THE GENETIC AND BEHAVIOURAL UNDERPINNINGS OF SOCIAL BEHAVIOUR

THE GENETIC AND BEHAVIOURAL UNDERPINNINGS OF NATURAL VARIATION IN SOCIAL BEHAVIOUR

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LAY ABSTRACT

Individual animals tend to vary in many traits including social behaviours. Using fruit flies, my goal was to understand what causes individuals to vary in two social behaviours: sociability and sexual aggression. I found that highly sociable flies tended to influence other flies to become more sociable due to a change in how much these flies interacted. I also found that individual differences in sociability are moderately heritable, and the genetic variation contributing to this is different between the sexes. Also, less sociable flies tended to be more aggressive than highly sociable flies. Finally, for sexual aggression, I showed that variation in a male's success in forcibly mating with a female was associated with changes in the expression of hundreds of genes, but these changes were mostly unique for evolved versus environmentally induced variation. Future work will similarly look to identify genes involved with individual differences in sociability.

ABSTRACT

A rich diversity of social behaviours exists in the animal kingdom, and these behaviours have evolved to perform a variety of adaptive functions. Social behaviours show variation both among and within species, however the mechanisms that give rise to this variation are not well understood. Using fruit flies (*Drosophila melanogaster*), my goal was to uncover the genetic and behavioural mechanisms that underpin natural variation in two different social behaviours: sociability and sexual aggression. First, I showed that sociability, which is the tendency of animals to engage in friendly activities together, is influenced by indirect genetic effects (IGEs), and that encounters among individuals drive these effects (Chapter 2). I then showed that sociability and social plasticity have low-moderate heritability (Chapter 3), and sociability is not correlated between the sexes or with activity. I then generated lineages of flies with high and low sociability using artificial selection (Chapter 4). The evolved lineages had significantly diverged sociability which was not associated with fitness measures or nearest-neighbor distances, but was negatively correlated with intrasexual aggression (Chapter 4). Finally, in sexual aggression, which I quantified as male forced copulation rate, I showed that evolved differences and differences due to social plasticity were both associated with the differential expression of many genes, but only a few of these genes were significant in both (Chapter 5). I also showed that these sets of genes are enriched in neuropeptide hormone and serotonin gene ontology categories, and that 4 of 7 chosen genes were validated for their effects on sexual aggression. Overall, this thesis sheds light on the complex mechanisms that underlie variation in these social behaviours, and it paves the way for future research to further elucidate some of these mechanisms, especially on the genetic basis of sociability using the evolved lineages I generated.

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DECLARATION OF ACADEMIC ACHIEVEMENT

This dissertation is organized according to McMaster University's approved sandwich thesis format, and consists of six chapters. Chapter 1 is an introduction to the thesis as a whole, and provides an overview of the subsequent data chapters. Chapters 2 and 3 are published manuscripts. Chapter 4 is a submitted manuscript currently in review. Chapter 5 is a manuscript in preparation for submission. Chapter 6 is an overall discussion connecting the results of the data chapters, and provides an outline of future prospects for this research.

CHAPTER 1 – Introduction
Author: Andrew M. Scott

CHAPTER 2 – Indirect genetic effects of several group members
Authors: Blake B. Anderson, Andrew M. Scott, and Reuven Dukas
Publication: Animal Behaviour, 123: 101-106 (2017).
Comments: B.B.A., A.M.S., and R.D. conceived this study. B.B.A. performed the preliminary experiment and experiment 1; A.M.S. performed experiment 2. B.B.A. and A.M.S. analyzed the data. B.B.A., A.M.S., and R.D wrote the manuscript.

CHAPTER 3 – Sociability in fruit flies: genetic variation, heritability and plasticity
Authors: Andrew M. Scott, Ian Dworkin, and Reuven Dukas
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CHAPTER 4 – Evolution of sociability by artificial selection
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CHAPTER 6 – Discussion
Author: Andrew M. Scott

CHAPTER 1 – INTRODUCTION

1.1 General Introduction

Broadly, social behaviours are behaviours that require interactions between conspecifics. Unless an animal's mode of reproduction does not require interacting with a mate (e.g., asexual reproducers, externally fertilizing sessile marine animals), all sexually reproducing animals must involve themselves in at least some social behaviour for the purposes of mating. Additional social behaviours may occur before mating, such as male courtship of females, and others after mating through parents feeding their offspring. Furthermore, social behaviours play an important role in optimizing foraging through cooperation (Clark and Mangel 1986) or local enhancement (Galef and Giraldeau 2001), and avoiding predators, for example through vigilance and "predator confusion" by schooling or swarming (Milinski and Heller 1978; Jeschke and Tollrian 2007). Even further, social behaviours may make up a majority of an animal's life, and involve many complex interactions critical for the survival of one's kin, as in eusocial insects (Wilson 1971). As humans, we can see the product of our history of social behaviour in all of modern life, in the accumulation of knowledge and technology through generations via social learning and cultural transmission, and the rich variety of cultures that have developed as a result of adaptation to different environments (Chang et al. 2011). Social behaviours are clearly an important target of selection, as evolution has generated an amazing diversity of such behaviours in function and complexity, and it is no wonder that evolutionary biologists have spent a great deal of time and effort to understand how these behaviours have evolved and endured (Wilson 1975; Clutton-Brock 2016; Ward and Webster 2016).

In addition to this wide variation in social behaviours among species, there also exists variation among populations within a species that have evolved in different environments. Such among-population variation has been very useful in understanding the selection pressures that drive social behaviour evolution. For example, in guppies (*Poecilia reticulata*) from different populations, shoaling behaviour in response to predators in a

laboratory setting was influenced by the level of predation experienced in the environments they evolved in (Botham et al. 2008). In the communal spider (*Anelosimus studiosus*), variation in social structure from solitary to multifemale communal nesting is associated with increasing latitude, with nest transplant experiments suggesting that this variation is not due to environmental plasticity, but instead is likely an evolved social behavioural polymorphism (Riechert and Jones 2008).

Further, to get at the fundamental source of variation that selection acts upon within a population, researchers have also recently explored individual variation in social behaviour, and the mechanisms that underly this variation. Individual variation in all kinds of social behaviours has been observed, from variation in mating strategies (Fraser et al. 2014) and shoaling behaviour (Cote et al. 2012) in fish, to sexual aggression (Baxter et al. 2019) and social aggregation (Anderson et al. 2016) in fruit flies (*Drosophila melanogaster*), and to cooperative breeding in western bluebirds (*Sialia mexicana*) (Charmantier et al. 2007), and cooperative hunting in bottlenose dolphins (*Tursiops truncates*) (Gazda et al. 2005). Such individual variation may be the product of segregating genetic variation in a population, environmentally induced plasticity, or a combination of (or interaction between) the two. Separating the genetic component contributing to this variation from environmental influences in order to gain a better understanding of the genetic architecture relevant for selection can be difficult, and often requires the use of model systems in a laboratory setting.

