., 2012; Wise et al., 2015). Mutations in the human ortholog of *rg*, *neurobeachin* (*NBEA*), are associated with autism spectrum disorder (Castermans et al., 2003; Volders et al., 2011).

Intriguingly, several of our top candidate sociability genes influence WNT signaling. WNT signaling has broad pleiotropic effects across development, including multiple aspects of neural development (Wodarz & Nusse, 1998). In humans, impaired WNT signaling has been linked to autism spectrum disorder (Caracci et al., 2021; Kalkman, 2012). Moreover, *WNT3* was among the top 15 genes significantly associated with autism in a recent genome-wide association meta-analysis (Grove et al., 2019).

Comparison to other relevant studies

We were surprised to see no overlap in candidate genes identified previously based on differences in gene expression across the selective treatments (Torabi-Marashi et al., 2025), and the genes identified based on changes in allele frequencies in this study. This is all the more puzzling as

18/19 of the genes we functionally tested using RNAi in the current study impacted sociability. A priori, it would have seemed likely that some of the genes whose expression was changing would be due to cis-regulatory variants influencing their gene expression. While we do not yet fully understand the lack of overlap, there are likely a few contributing factors. In our previous study, we examined changes in genome-wide patterns of gene expression in adult heads among the lineages artificially selected for low or high sociability. However, if the evolutionary changes impacted brain development, then potentially a substantial fraction of genes we identified as being differentially expressed are consequences of earlier developmental changes, but do not directly mediate variation in sociability. This may partially explain the moderate rate of functional validation in the previous study. That is, some candidate genes whose expression was knocked down were actually consequences of developmentally induced changes in the brain, but do not directly mediate variation in sociability. Yet some of the genes we functionally evaluated, have clear and replicable impacts. For instance, the RNAi knockdown of *sec5* showed a substantial reduction in sociability both in our initial assessment (Torabi-Marashi et al., 2025) and in a more recent assessment of its impacts on the dynamics of group formation (Dukas, 2025). In contrast, our current study and our list of candidate genes were based on allele frequency changes in or near genes. While 18 out of 19 of the genes we functionally tested influenced sociability, it is also possible that we incorrectly associated some variants with particular genes based on physical proximity. For example, a noncoding variant could influence a gene that is not directly proximal to the variant position (Jack et al., 1991). We would, however, require additional experimental work to understand this further.

In the comparison to the candidate social behavior genes identified in two previous studies on humans (Bralten et al., 2021) and honey bees (Shpigler et al., 2017), we found no more overlapping genes than expected by chance. Our results are in agreement with a recent study that found no evidence of overlap between sociability genes in *C. elegans* and humans (Roozen & Kas, 2025). Our results, however, are inconsistent with those of Shpigler et al. (2017), which suggested considerable overlap between genes associated with social behaviors in honeybees and genes associated with autism spectrum disorder in humans. It is likely that the variation in conclusions among the small number of studies is due to differences in the accuracy of assessments of orthologous genes between species, and in how these are accounted for in simulations. We discuss the topic of conserved molecular mechanisms for social behaviors across taxa in greater detail below.

Our analysis of SNPs in the *dokb* gene, which affects social network properties in fruit flies (Rooke et al., 2024), revealed that the allele identified by Rooke et al. (2024) showed no evidence of association with the divergence in sociability amongst our lineages (Figure S3A). However, another nonsynonymous SNP 30 bp proximal was consistently diverged among our sociability lineages (Figure S3B). It is not clear why we observed these differences. One possibility is that the two different SNPs influence distinct aspects of social behavior captured in the different protocols that Rooke et al. (2024) and we have employed. Another interesting possibility is that these two segregating alleles in *dokb* may show intragenic epistatic interactions. Given these possibilities, we intend to test in our sociability arenas *dokb* loss of function mutants as well as allelic combinations of the segregating variants.

