THE GUT MICROBIOTA'S EFFECT ON SOCIABILITY IN FRUIT FLIES

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By

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Lay Abstract

Many things in our internal and external environments can affect our social behaviours, including our gut microbiome. The gut microbiome could potentially affect social behaviours by interacting with the immune and nervous systems. Research has investigated the link between social behaviours and the gut microbiome in rodents mostly, but fruit flies are simple enough to be useful for this work. We worked with fruit flies to see how the gut microbiome affects sociability, a type of social behaviour that involves non-aggressive interactions with others. We removed the gut microbiomes of developing flies, and evaluated their sociability as adults. At first, we found that females without microbes were more sociable than normal females, but did not see this upon testing again. This difference could have been due to atmospheric and diet variations in the two experiments, and future research should see if these factors can affect social behaviours in flies.

Abstract

Social behaviour is defined as interactions between conspecifics. One facet of it, sociability, involves non-aggression interactions between conspecifics. Little research has investigated the genetics of sociability; more emphasis has been placed on its modulation by the environment. One component of the environment that has been of particular interest is the gut microbiome. Prior research indicates that the gut microbiome likely affects the behaviour of hosts via local manipulation and as an offshoot of metabolic processes, via potential channels including the vagus nerve and the immune system. The effect of the gut microbiome on social behaviour has mostly been investigated using rodent models. Fruit flies would be a useful model, given the simplicity of their gut microbiome and protocols to manipulate to it. There has been insufficient research on gut microbiome modulation of social behaviour in fruit flies (*Drosophila melanogaster*). We investigated the effects of the gut microbiome on adult fly sociability by generating germ-free wild-type flies and raising them on a germ-free food medium. After housing the flies in mixed-sex groups for 72 hours, we scored their sociability using a sociability assay developed in the Dukas lab at McMaster University. We found that germ-free females were more sociable than control females. A follow-up experiment did not confirm this effect. We postulate that this inconsistency in the results may have been due to reasons such as variation in food quality and atmospheric conditions, or a lack of robustness in the effects of microbes on sociability. Future work in this area would benefit from access to better-regulated microbial work facilities, and should focus on simulating environmental variation in diet and atmospheric conditions to discern its impacts on fly social behaviour.

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List of all Abbreviations and Symbols

Abbreviation:	Meaning:
ASD	autism spectrum disorder
AR	axenic room
CH	cuticular hydrocarbon
CVS	chronic variable stress
DGRP	*Drosophila* Reference Panel
dom	dominant
eAA	essential amino acid
ENS	enteric nervous system
GWAS	genome-wide association study
LB	Luria-Bertani
MFHD	maternal high-fat diet
MRD	maternal regular diet
NAR	non-axenic room
NOD	non-obese diabetic
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
rRNA	ribosomal ribonucleic acid
SCFAs	short-chain fatty acids
sub	submissive
UV	ultraviolet
VFA	volatile fatty acid
VPA	valproic acid

Declaration of Academic Achievement

I, Rajat Bhargava, was responsible for all microbial work, the majority of the behavioural work, all statistical analysis, and the academic writing. Reuven Dukas provided significant input on the experimental design, and significant feedback on all academic writing. Jianping Xu provided significant input on the microbial aspect of the experimental design, and contributed feedback that helped shape the final written thesis. Ben Evans and Ian Dworkin helped provide feedback that was useful for the final written thesis. Undergraduate students Aldruen Pates, Yatharth Patel, Andrew Roth, and Harmil Kalia greatly assisted with setup and observation on test days of experiments.

INTRODUCTION

Introduction to social behaviour

Social behaviour, which is defined broadly defined as interactions between conspecifics, has attracted attention across a plethora of species for many decades (Tinbergen 1953; Barnett 1958; Kruuk 1976; McGuire & Raleigh 1987; Heinrichs & Domes 2008; Anderson 2016). A vital facet of social behaviour is sociability, which is defined as the non-aggressive interactions between conspecifics. Examples of this include traveling in a group, roosting together, or feeding together (Scott et al. 2018).

The genetic and environmental impact on sociability

Prior research has investigated the genetic component of sociability in various species. Bralten et al. (2021) performed a genome-wide association study (GWAS) of sociability based on social functioning-oriented questions from adult humans (*Homo sapiens*) in the UK biobank. Through genetic correlation analyses, they looked at the genetic associations between their sociability scores and psychiatric disorders. They determined that population sociability's variation has a significant genetic component, and it is pertinent to various psychiatric illnesses. Petelle et al. (2015) determined the genetic variances of four characteristics-sociability, docility, activity, and exploration, in a wild-type population of yellow-bellied marmots (*Marmota flaviventris*). These traits showed significant additive genetic variance, and activity and sociability were positively correlated. Lee et al. (2021) disturbed and then restored expression of the Autism Spectrum Disorder (ASD)-associated gene *Shank3* in adult male mice (*Mus musculus*).

Restoration of *Shank3* in the medial prefrontal cortex resulted in improved sociability (assayed as a mouse's preference for an unfamiliar conspecific as opposed to a novel inanimate object) over a period of 5-8 weeks. Scott et al. (2018) developed novel arenas to assay sociability in fruit flies (*Drosophila melanogaster*), and found significant genetic variation in sociability in both sexes, and genetic variation in social plasticity amongst different genetic hybrid lines. They followed this up by artificially selecting on sociability in fruit flies (Scott et al. 2022), and found that after selecting for 25 generations, the high-sociability lineages had sociability scores about 50% higher than those of the low-sociability lineages. These lineages showed no differences in mating success, and an absence of relaxed selection after ten generations indicated no cost to maintaining high and low sociability. There is still a dearth of knowledge regarding the genetic underpinnings of sociability.

Prior research has also investigated the effect of the environment on sociability. Tõnissaar et al. (2008) used Sprague–Dawley rats (*Rattus norvegicus*) with high and low sociability to study the effects of chronic variable stress (CVS) on sociability. Cold water, tail pinch with a clothespin, etc. were the applied stressors. After three weeks, high sociability rats exposed to CVS showed lessened intake of sucrose in comparison with the baseline, suggesting that chronic exposure to stress makes highly social entities more easily anhedonic. Trezza & Vanderschuren (2008) were interested in seeing the effects of cannabinoid agonists on social play behaviour in adolescent male Wister rats (*Rattus norwegicus*). Their results indicated that cannabinoid neurotransmission can both enhance and inhibit social play in adolescent rats, depending on how the endocannabionoid system

is stimulated. Campolongo et al. (2018) found that mice exposed to valproic acid (VPA) during gestation, and later reared with VPA mice, engaged in less play as juveniles and less frequent social interaction as adults. Interestingly, these behavioural modifications were not observed for VPA mice reared with control mice. Their results support the notion that social enrichment during early life can restore sociability in a mouse ASD model. Hewlett et al. (2018) similarly found evidence for early social enrichment and its benefit on sociability. Western honey bees (*Apis mellifera*) showed impairment in their sociability due to early adulthood isolation, but isolated bees even up to the age of six days exposed to their hive for the period of a day recovered significant sociability.

Evolutionary theory of parasitic manipulation and the gut microbiome

An aspect of the environment that has become a hotbed for research is the gut microbiome. The gut microbiome is increasingly being understood to influence brain physiology and behaviour, and studies are starting to show how it can affect the central nervous system (CNS) through a conceptualized bidirectional gut microbiome-brain axis (Davidson et al. 2020). The idea of microbes as parasites that are capable of manipulating host behaviour at the global level is widely accepted. The proponents of this idea cite examples such as ants infected with *Ophiocordyceps* or *Pandora* fungi leaving behind their maternal nesting area to perish in areas that are most conducive to fungal spread (Andersen et al. 2009; Csata et al. 2021), flies infected by the fungus *Entomophthora muscae* moving to elevations promoting fungal sporulation and dying there soon thereafter (Elya et al. 2022), *or* the protozoan *Toxoplasma gondii* eliciting a decreased aversion of rodents towards cat odours (Vyas et al. 2007). Johnson and Foster (2018)

provide compelling evidence against this line of thinking, by applying evolutionary theory of parasitic manipulation and host-symbiont interactions to the microbiome. They posit that influences on host behaviour via manipulation of functions at the local level and as a by-product of microbial metabolism are more plausible than host level behavioural manipulation. I will cover their main points here.