One excellent model system is the fruit fly (*Drosophila melanogaster*), which is not only highly genetically tractable, but also exhibits a surprisingly rich social life. For example, male flies court females and such courtship may involve interference between competing males (Baxter et al. 2018), they show intrasexual aggression in both males (Dierick and Greenspan 2006; Baxter and Dukas 2017) and females (Ueda and Kidokoro 2002; Bath et al. 2017), they exhibit social learning (Durisko and Dukas 2013; Durisko et al. 2014a; Duménil et al. 2016), pheromonal mediation of social behaviour (Bartelt et al. 1985; Lin et al. 2015), social synchronization (Levine et al. 2002), social information use (Sarin and Dukas 2009; Battesti et al. 2012; Malek and Long 2020), and social aggregation

in both the larval and adult stages (Saltz 2011; Schneider et al. 2012; Durisko et al. 2014b; Anderson et al. 2016; Brenman-Suttner et al. 2018). Investigating the behavioural and genetic mechanisms underlying social behaviours in fruit flies can be fruitful not just in identifying variation that may be relevant to selection in fruit flies themselves, but also in extrapolation to the genetic and behavioural underpinnings in other animals including humans. A large proportion of human disease-causing genes and mental disorder-implicated genes have orthologs in fruit flies (Rubin et al. 2000; Inlow and Restifo 2004), and many of the neural circuits involved in social behaviour that are influenced by relevant variation may be highly conserved among species (Tierney 1995; Thor and Thomas 2002).

Over the course of my graduate studies, I have used fruit flies as a model to better understand the genetic and behavioural mechanisms that underlie variation in two social behaviours: sociability and sexual aggression. In the next two sections, I will introduce these social behaviours and discuss the prior research that has formed the basis for my research questions.

1.2 Sociability

An animal's sociability is its tendency to engage in non-aggressive activities with conspecifics, such as feeding together, roosting together, and travelling together. While sociability may be related to other social behaviours such as aggregation or social spacing, it is different in that it is specifically assessing an animal's decision-making about whether to join others in performing activities or not, rather than simply how close animals are willing to be to one another (i.e., their social space preference). An animal's sociability can have important consequences on a number of important ecological factors. For example, highly sociable animals have increased foraging efficiency (Clark and Mangel 1986; Durisko et al. 2014b), reduced need for anti-predatory behaviours through diffused vigilance responsibility amongst the group (Treves 2000), and access to information that can result in social learning (Sarin and Dukas 2009; Durisko and Dukas 2013) reducing the need for trial and error. On the other hand, there are a number of disadvantages to being

highly sociable, such as increased competition for resources (Wertheim et al. 2002) and increased pathogen and parasite transmission (Han et al. 2015). The varying selection pressures generated by the relative influence of these and other consequences of high or low sociability has led to the evolution of a wide variation in sociability among animal species. For example, in the bees (Apoidea), there is diversity in sociability from solitary species that only interact with conspecifics to mate, to communal group-living species, and further to those with eusocial reproductive caste-based societies (Wilson 1971; Michener 1974; Wcislo and Fewell 2017).

Recently, there has been great interest in studying sociability from a variety of perspectives, including neurobiological (e.g., Ferreira and Moita 2019), behavioural (e.g., Durisko et al. 2014), and evolutionary (e.g., Kurvers et al. 2014). Part of this interest is likely due to the critical role that social interactions play in human life and the effort to understand the causes of variation in sociability in humans (Bralten et al. 2019; Day et al. 2018), the extremes of which may be considered social disorders. Insights from model systems can play a role in helping to uncover some of the risk factors of these disorders at a mechanistic level. As discussed in the previous section, fruit flies have proven to be a useful model system in dissecting the genetic and mechanistic underpinnings of social behaviour, and recently sociability specifically. For example, the putative autism-spectrum neuroligin gene NLGN3 in humans (Jamain et al. 2003) has been shown to cause abnormal social phenotypes in fruit flies when its fly analog, *nlg3*, is knocked-down (Yost et al. 2020), providing support for analogous genetic disease-causing genes among distantly related species. However, these kinds of studies do not provide any information about evolutionarily relevant genes that may underpin natural variation in sociability.

As discussed in the previous section, in order to study the variation in sociability that is relevant for selection, we need to quantify it at an individual level. Such individual variation in sociability within populations certainly exists, and has been shown in a few cases, for example, in mosquitofish (*Gambusia affini*) (Cote et al. 2012), and in humans (Fowler et al. 2011; Day et al. 2018; Bralten et al. 2019), however there has not yet been a thorough analysis of individual variation in sociability in a genetically tractable model

system. In order to answer evolutionary questions on the genetic basis of natural variation in sociability, and the ecological forces contributing to the maintenance of this variation, we need to quantify the natural heritable variation in sociability, how this variation is associated with fitness, how sociability is genetically correlated with other traits, and what the underlying genetic architecture of sociability is.

Using fruit flies as a model system, I have taken a few experimental approaches to address these questions. First, to understand the degree of variation in sociability (i.e., the heritability), we used a powerful tool (The *Drosophila* Genetic Reference Panel (DGRP), Mackay et al. 2012) available for fruit fly researchers that allowed us to quantify variation in sociability among a large number of genotypes that together represent a typical natural population. Using the DGRP also allowed us to quantify variation within each of those genotypes as they are maintained as clonal lines. This allowed us to quantify both genetic and environmental influences on variation in sociability, and allowed us to estimate heritability. However, as sociability is, of course, a social trait, it was important for us to recognize that heritable variation in conspecific sociability influences the social environment, which can then in turn influence sociability in addition to one's own genetic makeup and the non-social influences of the environment. Therefore, I also wished to understand if these indirect genetic effects (IGEs, Moore et al. 1997) contribute to overall environmental variation, and if so, what behavioural mechanisms underlie these effects.

After quantifying the heritability of sociability, I then took a second approach aimed at uncovering the underlying genetic architecture contributing to this variation. This approach involved performing 25 generations of artificial selection on fruit fly sociability, generating lineages of flies with diverged sociability. Owing to time constraints and COVID-19 delays, my thesis does not include the genetic component. Nevertheless, this "evolve-and-resequence" approach will give us considerable power to investigate the genetic underpinnings of such evolved divergence in sociability (Schlötterer et al. 2015). Artificial selection studies have been successfully used in fruit flies to look at behaviours including mating (Mackay et al. 2005), courtship song (Turner and Miller 2012), odour-guided behaviour (Brown et al. 2017), colour preference (Marcus et al. 2018), resource

defense (Hoffmann 1988), learning (Mery and Kawecki 2002) and sexual aggression (Dukas et al. 2020). In addition to the genetic aspect, having access to populations with diverged levels of sociability allows us to investigate correlated responses to selection that may provide insight on the behavioural mechanisms underlying sociability and its fitness consequences. For example, we still do not understand the association between sociability and aggression. While one would intuitively predict a negative correlation between the two, an intriguing finding indicates higher levels of lethal aggression in social than in solitary mammals (Gómez et al. 2016). If a negative correlation does exist between sociability and aggression, low sociable males may then have the opportunity to monopolize food patches and any matings with females that visit their food patch (Hoffmann and Cacoyianni 1989). Conversely, highly aggressive males tend to have low mating success compared to controls (Dierick and Greenspan 2006; Penn et al. 2010), which may attenuate the fitness gains from this resource monopolization. Generating flies with low and high sociability gave us the opportunity to investigate such questions. The investigation of the relationship between sociability and aggression also led me to begin investigating more closely the genetic underpinnings of natural variation in another behaviour: sexual aggression.