Candidate gene validation

18 of the 19 candidate genes that we knocked down via RNA interference showed significant effects on sociability in at least one sex (Figure 2; Figures S4 and S5). The large number of trials necessary to quantify sociability and the necessity for leaving flies undisturbed when they settle in the sociability arenas did not allow us to quantify the dynamics that led to distinct sociability scores in flies with knockdown genes versus control flies. Follow up experiments on the artificially selected low and high sociability lineages, however, revealed significantly higher frequency of aggressive behaviors in both females and males from the low than high sociability lineages. Fly aggression was primarily lunging, defined as a fly's abrupt movement towards a nearby fly (Figure 4 in Scott et al., 2022). In general, however, social group formation involves some combination of attraction and reaction. Flies may be attracted to others and prefer to remain with others (Prokopy & Roitberg, 2001; Ward & Webster, 2016). We still do not know the relative contribution of attraction and reaction to social group formation in wild fruit flies. Moreover, it is likely that the proportional weight of attraction and reaction would vary among different sociability genes. We intend to quantify these parameters in future research.

Interestingly, many of the effects of knocking down candidate sociability genes differed between the sexes. Sex differences in behavior are prevalent among animals (Ngun et al., 2011; Nilsen et al., 2004). Indeed, on average, female sociability scores were about twice as high as those of males in both our current study (Figure S4) and our previous work with gene knockdowns (Figure S14 in Torabi-Marashi et al., 2025). We should note, however, that females had lower sociability scores than males in two previous studies from our lab (Scott et al., 2018; Scott et al., 2022), which used either recent descendants of wild fruit flies or 59 inbred lines from the *Drosophila* Genetic Reference Panel (Mackay et al., 2012). Moreover, we documented line by sex interactions (Scott et al., 2018). Apparently, the genetic mechanisms underlying sociability are influenced by both sex and genetic background. In fruit flies, sex-specific behaviors including aggression have been linked to *doublesex* (*dsx*) and *fruitless* (*fru*) (Dickson, 2008; Siwicki & Kravitz, 2009; Vrontou et al., 2006). Hence, the role of *dsx* and *fru* in modulating sociability warrants future study.

In addition to the genes discussed above, notable genes that we tested and found to have moderate to large effects on sociability included *Ptp99A*, *CG32264*, *Cpsf100*, *cnc*, *kek6*, *S6KL*, and *dpr8*. *Ptp99A* (Protein tyrosine phosphatase 99A) has roles in neural development (Hatzihristidis et al., 2015). In humans, members of the *PTP* gene family have been linked to many pathologies including autism and schizophrenia (Hendriks et al., 2013). In fruit flies, *Ptp99A* may influence visual responsiveness to conspecifics (Sato & Takahashi, 2025). While little is known about *CG32264*, its human orthologs, *PHACTR1* and *PHACTR2* have been linked to neurological disorders including epileptic encephalopathy and Parkinson's disease (Takai et al.,

2020; Wider et al., 2009; Xu et al., 2024). *Cpsf100* is part of the mRNA cleavage and polyadenylation specificity factor complex (Michalski & Steiniger, 2015) and has no known link to either neuroanatomy of neurophysiology. *cnc* (capn-collar) and its human ortholog, *NFE2L2*, encode a transcription factor, which plays a crucial role in dendrite pruning (Chew et al., 2021; Tan et al., 2024). Dysregulation in neuronal pruning is implicated in many neurodevelopmental disorders including autism spectrum disorders (Faust et al., 2021). *kek6* encodes a receptor for *Drosophila* neurotrophin 2 (DNT2), which regulates structural synaptic plasticity (Ulian-Benitez et al., 2017). Closely related receptors in humans have similar functions (Mandai et al., 2009; Tessarollo & Yanpallewar, 2022). *S6KL* (S6 Kinase Like) is part of the BMP signaling pathway, where it interacts with Ube3a. In humans, both loss and gain of *UBE3A* function are associated with neurodevelopmental and cognitive defects including Angelman syndrome and autism (Akiyama et al., 2024; Li et al., 2016). *dpr8* (defective proboscis extension response 8) belongs to the *dpr* gene family, which has neuronal wiring functions (Cheng et al., 2019).