The evolution of the manipulation of host behaviour at a global level is only expected to occur in the absence of other competing non-manipulative microbial strains in the gut microbiome - which would reap the benefits of the manipulation without any costs, unlike the manipulative bacteria strain - and when the benefits of host manipulation outweigh the costs of manipulation (Johnson & Foster 2018). Such conditions are not likely to occur (Vickery & Poulin 2010). The evolution of costly manipulation mechanisms is not favoured when a microbial strain competes with other strains, as these will hinder the strain's ability to survive in the gut niche (Johnson & Foster 2018).

The local manipulation explanation for how the microbiota affects host behaviour says that symbiotic microbes are naturally selected to manipulate their immediate gut surroundings in a way that has salient benefits for them, and this in turn influences the host's CNS and behaviour as a side effect. A potential mechanism for how this may work is local modifications to the host's enteric nervous system (ENS), which could then affect the host's behaviour via correspondence between it and the CNS (Rao et al. 2016). The immune system and autonomic nervous system are extensively connected (Kenney & Ganta 2014), and so it is possible that microbial effects on the immune system could lead to behavioural alterations. Local manipulation seems more evolutionarily plausible than

global manipulation of host phenotypes, but still, there is a simpler explanation for how microbes can affect the behaviour of the host.

The simplest explanation for how microbes manipulate host behaviour is that they create by-products through their regular metabolic activities, which can initiate a cascade that ultimately affects the host behaviour (Johnson & Foster 2018). Examples of such metabolites are short-chain fatty acids (SCFAs), which can elicit immune responses (Corrêa-Oliveira et al. 2016), interact with the ENS and induce secretions (Mirzaei et al. 2021), etc. Another plausible explanation for host behaviour changes as they relate to the effects of microbes is what is known as 'evolved dependence', which states that hosts that evolve alongside a symbiotic microbe may come to rely on it in terms of their physiological functioning (Weinersmith & Early 2016). In the absence of particular microorganisms, host physiology may be compromised, leading to effects on behaviour (Johnson & Foster 2018).

Physiological mechanisms underlying the bidirectional gut-brain axis

Sarkar et al (2020) uses Johnson and Foster (2018)'s line of thinking, and expands by thoroughly discussing available research on the actual physiological mechanisms that mediate the bidirectional gut-brain axis. Sylvia et al. (2018) also proposes potential mechanisms for this. I will briefly cover some of their main points here, supplementing them with additional studies that I found in the literature. There is a burgeoning literature indicating the immune system as being a potential channel through which the gut microbiota interacts with the brain. Bailey et al. (2011) for instance presented a social

stressor to CD-1 mice, and found an increase in interleukin-6 (IL-6), which was associated with stressor-induced changes in some bacterial genera. Antibiotic use significantly reduced the number of bacteria in the mice, as well as prevented the increase in IL-6. Agranyoni et al. (2021) performed selective breeding using the Sabra outbred mice strain to generate dominant (dom) and submissive (sub) mice, and transferred their gut microbiomes to male Swiss Webster mice using fecal transplantation. They found an uptick in numerous adipokines in both sub mice and germ-free mice that received the sub fecal transplant, in comparison to dom mice and germ-free mice that received the dom fecal transplant. They also found that germ-free/sub mice adopted the anti-social characteristics of sub mice. The upregulation of adipokines in both sub and germ-free/sub mice provides support for the role of the gut microbiota in adipocyte inflammation, which may affect social behaviours.

The vagus nerve has been suggested as a facilitator of gut microbiota brain cross-talk. Studies like Sgritta et al. (2019), where a vagotomy (vagus nerve removal) eliminated benefits of a probiotic, lend credence to this idea. The gut microbiota could be affecting the social brain by regulating signalling molecules. Microbial metabolites such as SCFAs can modulate the activity of host cells that produce signalling molecules, or microbes can directly activate signalling molecules (Sarkar et al. 2020). An example of such molecules would be glucocorticoids. Glucocorticoids can cross the blood-brain barrier, and germ-free (axenic) rodents have shown increased levels of corticosterone when exposed to stress in comparison to controls (Crumeyrolle-Arias et al. 2014). There is evidence for the gut microbiota also modulating the signaling of a neuropeptide

hormone often associated with sociability, oxytocin. Desbonnet et al. (2015) for instance found that antibiotic use in mice reduced hypothalamic oxytocin levels. Sgritta et al. (2019) interestingly found that treatment of *Shank3*-knockout mice with *Lactobacillus reuteri* increased oxytocin expression in the paraventricular nucleus. Buffington et al. (2016) also interesting found the same result as Sgritta et al. (2019), in the offspring of mothers on a high-fat diet.

Another mechanism through which the microbiome could be affecting behaviour is olfaction, in what can be called the microbiome-olfaction-behaviour pathway. The fermentation hypothesis posits that olfactory cues are bacterial metabolites that are used for chemical communication (Archie & Theis 2011). Bacteria make many odourants. Since there is the understanding that bacterial products can function as signalling molecules that directly or indirectly elicit behavioural change, there is a dire need to include olfaction into studies of the gut-brain axis (Bienenstock et al. 2018). Theis et al. (2013) sequenced the bacterial communities in pastes (scent markings) of wild spotted and striped hyenas (*Crocuta crocuta*). They found that the hyena pastes were teeming with odour-producing fermentative bacteria, and bacterial communities there had structures that covaried with volatile fatty acid (VFA) profiles (the main constituents of pastes) in the pastes. As well, the spotted and striped hyenas had different VFA and bacterial profiles, and these profiles varied with the sex and reproductive status of spotted hyenas occupying the same group. This study supports the fermentation hypothesis. Grieves et al. (2021) looked at the preen oil chemical composition and preen gland bacterial composition in song sparrows (*Melospiza melodia*). Bird body odour mostly

consists of preen oil. They found that the preen oil's chemical makeup differed across populations and between sexes. They also found population differences but no sex-based variations in the microbial community structure of the preen gland. There was no association between preen gland microbiota and preen oil chemicals overall. This study does not corroborate the fermentation hypothesis. As Sarkar et al. (2020) notes, overall, there is little and often conflicting evidence regarding physiological pathways that mediate microbial contributions to host social behaviours. More well-designed experiments to elucidate these physiological links are required.

Effect of the gut microbiome on social behaviour in rodent models and other animals

The effect of the gut microbiome on social behaviour has mostly been investigated using rodent models thus far. Three of the most common laboratory techniques used in conducting this research are the use of pharmacological reagents (such as antibiotics, probiotics, and prebiotics), germ-free (axenic) models, and microbiota fecal transplantation. These are covered in detail elsewhere (Sarkar et al. 2018). Mice studies have shown that axenic mice display deficits in sociability (Hsiao et al. 2013; Desbonnet et al. 2014; Stilling et al. 2018; Buffington et al. 2016), which microbiota re-integration can partially restore (Stilling et al. 2018; Buffington et al. 2016). A study by Arentsen et al. (2015) interestingly found that an absence of the gut microbiome actually increased sociability in adult male mice; it should be noted that these researchers speculate the discrepancy between their results and prior literature to be potentially due to the particular