1.3 Sexual Aggression

As I was performing a large-scale artificial selection experiment on sociability, others in our lab were simultaneously performing artificial selection on sexual aggression in fruit flies to understand its fitness consequences, as well as physical and behavioural correlated responses to selection (Dukas et al. 2020). As I was unable to perform the final genetic analysis on the diverged sociability populations due to COVID-19, the rapidly diverged lineages in this sexual aggression experiment gave me a great opportunity to investigate the evolved differences in gene expression between flies with low and high levels of sexual aggression. As my observed relationship between sociability and aggression in general suggest (Chapter 3), these analyses could additionally be insightful in uncovering the genetic differences between flies with low and high sociability. Further, the degree of

shared genetic underpinnings between sexual aggression and other forms of aggression (such as intrasexual aggression, whose genetic underpinnings have been extensively studied; Dierick and Greenspan 2006; Edwards et al. 2006; Gammie et al. 2007; Wang et al. 2008) is unclear, and exploring the genetic basis of sexual aggression can help elucidate this relationship.

Sexual aggression is a form of sexual conflict between males and females in which males attempt to physically coerce or otherwise force females into mating, which presents a clear benefit to males at a potentially severe cost to females. Examples of sexual aggression in the form of male forced copulation of females have been observed in fish (Farr et al. 1986; Fraser et al. 2014) and waterfowl (McKinney et al. 1983; McKinney and Evarts 1998), and may represent an alternative reproductive strategy in some species. In fruit flies, sexual aggression occurs in the form of male forced copulation of teneral females, which are recently-eclosed, have a soft cuticle, cannot prevent intromission, and cannot fly. Such forced copulation occurs in the wild (Markow 2000) and benefits males as these females do produce offspring, however this sexual aggression is costly to females (Seeley and Dukas 2011; Dukas and Jongsma 2012). There is no clear distinction in fruit flies between males that do and males that do not forcibly mate, as there is in some fish species where there are clear morphological differences between males with different behavioural strategies (Farr et al. 1986). However, there is still considerable genetic variation in the frequency that male fruit flies will perform this sexual aggression (Baxter et al. 2019). As with our question in the sociability research about what genetic mechanisms contribute to some flies choosing to perform activities with others and some choosing to be alone, we also asked what genetic mechanisms underlie some males forcibly mating and some not. There are a number of possibilities, for example differences in genes underlying motivation, or differences in genes underlying physical ability. Access to populations of fruit flies with diverged levels of sexual aggression gave us the opportunity to gain insight into these questions.

In addition to having access to populations with evolved differences in forced copulation tendency, we were also able to generate male flies that show a similar

divergence due to variation in the social environment experienced before exposure to teneral females (Baxter and Dukas 2017). This gave us an additional opportunity to ask whether evolved differences in the genetic mechanisms (i.e., gene expression) underlying sexual aggression tendency are similar to those mechanisms that underlie plastic differences. Such comparisons between evolved and plastic effects on behaviour can be highly informative regarding the evolutionary history of these traits, for example in elucidating whether genetic assimilation may have allowed for adaptive evolution through co-option of plastic gene expression (e.g., Scoville and Pfrender 2010).

1.4 Structure of the Thesis

In this section, I will briefly overview the structure of the next four chapters, which represent published, submitted, or in-prep manuscripts, and the logical flow between them in relation to my overall research goal of uncovering the genetic underpinnings of social behaviour.

First, in continuing with the work of a previous graduate student Blake Anderson, we wished to understand the degree to which the sociability of group members can influence each other through the social environment, and the mechanisms that underlie this indirect genetic effect (Chapter 2). We found that, indeed the social environment does play a role in shaping an individual's sociability, and this is mediated through simple social interactions. Therefore, to accurately estimate the heritability of sociability in wild populations, in the next study we used clonal flies from the *Drosophila* Genetic Reference Panel (DGRP), which allowed us to keep genetic variation in the social group to ~0. We estimated the broad sense heritability of sociability to be moderate, about 0.21 in females and 0.24 in males, with little correlation between the sexes or with activity (Chapter 3). We also estimated the heritability of plastic effects on sociability to be about 0.24 in males. While we had initially planned to use available genetic information from the DGRP to identify significant genetic variants associated with variation in sociability through genome-wide association, this approach proved to not be powerful enough. Consequently,

we utilized an artificial selection approach which will not only (eventually) give us the power we need to confidently choose candidate genes associated with variation in sociability, but also gave us the chance to directly assess the fitness consequences and correlated responses to selection in other social behaviours such as aggression (Chapter 4). We were able to generate lineages with significantly diverged sociability in both males and females after 25 generations, and further found that this divergence was not associated with variation in fitness for either sex. Interestingly, we found that selection on sociability also resulted in a correlated response in intrasexual aggression, providing evidence for a potential shared genetic underpinning for "friendly" and antagonistic social behaviours. While COVID-related delays did prevent further progress on the genetic work for these sociability-selected lineages, I did continue work on uncovering the genetic underpinnings of intersexual aggression, a social behaviour whose correlation with sociability and other forms of aggression remains unclear. Gene expression analyses revealed that both evolution of divergent male sexual aggression and variation in male sexual aggression generated through social plasticity are associated with the differential expression of hundreds of genes (Chapter 5). However, only a small proportion of these genes were implicated in both the evolved and plastic effects on sexual aggression, indicating that these mechanisms of variation are relatively independent for this form of sexual aggression. Of the potentially core genes that are important for both evolved and plastic differences, several genes involved in a variety of molecular functions (e.g., neuromuscular processes, vision, and potentially memory-related endopeptidase activity) were validated in their effects on sexual aggression.

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CHAPTER 2 – INDIRECT GENETIC EFFECTS ON THE SOCIABILITY OF SEVERAL GROUP MEMBERS

Anderson, B. B., Scott, A. M., Dukas, R. (2017). Indirect genetic effects of several group members. *Animal Behaviour, 123,* 101-106.