Genetics of sociability

In our current study and a related, recent one (Torabi-Marashi et al., 2025), we have identified a few hundred candidate sociability genes. We also tested the influence on sociability of 35 of the candidate genes and found that 28 of them had significant effects. While we are still far from having a full picture of the genetics of sociability, our rich data set is highly informative and provides a solid foundation for further research in our and other laboratories. For example, we are now in the process of examining the processes that determine the distinct dynamics of group formation in a few of our top validated sociability genes. We do this through detailed behavioral scoring of marked individuals in groups comprising either control flies or gene knockdown flies. As another example, the gene with the largest effect on sociability in our recent analysis (Torabi-Marashi et al., 2025) was *Sec5* (*Secretory 5*). Both *Sec5* and its human ortholog, *EXOC2*, encode proteins that are part of the exocyst complex, which has critical roles in neuronal development and function (Murthy et al., 2003; Swope et al., 2022). Moreover, mutations in *EXOC* genes have been linked to autism spectrum disorders (Halim et al., 2023; Li et al., 2014; Van Bergen et al., 2020). In an effort to translate our fruit fly work to mammals, we are now in the process of investigating the role of *EXOC2* in mouse sociability. We leave it to other laboratories to examine the developmental and neurobiological mechanisms that lead to distinct sociability as this is outside our expertise.

A still open question is to what degree the mechanisms that determine sociability are conserved across species. While some studies suggested genetic conservation of social behavior, others, including ours, have not indicated so (Roozen & Kas, 2025; Shpigler et al., 2017; Torabi-Marashi et al., 2025). One difficulty in searching for genetic conservation of a behavioral trait is having a clearly characterized and quantifiable behavior that can be compared among distant species. Clearly, social behavior, defined as interactions among conspecifics is too broad to be informative. This means that any attempt to search for genetic conservation would be limited because different studies might capture distinct social behaviors mediated by different genetic mechanisms. For example, Bralten et al. (2021) provided an admirable genetic analysis of human social behavior, which they defined as the "inclination to seek or enjoy social interaction." Unlike their thorough genetic analysis, however, they did not employ a validated scale for quantifying sociability. Indeed, one can readily question the strength of their 4-question scale, which included one question about loneliness and another about social embarrassment. To avoid the inherent ambiguity of the term "social behavior," we have focused on a specific and central social trait, sociability, which we define as individuals' tendencies to affiliate with others. Sociability can readily be quantified in many species including humans.

While our quantitative analyses comparing shared social behavior genes across species found no more common genes than expected by chance, it is clear from our discussion above that there is a fair number of conserved genes that modulate features of sociability in both fruit flies and mammals. These include *axo*/*CNTNAP2*, *rg*/*NBEA*, the *Nlg* gene group, genes involved in WNT signaling, and *cnc*/*NFE2L2*. Hence, further research on fruit flies can enhance our general understanding of the evolutionary biology, genetics, and neurobiology of natural variation in sociability.

Supplementary material

Supplementary material is available online at *Evolution*.

Data availability

Raw sequence data are available in the NCBI SRA database under BioProject: PRJNA1311514, with sample accessions: SAMN52927823–SAMN52927834 & SAMN53296674-SAMN53296677. Intermediate data and scripts for analysis are available at https://doi.org/10.6084/m9.figshare.30500915.v1 and https://github.com/DworkinLab/DrosophilaSociabilityGenomeScan

Author contributions

A.M.S., I.D., and R.D. designed, and A.M.S. ran the artificial selection experiment, A.T.M., T.A., and I.D. conducted the genomic analyses, I.D., R.D., J.R.R., and S.A. designed and J.R.S. and S.A. conducted the gene validation tests, A.T.M., R.D., and I.D. and wrote the manuscript.

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Conflict of interest

The authors have no conflict of interest to declare.

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