strain of the stimulus mice used in their three-chamber social approach test. Some studies have looked at the impact of parental diet of rodents on the gut microbiome of offspring, along with their social behaviour. Buffington et al. (2016) created maternal high-fat diet (MFHD) C57Bl6/J mice offspring, by feeding mothers with high-fat diets. These offspring showed reduced preference for social novelty and sociability. Since mice exchange gut microbiota via the fecal-oral route (Ridaura et al. 2013), they co-housed MFHD offspring with MRD (maternal regular diet) offspring for about 4-5 weeks, and interestingly found that these MFHD mice exhibited normal sociability and preference social novelty. Gacias et al. (2016) worked with two genetically different mouse strains, non-obese diabetic (NOD) mice and C57BL/6 mice. They found that daily administration of vehicle (dH_2O) in NOD mice made them socially avoidant. Transfer of gut microbiota from NOD mice that underwent vehicle administration to recipients treated with antibiotics made them socially avoidant, and led to their microbiota having an abundance of *Clostridiales*. Afroz et al. (2021) worked with C57Bl/6 mice and found that a high salt parental diet reliably altered the gut microbiome of offspring. It also resulted in ASD-like behavioural abnormalities in adult male mice, including increased hyperactivity, increased repetitive behaviours, and reduced sociability. Fecal microbiota transplantation with rodents has been used in the context of investigating other aspects of social behaviour as well. Agranyoni et al. (2021) found that adult mice that share closely related genetic backgrounds but exhibit a distinct social characteristic of dominance or submissiveness possess significantly different gut microbiota compositions, which correspond to their social behaviours. The role of the gut microbiome in shaping social

behaviour was corroborated by a follow up fecal microbiota transplantation experiment, during which a single transplantation from submissive donor mice caused axenic mice to adopt submissive behavioural patterns. Watanabe et al. (2021) found that axenic BALB/c mice were more aggressive than controls, and transplanting control mice feces in 0-week-old germ-free mice significantly reduced their aggression.

The effect of the gut microbiome on social behaviour has been investigated in other species as well. Perofsky et al. (2018) looked at Verreaux's sifaka (*Propithecus verreauxi*), a type of lemur, and found that a social group's more gregarious individuals have more diverse gut microbiota. Supplementation of honey bees with *Lactobacillus* and *Bifidobacterium* increased eusocial cooperative behaviours (Alberoni et al. 2018). Sylvia et al. (2017) used Siberian hamsters (*Phodopus sungorus*) to investigate the effects on social behaviour of administering a broad-spectrum antibiotic over a short period of time. They found that a single antibiotic treatment markedly reduced aggression in females. Cusick et al. (2022) used Siberian hamsters to look at interactions between maternal stress and maternal microbiome manipulations on offspring social behaviour. They found that female offspring pertaining to stressed mothers exhibited elevated aggression in comparison to control female offspring, whereas males showed no difference in comparison to control male offspring. The combination of stress and antibiotic use yielded interesting results, where female offspring showed similar levels of aggression to control female offspring, and male offspring showed slightly more aggression than control offspring. The alteration of the maternal microbiome modulated prenatal stress effects in a sex-specific manner evidently.

The effects of social behaviours and social environments on the gut microbiome

The bidirectionality of the gut-brain axis implies that social behaviour can initiate a cascade that affects the gut microbiota. Some research has indeed looked at the microbiome-social behaviour relationship in the opposite direction, where social behaviour may affect the gut microbiome. It should be noted that this research is often correlational, making it difficult to say anything definitive. Antwis et al. (2018) performed social network analysis and 16s ribosomal ribonucleic acid (rRNA) gene amplicon sequencing in Carneddau Welsh mountain ponies (*Equus ferus caballus*). They found that social interactions, such as stallion-mare and mother-offspring interactions, spatial structuring, and network ties affect the composition of the gut microbiome. Individuals in this species impact each other's gut microbiome, and ultimately also impact that of the entire group. Tung et al. (2015) looked at two social groups, comprising wild adult yellow baboons (*Papio cynocephalus*) from the Amboseli ecosystem in southern Kenya. They found that an individual baboon's microbiome composition were predicted by membership in a given social group, as well as its contacts in a grooming-based social network. Since the two social groups lived in a homogenized environment and had a shared diet, researchers were able to exclude diet as the reason for the association between social proximity and gut microbial composition. Their findings suggest that social interactions of groups are crucial for the transmission of gut microbiomes between members of the same group. Amaral et al. (2017) placed mother-reared infant rhesus monkeys (*Macaca mulatta*) in small social groups, and found there to

be homogenization of their gut microbiota within 2 weeks. Microbial communities were more similar within peer groups than across groups. Moeller et al. (2016) looked at Kasekala chimpanzees (*Pan troglodytes*) from Gombe, Tanzania, and found that gut microbiomes of the chimpanzees were more homogenized during seasons of increased sociability. They also found that regular social interaction yielded increased diversity within individual microbiomes. Taken together, their results indicate that social behaviour may play a role in the generation of the gut microbiome in this species, and in the maintenance of the richness of microbial species. Kwong et al. (2017) collected bees at several sites around the world, and found that five central gut bacterial lineages exist in corbiculate bees (*Hymenoptera: Apidae*)-a clade consisting of pollinators- the acquisition of which aligned with the appearance of their eusociality. Their results indicate that vertical transfer of microbes through social contact may have had a pivotal role in shaping the corbiculate bee microbiome over the clade's evolutionary history.

Some studies have found a negative association of social behaviour with the gut microbiome. Raulo et al. (2018) worked with red-bellied lemurs (*Eulemur rubriventer*) to see if gut microbiome was associated with various social factors, including individual sociability, group membership, and social network position. They found that social network position and group membership predicted gut microbiota composition, but surprisingly there was a negative correlation between the diversity of the gut microbiome and individual sociability. Powell et al. (2018) and Copeland et al. (2022) both looked at queen bees in Western honey bees, and found counterintuitively that deficient social environments led to increased gut microbiome size and diversity. Their results undermine

the importance of the social environment for maintaining the diversity of the gut microbiome, but nonetheless point towards social behaviour possibly affecting the gut microbiome.

The case for fruit flies as a model for studying the effects of the gut microbiome on social behaviour

To date, non-human studies looking at the effects of the gut microbiome and social behaviour have focused on mostly rodent models (Sherwin et al. 2019). Our comprehension of how microbes interact and affect social behaviour is limited by our use of a select few animal models, and other model organisms should be considered. Soares et al. (2019) suggest the use of fish models like zebrafish to look at the bidirectional relationship between the gut microbiome and social behaviour. The gut microbiome of fish is instrumental in the innate immune response (de Bruijn et al. 2018), and exhibits shifts in its composition over the course of development (Shin et al. 2015). The limitations with using fish models for this research is that living in aquation conditions could increase the possibility of microbial contamination through the water (Soares et al. 2019). Insects present intriguing models, as they vary markedly in terms of the attributes of their gut microbiome, as well with regards to how sociable they are (Liberti et al. 2020). There is a host of insect literature that points towards microbes affecting odour profiles to impact conspecific social interactions and chemical signaling (Wada-Katsumata et al. 2015; Venu et al. 2014; Sharon et al. 2010; Aguilera-Olivares et al. 2016), which further makes insects an intriguing prospect. Fruit flies emerge as a viable

simple animal model that can be used in gut microbiome research. Relative to mammals, this simple system allows for unambiguous methodology to alter the gut microbiome, and ascertain the function of individual microbial strains. Axenic fruit flies growing on nutrient-rich media can persist for many generations, and likely indefinitely. In contrast, experimentation with axenic roundworms (*Caenorhabditis elegans*) and zebra fish (*Danio rerio*) (other simple animal models) is usually confined to the larval stage, as roundworms require bacteria to fully develop, and zebra fish axenic protocols are costly (Douglas 2019). This, alongside recent discovery of stable colonizing microbial strains in fruit flies (Ludington & Ja 2020) makes the fruit fly an intriguing model for gut microbiome research.

Gut microbiome research in fruit flies

Gut microbiome research with fruit flies has investigated topics including the role of the gut microbiome in locomotion and activity modulation (Schretter et al. 2018; Selkrig et al. 2018), its effects on memory (Silva et al. 2021), sleep (Selkrig et al. 2018; Silva et al. 2021), olfaction-based foraging (Wong et al. 2017; Qiao et al. 2019), physiological changes during aging (Ren et al. 2007; Clark et al. 2015; Lee et al. 2019) and in the modeling of various neurodegenerative diseases (Feltzin et al. 2019; Wu et al. 2017; Westfall et al. 2019; Kong et al. 2018).