2.1 Abstract

Indirect genetic effects (IGEs) are a major driver of social evolution, but much of the experimental work pertaining to IGEs on social behaviour has focused on the effect of stimulus individuals on single focal individuals. We extended IGE research to examine how stimulus individuals influence social interactions among several focal individuals. Specifically, we relied on recent work on social behaviour in fruit flies to examine whether IGEs cause 12 stimulus flies of distinct genotypes to alter social interactions within groups of 6 focal flies. The social behaviour of focals was significantly affected by the genotype of the stimulus flies. Focals were closer together when grouped with stimulus flies from genotypes that were close together than when grouped with stimulus flies from genotypes that were farther apart. A mechanism mediating this effect was the encounter rate between focal flies, which was lowest when the focal flies were grouped with stimulus flies of the more cohesive genotypes.

2.2 Introduction

It has long been recognized that the observed behavior of a social group reflects the characteristics of its individual members, and that some individuals might disproportionally determine group performance (Allee 1938; Modlmeier, Keiser, Watters, Sih & Pruitt 2014; Pentland 2014). For example, the average social sensitivity of group members was the best predictor of performance on a variety of collective tasks by human groups (Woolley, Chabris, Pentland, Hashmi & Malone 2010). And in the social spider, *Stegodyphus*

dumicola, the presence of a few mature females increased the frequency of attacking prey in small juvenile groups and decreased attack latencies in large juvenile groups (Modlmeier et al. 2015).

When individual traits that influence social behavior are heritable, the performance of one group member is partially determined by the genotypes of other members. Such indirect genetic effects (IGE) (Griffing 1967; Scott 1977; Moore, Brodie & Jason 1997) have been documented in a variety of traits and taxa including aggression in deer mice (*Peromyscus maniculatus*) (Wilson, Gelin, Perron & Réale 2009), domestic pigs (*Sus scrofa*) (Camerlink, Ursinus, Bijma, Kemp & Bolhuis 2015) and fruit flies (*Drosophila melanogaster*) (Saltz 2013), mate choice in field crickets (*Teleogryllus oceanicus*) (Bailey & Zuk 2012) and tree hoppers (*Enchenopa binotata*) (Rebar & Rodríguez 2013), chemical signaling in fruit flies (*Drosophila spp.*) (Petfield, Chenoweth, Rundle & Blows 2005; Kent, Azanchi, Smith, Formosa & Levine 2008), and anti-predatory behaviour in guppies (*Poecilia reticulata*) (Bleakley & Brodie 2009).

Much of the experimental work on IGEs on social behaviour has focused on the effect of stimulus individuals on focals. The only exception we know of (Saltz 2013) considered the effect of an stimulus individual on interactions between two focal individuals. Saltz (2013) termed the classically considered interactions between the stimulus and focal individual 'first order IGEs', and the effect of the stimulus individual on interactions between the two focals 'second order IGEs'. Social behaviour often involves many individuals. Because theory indicates that IGEs can profoundly influence both the rate and direction of the evolution of social traits (Moore et al. 1997; Wolf & Moore 2010), it is pertinent that we examine IGEs of stimulus individuals on social interactions among several focal individuals. To this end, we relied on the recent work on social behaviour in fruit flies (Krupp et al. 2008; Sarin & Dukas 2009; Saltz 2011; Battesti, Moreno, Joly & Mery 2012; Simon et al. 2012) and on our own research documenting significant genetic variation in social behaviour in fruit flies (Anderson, Scott & Dukas 2016) to test whether stimulus flies of distinct social genotypes determine social features among groups of 6 focal flies. While there are different ways to define and measure social behaviour, our focus here

is on the tendency of conspecifics to be close to others (Ward & Webster 2016). Specifically, we predicted that 6 focal flies would be closer together when grouped with 12 flies of stimulus genotypes that were close together than when grouped with 12 flies of stimulus genotypes that were farther apart. In a follow up experiment, we examined the behavioural mechanism mediating the IGEs.

2.3 Methods

2.3.1 General methods

We maintained all populations at low density in 40 ml vials each containing 5 ml standard food (one liter of which contained 90 g sucrose, 32 g yeast, 75 g cornmeal, 20 g agar, and 2 g of methyl paraben), at 25°C and 60% relative humidity, on a 12:12 light cycle with lights on at 10 am. These conditions are optimal for fruit fly well being. Furthermore, we handled flies either by gentle aspiration or with a soft brush following brief anesthetization with CO_2, and applied no harmful manipulations. Our focal flies belonged to an inbred line of Canton-S, which has been in captivity for decades and in our laboratory for 6 years. Our 3 stimulus fly lines were 2 lines of the *Drosophila* Genetic Reference Panel (DGRP; Mackay et al. 2012) and the Canton-S line. We chose the 2 DGRP lines (304 and 427) based on our previous work (Anderson et al. 2016) as well as the preliminary experiment described below.

2.3.2 Preliminary experiment

We collected flies within 8 h of eclosion on day 1 and housed them in mixed-sex vials each containing 20 males and 20 females. On day 4 at 8 AM, we transferred groups of 18 males from each line each into an 85 mm food dish. The petri dishes contained standard food, with corn meal omitted to minimize variation in surface texture. The volume of food was sufficient to minimize headspace, such that flies were constrained to 2 dimensions during

observations. At 1 pm, we placed the dishes inside test boxes (53 cm x 31 cm x 30 cm; length x width x height) made of semi-opaque plastic and illuminated by diffused room light. After an additional 2 hours of acclimatization, we video recorded the dishes for 1 hour with high resolution webcams (Logitech C920) through a hole in the center of each box lid.

During video analyses, we sampled Cartesian coordinates of each fly at 30 second intervals and calculated a single nearest neighbour index for the 18 flies in each dish. The nearest neighbour index is defined by the ratio between the mean observed nearest neighbour distance and that expected by chance at the given density. Nearest neighbour indices range from 0, where all points occupy the same region in space, to 2.15, which represents a perfectly uniform distribution (Clark & Evans 1954; Anderson et al. 2016). Calculations were similar to those illustrated in Fig. 2.1a for experiment 1 but were based on 18 flies belonging to a single line. Similar measures have been used successfully in numerous studies on social behaviour in a variety of species (White & Chapman 1994; Evans & Harris 2008; Durisko, Kemp, Mubasher & Dukas 2014). The distance among individuals reflects some balance between the degree of attraction to and avoidance of others, with the latter being either a response to the presence of a nearby individual or a result of some aggressive interactions (Conder 1949; Brown & Orians 1970). Hence the average nearest neighbour distance in a group provides us with a comprehensive and objective measure for comparisons between genotypes and treatments of the outcomes of social interactions among individuals. Nevertheless, a complete characterization of social behaviour will benefit from using a variety of protocols (Saltz 2011; Schneider, Dickinson & Levine 2012).