The research on the gut microbiome and social behaviour in fruit flies is still scarce however. Venu et al. (2014), like Qiao et al. (2019) and Wong et al. (2017), looked at the role of the gut microbiota in olfactory-guided food decision-making, but looked at it through the lens of social attraction. They found evidence for fruit flies relying on larval

microbiome-derived volatile metabolites for attraction to profitable food patches found at long distances. Research has looked at the role of the gut microbiota in the mating of fruit flies. Lizé et al. (2014) looked at kin selection in three species of *Drosophila*, one of which was monandrous, another one lived in dense aggregations, and another one was a food generalist species. They wanted to know whether relatedness, familiarity, and food eaten during development modulated copulation investment in the three species. In the food generalist species, they found that the food consumed during development effectively masked true kin recognition. Food type affected copulation duration, and antibiotic treatment eliminated this effect, indicating the influence of the gut microbiota. Leitão-Gonçalves et al. (2017) looked at the effects of commensal bacteria on reproduction in fruit flies, and found that commensal bacteria provision reversed decreased reproduction due to essential amino acid (eAA) deprivation. Sharon et al. (2010) found an influence of commensal bacterial on fruit fly mating preferences. Depending on which diet the fly was raised (starch or corn-molasses-yeast), they showed a mating preference that continued for many generations. Antibiotic treatment removed this preference, indicating that it was facilitated by the gut microbiota. Further infection experiments with microbes from fly media before antibiotic treatment confirmed this. Analysis of cuticular hydrocarbon (CH) profiles in antibiotic treated and untreated flies indicated that the gut microbiota could be affecting the levels of sex pheromones. Najarro et al. (2015) successfully replicated the diet-induced mating preferences observed in Sharon et al. (2010) using a different fruit fly strain.

Some studies have shown contradictory results regarding the effects of the gut microbiome on social behaviours in fruit flies. Microbiota-based alterations in mating were reported in some studies (Sharon et al. 2010; Najarro 2015), but not others (Leftwich et al. 2017; Selkrig et al. 2018). Chiang et al. (2022) note that these studies all used different wild-type flies and had different methods for generating the axenic condition, which may have caused a variety of consequences on the host and led to these inconclusive results. Rosenberg et al. (2018), a letter from individuals involved in the work of Sharon et al. (2010), pointed out that while Leftwich et al. (2017) published a properly controlled, sufficiently powered experiment trying to replicate the work of Sharon et al. (2010), they may have failed to reproduce the mating preference results because they switched from 0.1% methyl paraben in the media used before the experiment to 0.3 % methyl paraben in the media during the experiment. They note that in their own work they found that methyl paraben essentially eliminates mating preference when it is present in too high concentrations. Other contradictory results can be seen with regards to aggression. Grinberg et al. (2022) found that antibiotic-treated male flies had heighted aggression (Grinberg et al. 2022), whereas Jia et al. (2021) found that axenic male flies showed a decrease in it (Jia et al. 2021). Grinberg et al. (2022) speculate that this discrepancy in results could be due to the procedure applied to remove fly microbes, facility-related effects on wild-type fly microbiomes, and differences in behavioural tests. Interestingly, there is evidence that *Wolbachia* infection can increase aggression in male flies (Rohrscheib et al. 2015). Another study (Arbuthnott et al. 2016) looked at the effect of *Wolbachia* infection of female attractiveness, and did not find a

significant effect. They note that it is imperative for studies to have proper controls, as if they did not have uninfected, antibiotic-treated lines in their assay, they would have incorrectly declared an effect of *Wolbachia* on female attractiveness.

CHAPTER 1: EFFECT OF GUT MICROBIOTA ON SOCIABILITY IN FLIES

1a. Does removing the gut microbiota affect the sociability of flies?

Rationale

There is an evident lack of research on the effects of the gut microbiome on social behaviour in fruit flies. In particular, there is a dire need to investigate how the gut microbiome affects sociability. This would give us a better informed evolutionary ecological perspective on how sociability can evolve and be maintained, and give us greater insight into underlying causes for deficiencies in sociability. Chen et al. (2019) did look at something that appears to be sociability, which they label as 'direct social contact'. Their protocol involved placing two same-sex flies into different layers of a two-layer chamber, with a plastic transparent barrier in place until the 20 minute direct social contact assay started. They then derived a direct social contact index, which provided a measure of the proportion of the total observation period that the two flies spent in direct contact with each other. Their protocol only looked at only two males or females at a time. Scott et al. (2022) used a sociability arena protocol that allows for the interaction of multiple same-sex flies at a time, which we find to be more ecologically valid. The protocol of Chen et al. (2019) also seems to have no clear metric for determining what constitutes close social proximity, whereas for Scott et al. (2022) the determination is

clear: flies in the same compartment are in close social proximity to each other. We wanted to focus our investigation on the effects of the gut microbiome on fruit fly sociability. We used the protocol of Venu et al. (2014) for creating axenic fruit flies, and a sociability selection arena protocol from Scott et al. (2022) to test this. We used a fly population sourced from wild-caught female flies from Hamilton, Ontario in 2018.

Protocol:

The procedure for creating the axenic cultures was adapted from Venu et al. (2014) and Brummel et al. (2014), along with insight from Silva et al. (2021) regarding when to set up axenic and control treatments. This procedure involves dechorionation, which removes the chorion (outer protective layer of the egg). Emerging larvae feed on the chorion and ingest the microbes on it, generating the basis of their microbiome. This is why it is imperative to remove the chorion to create truly axenic flies (Bakula 1967). The procedure for setting up and running the sociability test was adapted from Scott et al. (2018, 2022). We ran a total of five replicates.

Setting Up Vials for Axenic Flies

Preparing and Storing Stock Solutions for Antibiotics and Antifungals

We prepared stock solutions with concentrations of 4.16 mg/mL, 10.40 mg/mL, 2.60 mg/mL, 5 mg/mL, and 104.00 mg/mL for ampicillin, chloramphenicol, fluconazole, benomyl, and methyl paraben respectively. With the exception of ampicillin, which we dissolved in double distilled water, we dissolved everything in 100% ethanol. Before mixing and dissolving, we added the solvent for each stock solution to an autoclaved

glass container using a graduated cylinder. Once we added the solutes and mixed and dissolved them in their respective solvents, we used 45 µm single-use syringes and filters to sterilely add the stock volumes to new autoclaved glass containers. We stored the methyl paraben and fluconazole stock solutions at room temperature, the chloramphenicol and benomyl stock solutions at 2-8 °C in a refrigerator, and the ampicillin stock solution in a -20 °C freezer.

Preparing Antibiotic-Free LB Agar Plates

We used 15 mm diameter petri dishes. The concentration of Luria-Bertani (LB) broth and agar in the LB agar solution was 25 g/L and 18g/L respectively. We autoclaved the LB agar solution in a glass Pyrex container using a stir bar. After this, under a laminar flow cabinet with its blower turned on, we poured approximately 10 mL of the LB agar solution to each petri dish. We allowed the plates to dry overnight in the laminar flow cabinet before refrigeration at 2-8 degrees Celsius.

Sterilizing Equipment (Up Until Dechorionation)

We carefully sterilized equipment (plastic vials, brushes, fly mesh, etc.) up until the dechorionation stage using ethanol and ultraviolet (UV) irradiation before use. We rinsed almost everything with 70% ethanol, and allowed it to dry briefly. During that time, we wiped a level II biosafety cabinet down with 70% ethanol and cleaned it using UV irradiation for 15 minutes. After this, we placed the equipment in the level II biosafety cabinet and exposed it to UV radiation for 15 minutes. We were careful to avoid looking directly at the UV radiation, or exposing any skin to it.

Preparing Axenic Food

This was the same as the regular protocol for making standard food in the Dukas lab at McMaster University, Canada, with some modifications.