We intended to use in the main experiment and hence tested in the preliminary experiment 6 DGRP lines (304, 360, 362, 365, 427 and 437) as well as our Canton-S. We expected to observe two discrete levels of social behaviour from our DGRP lines based on our previous work, which employed a distinct protocol (Anderson et al., 2016). However, only line 304 expressed a social phenotype that was significantly different from the other DGRP lines (all $P < 0.001$, uncorrected pairwise T-tests). The nearest neighbour scores of

the remaining 5 DGRP lines were indistinguishable from one another (all $P > 0.77$), though line 427 was the least variable DGRP line tested. We thus proceeded using only lines 304, 427, and our Canton-S line, which was the least social line of the three (all $P < 0.05$; Fig. 2.1b).

2.3.3 Experiment 1

We collected flies within 8 h of eclosion on day 1 and housed stimulus and focal males in different mixed-sex vials each containing 14 males and 14 females. Focal and stimulus Canton-S flies always came from distinct vials. On day 4 at 8 AM, we marked focal and stimulus males with either pink or blue fluorescent powder, which was counterbalanced across days. An hour after marking, we briefly anesthetized the flies under light CO_2 and transferred 6 focal males from one vial and 12 stimulus males from another vial into each 85 mm petri dish with food as described above. That is, each experimental dish contained 18 males. At 1 pm, we transferred 6 dishes of flies into each of 4 test boxes described above. Following an additional 2 hours of acclimatization, we video recorded the flies for 60 minutes as described above. During video analyses, we sampled Cartesian coordinates of each fly at 60 second intervals. Observers blind to fly treatment verified the position of all 18 males and distinguished the 6 focals from the 12 stimulus males based on color.

To quantify social behavior, we calculated two nearest neighbour indices independently for each dish and time point: one for the 6 focal males and one for the 12 stimulus males (Fig. 2.1a). We observed a total of 126 dishes (N=42 per stimulus line), and analyzed the data in R version 3.2 (R-Core-Team 2014) with a linear mixed model with focal male nearest neighbour index as a response variable, stimulus genotype and focal color as fixed effects, day, box, and dish as random effects, and time as a repeated measure. Though there was a significant effect of color ($\chi^2_1 = = 14.38$, $P < 0.001$), there was no effect of day ($P = 1.0$), box ($P = 0.15$), nor changes over time ($\chi^2_1 = 0.01$, $P = 0.93$).

Our preliminary data indicated that the nearest neighbour index is sensitive to the number of individuals when a group of flies is divided into two subgroups of different sizes.

This was relevant here, as we observed 6 focal flies and 12 stimulus flies within the same dish. To verify this outcome, we performed a simulation in which we sampled dishes from our preliminary experiment (with replacement), randomly partitioning each dish into subgroups of 6 and 12 and calculating a nearest neighbour index for each subgroup. The nearest neighbour indices were greater for subgroups of 6 flies (1.0 [0.46, 1.38], mean and 95% CI) than for subgroups of 12 flies (0.89 [0.47, 1.14]). This most likely explains the difference in nearest neighbour indices between the 12 stimulus and 6 focal Canton-S flies observed when comparing figures 2.1c and 2.1d.

To quantify the magnitude of the indirect genetic effect on focal phenotype, we fit a second model to estimate the interaction coefficient (Ψ) based on the partial regression coefficient between focal and stimulus fly nearest neighbour indices (Moore et al., 1997; Equation 2b). This model was identical to our initial model, but included stimulus fly nearest neighbour index and its interaction with genotype as fixed effects. Though the IGE is presumably driven by the more numerous stimulus males, we corrected Ψ estimates to account for the possibility of a reciprocal IGE (Bijma 2014; Equation 12). The interaction between stimulus male nearest neighbour index and genotype was not significant (χ^2_2 = 1.05, P = 0.59), suggesting that the relative strength of the IGE was similar when observed with the related stimulus Canton-S and the unrelated stimulus DGRP.

2.3.4 Experiment 2

In experiment 2, we wished to test whether social interactions among the focals varied when grouped with stimulus flies from each of the 3 distinct genetic lines. To quantify the encounter rates between stimulus flies and focals, and between focals and focals, we used a protocol similar to that of experiment 1. We had 3 treatments, one for each line of stimulus flies. Each 85 mm dish contained 6 focal males and 12 stimulus males. We had 18 dishes, 6 for each stimulus fly treatment. We placed two dishes in each of 3 testing boxes, allowing videos of 6 dishes to be recorded each day over 3 days. Testing box, treatment day, and fly colour were counterbalanced for each dish treatment.

For each dish, observers blind to treatment recorded all encounters during the first 10 minutes of each video. We defined encounters as either a clear inspection by one fly of another (e.g. licking or prodding with legs), or the movement of one fly toward another with a clear reaction from the other fly (e.g. wing fluttering or moving away). We separately recorded encounters between stimulus flies and focals and between focals and focals. For encounters between stimulus flies and focals, encounters included stimulus flies moving toward stationary focals, focals moving toward stationary stimulus flies, and both stimulus and focal flies moving toward each other. For encounters between focals and focals, encounters included one focal moving toward a stationary focal, and two focals moving toward each other.

We analyzed the data with a generalized linear model with Poisson distribution and log link function and used sequential Bonferroni for pairwise comparisons. A non-parametric test revealed similar results. In the analysis of encounter rates *among focal flies*, focal color did not have a significant effect, but both box and day effects were significant (n=18, Wald χ^2_1=0.2, 13.6 and 30 and P=0.65, P<.001 and P<0.001 respectively). In the analysis of encounter rates *between stimulus and focal flies*, focal color, box and day effects were all significant (n=18, all *P's*<0.001).

2.4 Results

In the preliminary experiment, there was a significant effect of the stimulus fly genotypes on their average nearest neighbour index (χ^2_2 = 74.4, P < 0.001; Fig. 2.1b). A post-hoc analysis of experiment 1 with stimulus male nearest neighbour index as a response variable showed commensurate differences between genotypes (χ^2_2 = 141.0, P < 0.001, Fig. 2.1c). Most importantly, the average nearest neighbour index of focal flies differed significantly based on the genotype of the stimulus flies they were paired with, with focals adjusting their social behaviour in response to that of the stimulus flies (χ^2_2 = 6.06, P < 0.05; Fig. 2.1d). The corrected interaction coefficient (Ψ) was positive (partial regression coefficient = 0.084 ± 0.029 SE, adjusted Ψ = 0.042 ± 0.015 SE). In experiment 2, the stimulus flies

had a significant effect on the encounter rates *among focal flies*, which were highest when the stimulus flies had the highest nearest neighbour index (CS) and lowest when the stimulus flies had the lowest nearest neighbour index (DGRP 304) (Wald χ^2_2=60 n=18, P<0.001; P<0.01 for all pairwise comparisons, Fig. 2.2). The encounter rates *between stimulus and focal flies* were highest with the line with the highest nearest neighbour index (CS), lower with the intermediate line (DGRP 427) and lowest with the line with the lowest nearest neighbour index (DGRP 304) (Wald χ^2_2=341 n=18, *P*<0.001; *P*<0.001 for all pairwise comparisons, Fig. 2.2).

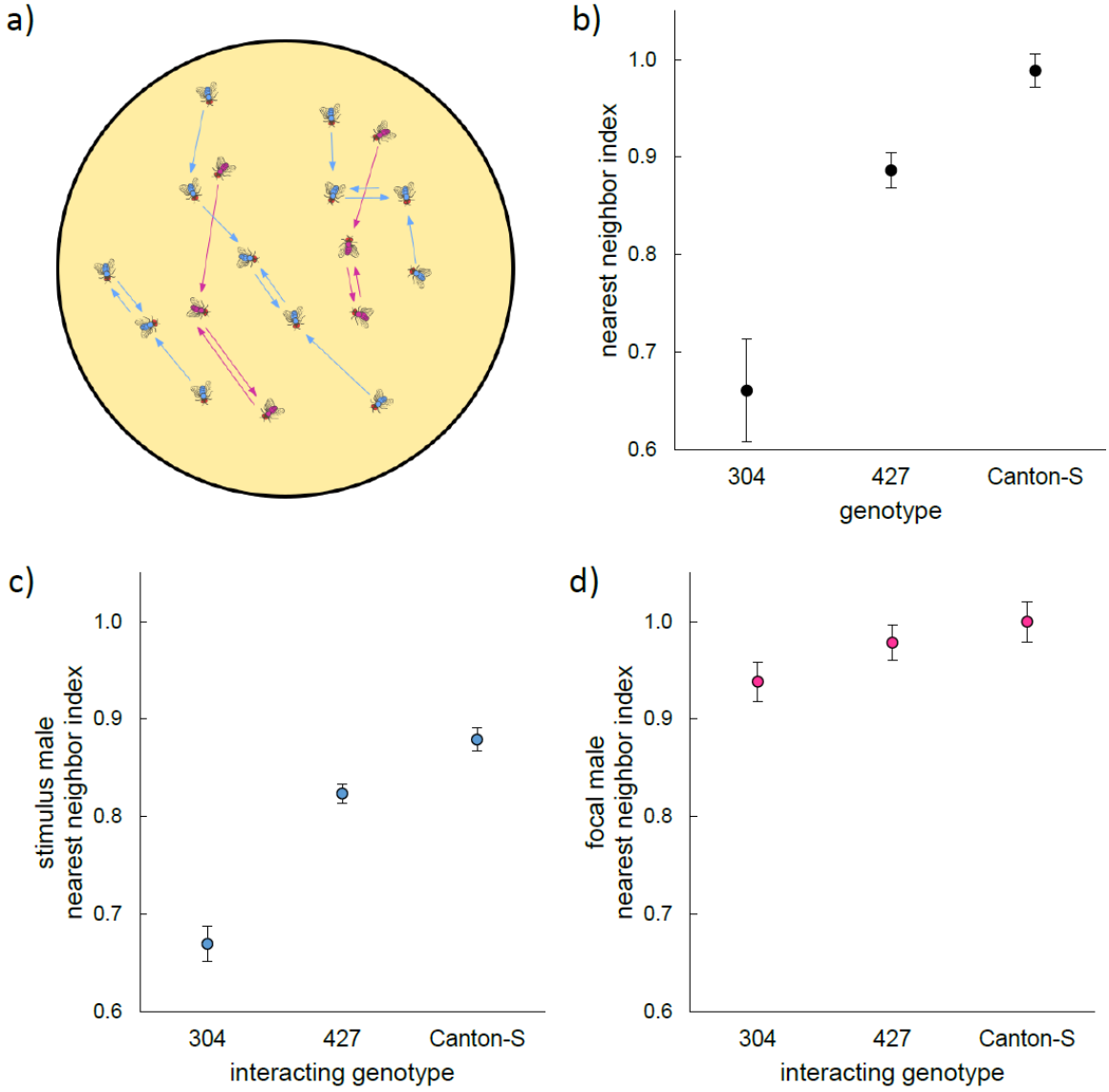

Figure 2.1

(a) Diagram illustrating nearest neighbor measurements amongst 6 focal (pink) and 12 stimulus (blue) flies. Note that, for clarity, flies are drawn larger than their actual size relative to the 85 mm dish. Also note that, in panels c and d, measurements for each focal are done without regard to stimulus flies' positions, and vice versa. (b) Mean ± SE nearest neighbor index among groups of 18 flies from each stimulus line during the preliminary experiment. (c) Mean ± SE nearest neighbor index measured amongst groups of 12 stimulus flies from the 3 different lines. (d) Mean ± SE nearest neighbor index measured amongst the 6 focal flies (Canton-S) when grouped with 12 flies from each of 3 distinct stimulus lines.