We prepared standard food (1 % w/v agar powder, 7.5% w/v cornmeal, 9% w/v sucrose, 3.2% w/vol baker's yeast (*Saccharomyces cerevisiae*)) in a glass Pyrex container placed on a hotplate. We used a whisk to break up any clumps, and a spatula to stir evenly. This needed to be stirred well to evenly distribute the yeast and to break up any clumps. Once we had added these ingredients by the 50 °C mark, we autoclaved the solution. During this time, we sterilized the spatula and thermometer using ethanol and irradiated with UV light for 15 minutes under a level II biosafety cabinet. At the conclusion of 30 minutes, we transferred the solution to a laminar flow cabinet and used a thermometer to observe it cooling to 50 degrees Celsius. When it reached this point, we used a 1000 μL micropipette to add antibiotics (2 g/L methylparaben, 10 mg/L fluconazole, and 5 mg/L benomyl) and antifungals (50 mg/L ampicillin and 20 mg/L chloramphenicol) to the food. We stirred the food thoroughly to make sure the antibiotics and antifungals were homogeneously spread throughout it. Immediately after this (to prevent over-cooling and solidification), we transferred the food to about 16 autoclaved glass vials in increments of roughly 10 mL/vial.

We exposed the vials to the blower in the biosafety cabinet for approximately four hours prior to dechorionating, to get rid of excess moisture.

Setting Up Egg-Laying + Retrieving Bottles

We set up bottles with standard food at around 9 p.m., creating grid pattern markings in the center that were about 2 mm deep into the food (this encourages egg-laying around the edges). We added one bottle to each cage of the focal wild-type population. At around 9 a.m. the next morning, we removed the bottles from their cages.

Checking for Contamination in the Axenic Vials

Before dechorionation, we spot checked for contamination by swabbing the food in a few sample axenic vials and plating on agar LB plates.

Dechorionation + Checking to See if it Worked

We cut down each bottle, so that the exterior plastic was only slightly above the level of the food inside. This simulated working with a petri dish.

We sterilized equipment as described before in "Sterilizing Equipment (Up Until Dechorionation)". We transferred everything over to a laminar flow cabinet and turned on the blower. We added distilled water to the surface of the bottle and gently wiped with a sterilized paintbrush to suspend the eggs. We then poured the slurry over the center of a mesh held over a waste container to collect the eggs.

We placed the center of the mesh with the eggs over a sterilized container, and poured 90 mL of a 2.7% sodium hypochlorite solution (two times diluted industrial grade bleach) through the mesh gently. We let the eggs soak in the solution for two minutes to remove the chorion from the embryo. We used sterilized forceps to gently dip the center

of the nylon mesh (with the eggs) in and out of the solution periodically (to resuspend the eggs) over the course of these two minutes.

We then set up a funnel over a waste container. We placed the center of the mesh into the funnel. We used 90 mL of distilled water twice and 90 mL of 70% ethanol twice to wash the eggs. Using a sterile brush, we transferred the eggs to the axenic vials in the laminar flow cabinet. We transferred 40-60 eggs to each axenic food vial. We left one axenic food vial without eggs and used it to spot test for contamination via plating at this point; we observed the LB plate over the course of 1-2 days to confirm that the food was axenic. We capped each vial (including the axenic food vial without eggs) with an autoclaved foam plug after we had added eggs to it. Once we had set up the vials, we transferred them to a clear plastic container designated for the axenic flies, with conditions of 25 °C, 50% relative humidity, and a 12:12 hour (regular) light:dark cycle.

Setting Up Vials for Control (Xenic) Flies

We did the egg-laying for the control flies approximately 48 hours after that for the axenic flies, based on the insight from Silva et al. (2021) that axenic flies reliably eclose 36-48 hours after control flies. Sterilization using ethanol and UV was not imperative here, like it was for setting up axenic fly vials.

Preparing Standard Food

This was the same as the protocol for preparing axenic food, except we added no antibiotic reagent, and the only antifungal reagent we added was methyl paraben.

Setting Up Egg-Laying + Retrieving Bottles

This was the same as the protocol for axenic flies.

Rinsing with Distilled Water

We cut the bottles so the plastic was slightly above the level of the food medium. We added double distilled water to the surface, and used a brush to suspend the eggs. We then poured the slurry over a mesh held over a waste container to collect the eggs. We inserted the mesh inside a funnel, and washed into the waste container with four rounds of double distilled water.

We then transferred the eggs using a brush to the standard food vials at a density of 40-60 eggs/vial. We left one standard food vial without eggs and used it to set up a positive control plate at this point; we inspected the LB plate over the course of 1-2 days. We capped all vials (including the standard food vial with no eggs) with foam plugs, and transferred them to a clear plastic container designated for the control flies, with conditions of 25 °C, 50% relative humidity, and a 12:12 hour (regular) light:dark cycle.

Plating and Sexing Axenic and Control Flies

We spot tested via plating from the two axenic and control vials set up without eggs. We observed these over the course of 1-2 days to confirm that the axenic food did not have microbial contamination and that the standard food was a proper positive control.

We discarded any axenic flies that eclosed less than 36-48 hours later than the time it took for control flies to eclose. This means that legitimate axenic flies and control

494 flies should have eclosed around the same time. We used a few flies from each axenic fly
495 vial with an equal sex ratio (Heys et al. 2018) to prepare homogenates on LB agar plates.
496 We observed them over the course of 1-2 days to check for any microbial growth, which
497 would indicate microbial contamination in particular vials, rendering them unusable. We
498 also prepared homogenates using a couple of flies with equal sex ratio from each control
499 fly vial, to set up positive control plates.

500 When the axenic and control flies eclosed, we cleared them and sexed them under
501 a laminar flow cabinet within 8 hours of clearing. We sexed them on ice, using a petri
502 dish and a paint brush that we had sterilized using ethanol and UV irradiation. We
503 transferred 10 axenic males and 10 axenic females to each of ~ 14 new axenic food vials
504 (extra vials set up to account for potential fly mortality or escape). We transferred 10
505 standard males and 10 standard females to each standard food vial. Interestingly, we
506 consistently observed that the axenic flies took on average a minute and a half less than
507 the control flies to knock out during ice exposure. We then transferred the vials to
508 designated plastic containers with conditions of 25 °C, 50% relative humidity, and a 12
509 hour: 12-hour light:dark cycle for approximately 72 hours.

510 **Preparation for Test**

511 12 hours before the 72-hour housing period had finished, we made axenic food and
512 control food at a thickness of 1 mm in petri dishes sterilized using ethanol and 15 minutes
513 of UV light exposure. We left these to dry using the blower of a level II biosafety cabinet

for 1.5 hours, before capping them with their covers and putting them in a refrigerator at 2-8 degrees Celsius.

A few hours before the 72-hour housing period had finished, we sterilized the sociability arenas, aspirators, spatulas, Tupperware containers, and metal stands that we used during the testing by using ethanol and 15 minutes of UV light exposure. Each Tupperware container was large enough to fit a metal stand that would hold sociability arenas on its horizontal surface. An hour before the 72-hour housing period had finished, we prepared an orange juice + yeast solution (3g yeast/100 mL), by autoclaving the orange juice and mixing the yeast into it under a laminar flow cabinet after it had cooled to 50 °C. We poured the solution over the food in petri dishes in a laminar flow cabinet so that a thin layer covered the food, and left it to refrigerate for a short time.

Sociability Arenas

The sociability arenas were 3D printed circular dishes that were 37 mm wide and 5 mm high. They had eight compartments, divided by thin walls that had 5 mm wide and 3 mm high openings. The arenas were capped with a Petri dish cover with a 3 mm hole. A schematic of a sociability arena is provided in figure 1.