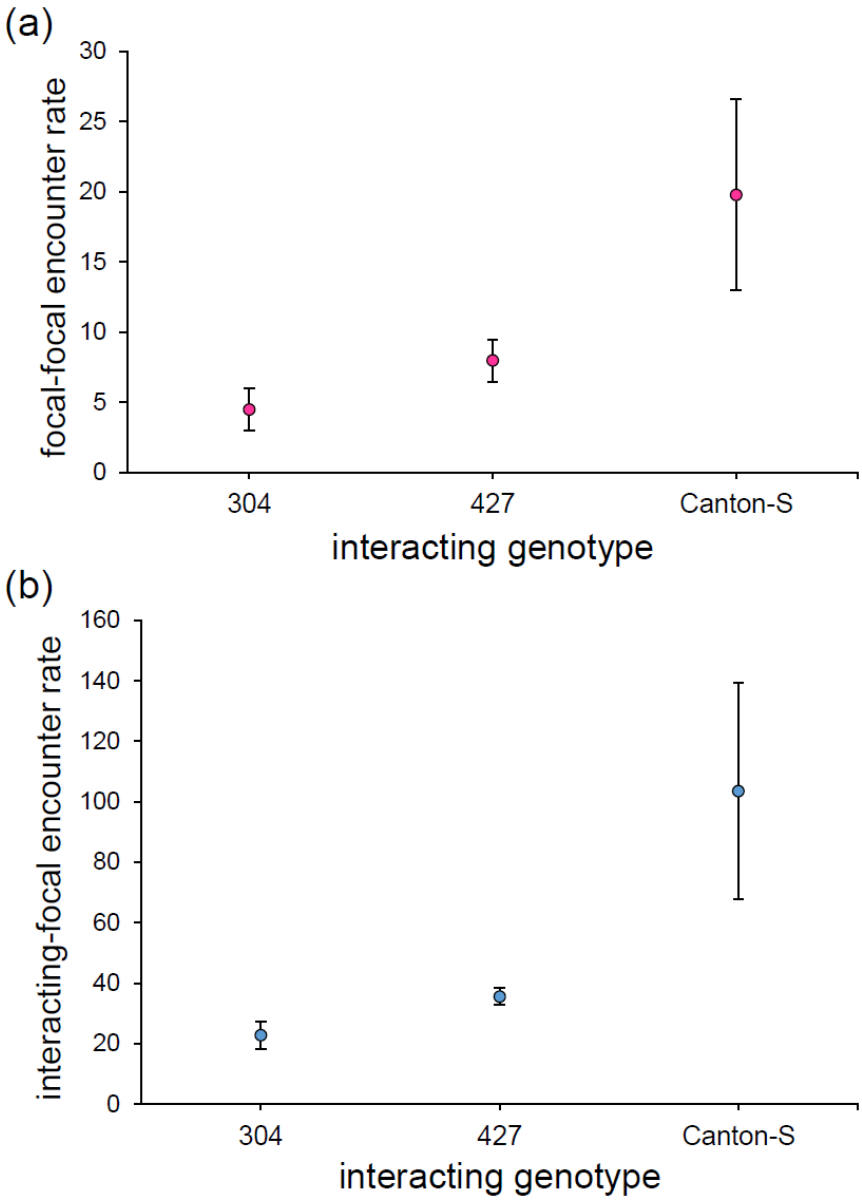

Figure 2.2

The average number of encounters per 10 min (a) among 6 focal flies and (b) between stimulus and focal flies in experiment 2.

2.5 Discussion

Our major finding was that social behaviour within a group of 6 focal group members varied as a function of the genotype of 12 other stimulus individuals (Fig. 2.1). As far as we know, this is the first study that documents IGEs caused by stimulus individuals on the social dynamics among several focal group members. The most relevant previous work involved applied research in a few species of farm animals housed in groups with the goal of reducing overall stress, injury and mortality, and increasing features such as growth rate and egg laying. The key emphasis of that work has been on estimating IGEs and designing the best artificial selection regimens to maximize group features, which are the most relevant to farmers (Muir 1996; Ellen, Visscher, van Arendonk & Bijma 2008; Bijma 2010; Wade, Bijma, Ellen & Muir 2010). While this body of applied research clearly illustrates the importance of IGEs, it has not provided data on how IGEs of stimulus individuals might influence social interactions among several focal individuals. The other relevant work already mentioned in the introduction indicated that levels of aggression by a stimulus male fruit fly influenced aggressive interactions between two focal male fruit flies (Saltz 2013).

To some degree, it is intuitive that stimulus individuals can change the social dynamics among several focals. For example, in humans, one can readily envision how a single person would alter the social dynamics at a holiday family dinner. And in the business world, it is widely agreed that a group leader can dramatically affect group performance via the nature of interactions among team members (Hackman 2002). When it comes to animal behavioural and evolutionary biology, however, we still know little about how IGEs by some individuals influence social interactions among several focals. Another highly relevant issue is the impact such IGEs would have on the rate and direction of social evolution. While we require further empirical data on that topic, it is likely that within group cohesion and the quantity and quality of its interactions can affect a variety of features linked to fitness. Such factors, which have been documented in fruit flies, include the exchange of social information (Sarin & Dukas 2009; Battesti et al. 2012),

longer lifespan owing to fewer antagonistic interactions (Carazo, Tan, Allen, Wigby & Pizzari 2014), suppression of microbial competitors and pathogens (Rohlfs 2005; Rohlfs, Obmann & Petersen 2005), enhancing the growth of favorable yeast species (Wertheim, Dicke & Vet 2002; Wertheim, Marchais, Vet & Dicke 2002; Stamps, Yang, Morales & Boundy-Mills 2012), locating the best available resources (Durisko & Dukas 2013) and improved larval digging (Durisko et al. 2014), which could reduce their predation risk (Rohlfs & Hoffmeister 2004).

We chose to use CS males as one of the stimulus fly treatments, meaning that, for that treatment, the focals (always CS males) and stimulus flies came from the same population, but from distinct vials. One would expect the higher relatedness between focal and stimulus males in this treatment to increase cohesion, perhaps through the expected reduced aggression between related males (Carazo et al. 2014; Martin & Long 2015). It appears, however, that the dominant effect was the tendency of CS males to be the most dispersed, as indicated by their highest nearest neighbour index (Fig. 2.1b, 1d). Furthermore, the fact that the interaction between stimulus male nearest neighbour index and genotype was not significant (see methods) suggests that the relative strength of IGEs was similar when the stimulus males were Canton-S and DGRP. Nevertheless, we cannot separate the possible effects of focals' relatedness to, and sociability of the stimulus flies. Another issue that we still cannot resolve is the occasional significant effect of the standard fluorescent powder that we use for marking flies.

We have identified one possible mechanism mediating the effect of stimulus flies on focals: the encounter rates *among focals* were highest when interacting with the least cohesive stimulus line (CS) and lowest when interacting with the most cohesive stimulus line (Fig. 2.2a). This is perhaps because the encounter rates between *stimulus flies and focals* were highest when the stimulus flies were the least cohesive and lowest when the stimulus flies were the most cohesive (DGRP 304; Fig. 2.2b). While it is clear that the quantity and quality of interactions determine a group's sociability, we still do not know how the encounter rate may affect our sociability score. We can rule out some artifact of

activity levels because our independent analyses indicated no correlation between activity levels and sociability in 29 isofemale lines (Anderson et al. 2016).

In both our previous and current work, we observed no overt aggressive interactions but we cannot preclude the role of either explicit aggression during the habituation period prior to video recording or subtle antagonism during video recording. For example, it is possible that the significant effect of encounter rate is associated with either subtle behavioural cues or odour signals. It is indeed known that olfaction plays a role in fruit fly social interactions (Schneider et al. 2012), and that cuticular hydrocarbons, which can mediate social interactions, may vary in response to social cues (Krupp et al. 2008; Gershman, Toumishey & Rundle 2014). Interestingly, encounters involving touch were the mechanism mediating both collective behaviour that enhanced avoidance of an aversive odour in fruit flies (Ramdya et al. 2015), and the switch from solitary to gregarious phase in desert locusts (*Schistocerca gregaria*) (Simpson, Despland, Hagele & Dodgson 2001). It thus appears that mechanosensory information has a special role in orchestrating social behaviour in insects.

IGEs are widely acknowledged as a major potential factor in social evolution due to their complex effects on the relationships between genotypes and phenotypes and the fact that they themselves can evolve (Wolf, Brodie Iii, Cheverud, Moore & Wade 1998). Although fruit flies are not typically considered among the multitude of species serving for research on the mechanisms of social behaviour, recent data (Saltz 2011; Battesti et al. 2012; Schneider et al. 2012; Durisko & Dukas 2013), our current demonstration of IGEs of stimulus individuals effecting social behaviour among several focals, and the numerous tools available for mechanistic and evolutionary research in this classic model system open up further fruitful directions for research on the role of IGEs in the evolution of social behaviour.