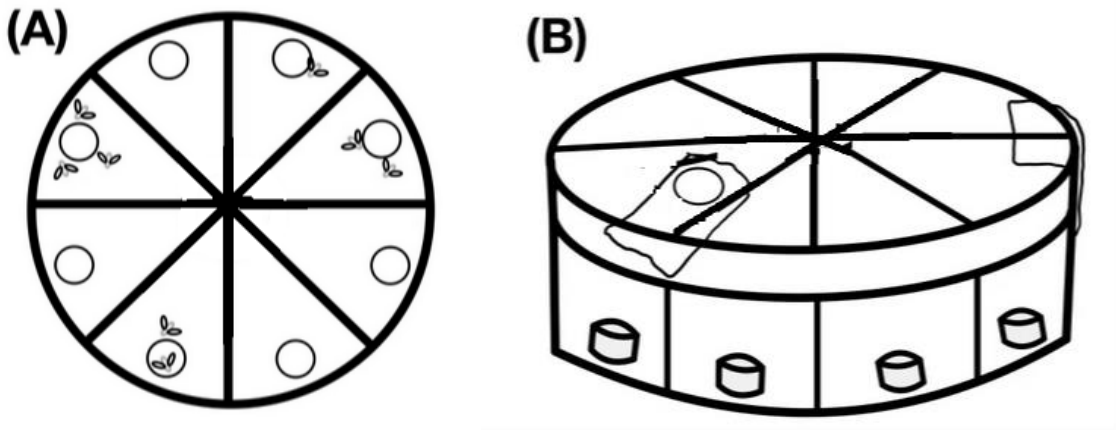

Figure 1: Diagrams of sociability arena. A) Top view without any lid. Circles represent food patches. B) Side view with a lid taped down on both sides (one piece of tape covers the opening in the lid through which flies are added).

Test

72 hours after we had transferred the flies to their individual housing conditions, we set up the sociability arenas. We created circular food patches 5 mm in diameter, and added one to each compartment in each sociability arena using a spatula. We added a thin piece of tape to two opposing sides of each arena's lid, to make sure that the lid did not shift after we added the flies. The individual adding the flies used scent-free mouthwash to try to reduce any transference of bacteria from them to the flies while aspirating. We placed the arenas on a metal rack with a flat surface, which we placed inside a large Tupperware container. We then placed a sterilized container filled with double deionized water below the metal rack, to maintain approximately 50% relative humidity. The relative humidity of the room where we ran our tests fluctuated day to day based on external atmospheric conditions, and thus the amount of water required to maintain a relative humidity of about 50% in the container varied between different test days.

We left the flies in their arenas for a 1.5-hour acclimation period, with the lid placed on top of the Tupperware container. After this, we observed how many flies were in each compartment at the start of a 1.5 observation period and every 10 minutes thereafter.

In this experiment, the total sample size for each of the four treatments was 30 arenas. There were no fly mortalities or escapes from the arenas during the tests.

Data Processing and Statistical Analysis

We used the observation values to calculate a sociability score based on an aggregation index, as done by Scott et al. (2018, 2022). The sociability score is equal to variance/mean. A score of zero would represent the lowest possible score, a score of one would mean that there is a random distribution, and a score of eight would represent the highest possible sociability score. We then averaged the data for each arena across the entire 1.5-hour observation window.

We used R (version 4.2.0; R Core Team 2022) to conduct the statistical analyses. We used the 'DHARMa' R package (version 0.4.6; Hartig & Hartig 2022) to make diagnostic plots, and constructed a linear mixed effect model using the 'lmer' function of the 'lme4' package (version 1.1-31; Bates et al. 2015). 'Treatment', 'sex', and the interaction between them were treated as fixed effects, 'sociability score' was the response variable, and 'box number', 'day', and 'arena number' were random effects. The "lmerTest" R package (version 3.1-3: Kuznetsova et al. 2017) was used to extract t-values and p-values from the linear mixed effect model. We used the 'emmeans' function of the 'emmeans' R package (version 1.8.2; Lenth 2022) to conduct pairwise comparisons between the different groups. The emmeans function uses the Tukey method to adjust p-values for multiple comparisons (Lenth 2022). We used the 'ggplot2' package (version 3.3.6; Wickham 2016) to create a boxplot.

Results:

We found a main effect of treatment, where axenic flies unexpectedly had higher sociability scores than control flies ($t_{(111)}$=2.660, p<0.01; Fig. 2). There was also a main effect of sex, where females had higher sociability scores than males ($t_{(110)}$=3.900, p <0.001; Fig. 2). There was also an interaction between treatment and sex ($t_{(111)}$=2.655, p<0.01; Fig. 2). Adjusting for multiple comparisons, axenic females were more sociable than axenic males ($t_{(110)}$= 3.900, p<0.001; Fig. 2), control females ($t_{(111)}$= 2.660, p <0.05; Fig. 2), and control males ($t_{(107)}$= 2.806, p<0.05; Fig. 2).

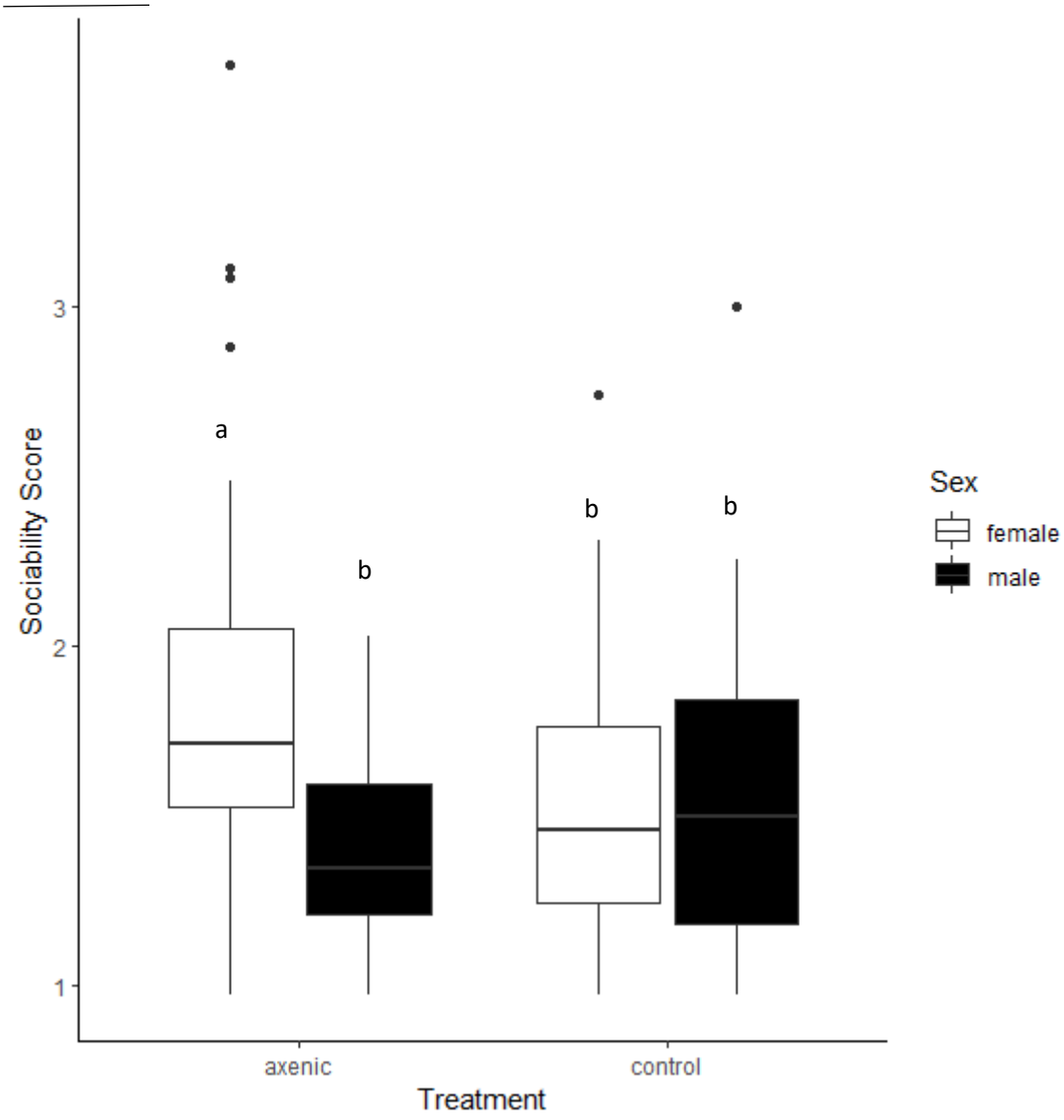

Figure 2: Boxplot of the sociability scores for arenas with axenic females (N=30 arenas), axenic males (N=30 arenas), control females (N=30 arenas), and control males (N=30 arenas). The measure of centrality depicted here is the median sociability score. The letters about the whiskers indicate which groups are statistically different or not statistically different from each other.

1b. Can we confirm the observed difference in sociability between control and axenic female flies?

Rationale:

We needed to see if the difference between control and axenic females in our first experiment could be verified by running a follow up experiment. The sample size for the control and axenic control female treatments was 30 arenas in the first experiment. To rule out there being a significant effect owing to a low sample size, we needed to run another experiment where the sample size for the control and axenic control female treatments would be higher.

Protocol:

We ran five replicates again. The protocol here was the same as it was for the first experiment, with some alterations. We switched to a different brand of baker's yeast, as there was no longer any available supply of the brand we used in the first experiment. We transferred eggs to 32 control vials and 32 axenic vials instead of 16 control vials and 16 axenic vials. The reason for this was that we intended to use twice as many females in our experiments than in the prior experiment.

We still added 10 males and 10 females to each vial to remain consistent with the first experiment. Thus, during the tests, we still released both males and females to fly cages that we aspirated females from during arena set up. We had to be careful to regularly remove males from the fly cages, to avoid a prolonged male-biased sex ratio for

the axenic or the control flies, which could have affected the female flies and confounded our results.

We should note there was an inordinate amount of mortality with the first replicate, but we still ran the test as we had enough females to run it. During the second replicate's test, flies in arena #19 escaped before the end of the observation period. For this reason, the total sample size for the control treatment was 59 arenas instead of 60. The total sample size for the axenic treatment was 60 arenas.

In terms of statistical analysis, we did things as we did for the first experiment, but did not include 'sex' as a fixed effect in our linear mixed model. We also did not use the emmeans package to conduct pairwise comparisons.

Results:

We did not see a significant difference between the axenic treatment and control treatment ($t_{(111)}$= 0.701, p=0.485; Fig. 3).

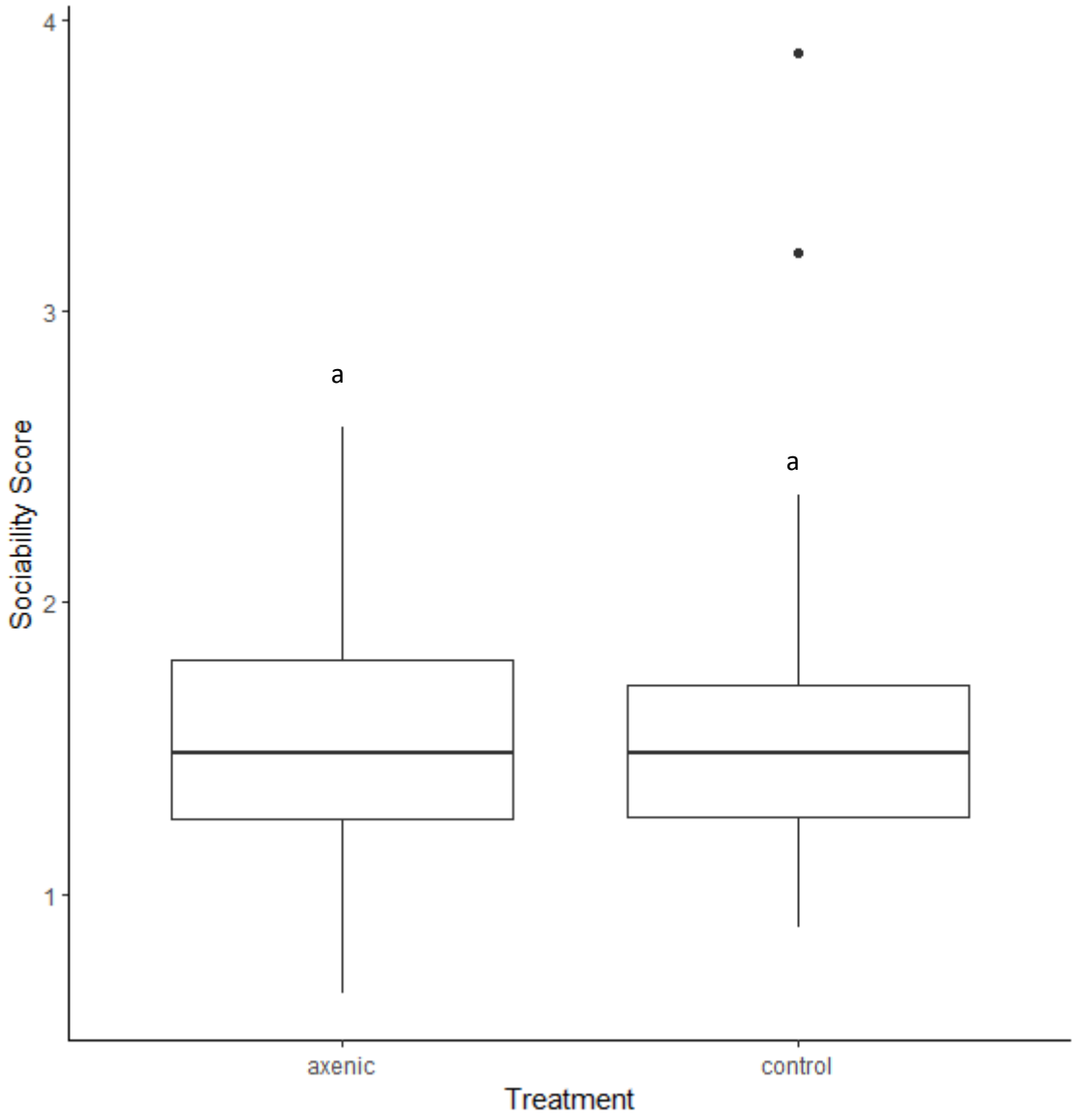

Figure 3: Boxplot of the sociability scores for arenas with axenic females (N=60 arenas) and control females (N=59 arenas). The measure of centrality depicted here is the median sociability score.

DISCUSSION

Sociability in males versus females

In our first experiment, we found that there was a main effect of sex, where females were more sociable than males. This effect was driven by the axenic females group, as control females did not show greater sociability in comparison to control males (Fig. 2). The difference between axenic females and males is subject to further experimentation, where we need to increase the sample size and see if the difference still holds up. Prior literature is mixed on the sex difference in sociability between males and females. Seltmann et al. (2019) found that in semi-captive Asian elephants (*Elephas maximus*), males tended to live in less social family units than females. Scott et al. (2018) looked at 60 *Wolbachia*-free fruit fly lines from the *Drosophila* Reference Panel (DGRP), and Scott et al. (2022) generated artificial selection lineages from approximately 600 wild-type fruit fly females caught in Hamilton, Ontario. They both found that males have higher sociability scores than females. Looking at rodent studies that have actually investigated the link between the gut microbiome and sociability, only Desbonnet et al. (2014), Hsiao et al. (2013), and Afroz et al. (2021) looked at both males and females, and they found no differences in sociability between the two sexes. Buffington et al. (2016) and Stilling et al. (2018) only looked at males. ASD is more prevalent in males than females (Werling & Geschwind 2013), and thus much of the literature using rodents as ASD models to explore potential links between the gut microbiome and markers of ASD like reduced sociability only use males. There is no clear picture of how the sociability of males compares to that of females across all species; future research using animal models to study ASD should

make a concerted effort to look at both males and females, to help shed light on potential sex differences with regards to sociability.

Possible explanations for the lack of treatment effect in follow-up experiment

In our first experiment, we found that axenic females were more sociable than control females (Fig. 2). Upon expanding the sample size in our second experiment, we did not find this difference between axenic females and control females (Fig. 3). Although we are not sure about why there was this discrepancy in our results, there are some possibilities to consider. One potential explanation for this is the phenomenon of regression to the mean, where there is a tendency for measurements following extreme measurements to be closer to the mean (Barnett et al. 2005). The axenic females mean sociability score in the first experiment could have been an extreme result attributable to the sample size of 30 arenas in the first experiment, which regressed towards the mean in the second experiment as the sample size was increased twofold.

Another possibility could be due to a change in the diet between the two experiments. As mentioned earlier on, we had to switch to another brand of baker's yeast during the second experiment. Douglas (2018) discusses the contradictory results of Fast et al. (2018). Fast et al. (2018) formed gnotobiotic flies - meaning their microbiota was removed and then selected microbes were introduced in a controlled manner - with various bacteria in their *Drosophila* cultures. This study had some unanticipated results based on prior literature, one of which was flies bearing *L. plantarum* - a probiotic that

has shown beneficial effects on growth rates in fruit fly larvae and juvenile mice before (Storelli et al. 2011; Schwarzer et al. 2016) - dying early than axenic flies. Douglas (2018) explains that the results of Fast et al. (2018) fit into the trend of inconsistent results between different studies, and posits that these inconsistencies are context-dependent: it depends on diet, bacterial strain, genotype, etc. For our experiments, there is a possibility that the two utilized baker's yeast brands differed in the quality of their strains, and thus there were contradictory results between the two experiments. Other literature has looked at the effects of yeast quality in fruit flies. Grangeteau et al. (2018) looked at the effects of different qualities of baker's yeast added to juvenile diet on adult life traits. They found that juvenile diets modulated adult survival, food preference, cuticular pheromones, and mating behaviour. With mating behaviour being a social behaviour and the evidence for cuticular pheromones being involved in the gut-brain axis, it would not be unfeasible for yeast quality to influence sociability. This is subject to further investigation.

Another possibility is that there was atmospheric variation during the time of testing between the first and second experiment, and the effect of the gut microbiota on fly sociability is not robust enough to be maintained regardless of surrounding conditions. As mentioned earlier on, the humidity outside varied between different test days, and this affected the humidity in the lab space where we conducted the sociability tests. This caused us to vary the amount of water added inside of the plastic containers containing the sociability arenas, in order to maintain a relative humidity of 50% inside of the container. In some cases, no water was added to the container, and in some cases, a lot

more than normal was added. One possibility with future investigations is to buffer against extreme atmospheric conditions by raising the flies under more extreme conditions. Bubliy et al. (2013) worked with a different species of fruit fly (*Drosophila simulans*) than the one we worked with, investigating how different combinations of humidity and temperature affect resistance in adults. They found that flies that were exposed to combined heat and humidity stress were able to effectively generate plastic responses to improve tolerance to both stresses. This may be worth experimenting with in future gut microbiota-behavioural work with *Drosophila melanogaster*, to see if the behaviour of flies reared under more extreme conditions is less affected under extreme atmospheric conditions.

Microbial workspace-related limitation

There was another limitation aside from the ones already discussed. We tried to determine the gut microbiota makeup in the control and axenic flies, to confirm that the axenic manipulation worked. We did reliably see a development delay - as described in Silva et al. (2021) - where flies from the axenic treatment took two days longer to eclose than flies from the control treatment, as well as a difference in incapacitation time between the axenic and control treatment while sexing on ice. But still, a quantitative means of confirming that the axenic treatment worked was required. We intended to use adult axenic and control flies collected from our second experiment to do intestine extraction, microbial DNA isolation, and subsequent polymerase chain reaction (PCR) amplification using the protocol provided by Fink et al. (2013). However, potentially owing to ongoing microbial work with soil in the microbiology facility we had access to, we had issues with

contamination and were not able to complete this work. In the future, it would be ideal for us to work in the absence of surrounding microbial work. Not only this, but it would be ideal for us to work exclusively in what is known as an axenic room (AR). An axenic room ensures maximum sterileness in the surrounding environment as opposed to a non-axenic room (NAR), which is what we were working in. Lebeuf et al. (2021) looked at microbial loads in cages housing mice over two weeks in an AR and a NAR. They found that AR management protocol led to a microbial load that was 1000 times lower than that found in the NAR. They also found that the proportion of bacteria sourced in the environment was significantly higher in NAR samples than AR samples; this could be due to things such as differences in air circulation. Evidently, working in an AR facility going forward would not only reduce contamination of developing axenic flies, but also reduce contamination issues with subsequent microbial quantitative PCR (qPCR).

Prospects

Our work adds to an existing body of literature investigating the effects of the gut microbiome on social behaviour, that has contradictory results and lacks clarity. Future investigations should simulate atmospheric variation in a controlled manner, and see how it affects behavioural assays in gut microbiome-social behaviour research. Aside from this, there is much more to be explored in this field of research. Our observation that axenic flies knocked out on ice faster than control flies calls for further inquiry through proper experimentation. Other literature has found that the gut microbiome affects the tolerance of temperature in fruit flies. Henry and Colinet (2018) found that axenic flies recovered more slowly from a chill coma protocol, and had lower survival upon cold

exposure than control flies. They did not find evidence for heat tolerance being affected by gut microbiome removal. A study in *Drosophila subobscura* however found that control flies that underwent mild heat exposure showed higher thermal tolerance than axenic flies (Jaramillo et al. 2021). Looking at rodents, Harshaw et al. (2022) worked with C57BL/6 mice and found that maternal antibiotic treatment of mothers produced offspring that were significantly less active in response to cold than the offspring of mothers from the control treatment. Investigation of the effects of cold exposure on incapacitation time in fruit flies would help contribute to the gut microbiome-thermal resistance literature.

Further investigations on the effect of the gut microbiome on sociability in fruit flies and other species should place a greater importance on looking at the repeatability of sociability. Consistent inter-individual variation in sociability is a facet of the variation in personality amongst animals (Gartland et al. 2022); it is thus important to look at if differences in sociability can be maintained over a period of time. Strickland & Frère (2018) looked at the repeatability of sociability in a natural population of Eastern water dragons (*Intellagama leseurii)*, and found that males showed significant repeatability across the years, while females did not. It would be interesting to look at sex differences in the repeatability of sociability in axenic fruit flies, in extension to the result in our first experiment that axenic females are more sociable than axenic males. We could look at sociability in axenic fruit flies over several generations, or even investigate it over the course of an axenic fly's lifetime. Sociability could be measured during larval development, and also later on at different timepoints post-eclosion. This would of course

be challenging, considering repeated behavioural measurements over time increases the chances of microbial contamination, but it is still worth considering.

Much work remains to be done with sociability in general. One potential avenue to further our understanding of it involves looking at the effects of the winner-loser effect on sociability in fruit flies. The winner-loser effect can be described as a higher chance of a winner winning an ensuing social conflict, and a loser losing an ensuing social conflict, regardless of what the opponent's identity is (Dugatkin 1997). Nakajo et al. (2020) created loser zebrafish (*Danio rerio*) that underwent repeated social defeat. Using a social preference test, they assayed sociability. They found no clear change in sociability in defeated zebrafish after the social defeat paradigm. Krishnan et al. (2007) however found evidence for reduced sociability in mice subjected to chronic social defeat. It would be interesting to combine the winner and loser generation protocol from Filice & Dukas (2019) and the sociability assay from Scott et al. (2022) to see how winning or losing affects sociability in fruit flies.

Concluding remarks

In conclusion, we found a main effect for sex in our first experiment, where females were more sociable than males. This was facilitated by the significant difference between axenic females and males, and there was no difference between control females and males. We also found a main effect for treatment in our first experiment, where axenic flies were more sociable than control flies. In our follow-up experiment where we tried to confirm the difference in sociability between axenic females and control females by

increasing the sample size, we did not detect the same difference. Our results contribute to existing literature looking at the effects of the gut microbiome on social behaviours, where results are often contradictory. Future research should focus on how environmental variation in diet and atmospheric conditions can affect social behaviours in flies.

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