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CHAPTER 3 – SOCIABILITY IN FRUIT FLIES: GENETIC VARIATION, HERITABILITY, AND PLASTICITY

Scott, A. M., Dworkin, I., Dukas, R. (2018). Sociability in fruit flies: genetic variation, heritability and plasticity. *Behavior Genetics*, *48*(3), 247-258.

3.1 Abstract

Sociability, defined as individuals' propensity to participate in non-aggressive activities with conspecifics, is a fundamental feature of behavior in many animals including humans. However, we still have a limited knowledge of the mechanisms and evolutionary biology of sociability. To enhance our understanding, we developed a new protocol to quantify sociability in fruit flies (*Drosophila melanogaster*). In a series of experiments with 59 F1 hybrids derived from inbred lines, we documented, first, significant genetic variation in sociability in both males and females, with broad-sense heritabilities of 0.24 and 0.21 respectively. Second, we observed little genetic correlation in sociability between the sexes. Third, we found genetic variation in social plasticity among the hybrids, with a broad-sense heritability of ~0.24. That is, genotypes differed in the degree of sociability after experiencing the same relevant social experience. Our data pave the way for further research on the mechanisms that underlie sociability as well as its ecological and evolutionary consequences.

3.2 Introduction

Social behavior, broadly defined as the interactions between conspecifics, has been subjected to extensive research in a broad range of organisms from bacteria to humans (Allee, 1931; Wilson, 1975; Ward and Webster, 2016). A key aspect of social behavior is sociability, defined as the tendency to engage in non-aggressive group activities. Examples include feeding or roosting together, and traveling in a group. Sociability varies widely

among animal species, between distinct ecological settings within a given species and among individuals within a population. For example, an analysis of social behavior among over 2500 mammalian species revealed a robust pattern of evolutionary transition from the ancestral solitary condition, which occurs in 68% of the species, to social monogamy (9%) and then to group living (23%) (Lukas and Clutton-Brock, 2013). Within some carnivore species such as red foxes (*Vulpes vulpes*) and grey wolves (*Canis lupus*), food abundance and distribution dramatically alters sociability (Macdonald, 1983; Johnson et al., 2002). Finally, Cote and colleagues (Cote and Clobert, 2007; Cote et al., 2012) documented individual variation in sociability in both lizards (*Lacerta vivipara*) and fish (*Gambusia affinis*).

There has recently been increased interest in using fruit flies (*Drosophila melanogaser*) as a model system for research on social behavior. Although fruit flies are traditionally classified as solitary insects, they actually show a variety of social behaviors including aggregation at food sources, which is actively modulated through pheromones (Bartelt et al., 1985; Wertheim et al., 2006; Lin et al., 2015), social synchronization of the circadian clock (Levine et al., 2002), reliance on social information gleaned from conspecifics (Sarin and Dukas, 2009; Battesti et al., 2012), and the formation of social groups (Saltz, 2011; Schneider et al., 2012; Simon et al., 2012; Anderson et al., 2016). While numerous taxa have been used successfully for research on social behavior, fruit flies are especially fruitful for such investigation owing to the abundance of tools that can facilitate all levels of biological analysis from genetics and neuroscience to behavioral and evolutionary biology (e.g. Ashburner, 1989; Greenspan, 2004; Zhang et al., 2010).

To enhance our knowledge of the evolutionary biology of sociability, we need further information about topics such as heritable variation in sociability, genetic correlations between life stages and sexes, and heritable variation in the plasticity of sociability. There are currently limited data regarding genetic variation in sociability and its mechanistic basis. By far, the most established research on the genetics of sociability involves mouse models of autism spectrum disorder. This line of research has identified a large variety of genes that influence social behavior (Moy and Nadler, 2008; Silverman et

al., 2010; Tuttle et al., 2017). Perhaps the best known case of natural genetic variation in sociability is the solitary and social forms of the nematode *Caenorhabditis elegans*. Solitary foragers disperse across a bacterial food substrate and feed alone, whereas social foragers aggregate and form clumps of up to several hundred individuals (De Bono and Bargmann, 1998). In fruit flies, individuals from five distinct genetic lines varied in their social environmental choice (Saltz, 2011), and work in our laboratory documented genetic variation in inter-individual distance among 29 distinct inbred lines (Anderson et al., 2016). Finally, in humans, personality traits associated with sociability including extraversion and the number of friends are highly heritable (Fowler et al., 2009; van den Berg et al., 2016). As noted earlier, sociability is also affected by the environment (Macdonald, 1983; Johnson et al., 2002). We know, however, of no research assessing genetic variation in the plasticity of sociability.

We developed a new apparatus to critically address sociability, defined as the tendency to engage in social activities with other individuals. A few laboratories, including ours, have used a variety of protocols to quantify social behavior in fruit flies (Tinette et al., 2004; Bolduc et al., 2010; Saltz, 2011; Saltz and Foley, 2011; Schneider et al., 2012; Lihoreau et al., 2016; Philippe et al., 2016; Anderson et al., 2017; Fernandez et al., 2017). Most notably, some protocols focused on social influences on food search behaviour (Tinette et al., 2004; Lihoreau et al., 2017). Other protocols measured inter-fly distance (Bolduc et al., 2010; Anderson et al., 2017; Fernandez et al., 2017). A few studies relied on social network analyses (Schneider et al., 2012; Pasquaretta et al., 2016). Finally, one study examined how male-male aggression influenced male and female fly distributions among food patches (Saltz and Foley, 2011). While the other protocols are highly illuminating, they do not provide the critical feature that we wished to quantify, which was individuals' decisions to either join others or be alone at a food patch while controlling for food searching and sexual interactions and including in the analyses all individuals in each arena. Our new protocol allowed groups of same-sex flies from a given genetic background to arrange themselves according to their social preference inside arenas with distinct compartments separated by traversable barriers (Figure 3.1).

We conducted a set of experiments addressing the following questions. First, what is the magnitude of genetic variation in sociability? Second, is there a genetic correlation in sociability between males and females? Third, are there key behavioral determinants, such as levels of activity, aggression, or non-aggressive interactions, that correlate with the observed genetic variation in sociability? Finally, do distinct genotypes respond differently to their social environment? That is, is there genetic variation in the plasticity of sociability?

3.3 Methods

3.3.1 General methods

We chose 60 Wolbachia-free lines from the *Drosophila* Genetic Reference Panel (DGRP). These lines were derived from mated females caught in Raleigh, North Carolina, USA, whose progeny were inbred through 20 generations of full-sibling mating (Mackay et al., 2012). We maintained these lines in vials with 5 mL of standard food medium (1L = 90 g sucrose, 75 g cornmeal, 10 g carrageenan, 32 g yeast, and 2 g methyl paraben dissolved in 20 mL ethanol) in an environment chamber at 25°C, 50% relative humidity, and on a 12:12 light cycle with lights on at 10 AM.

In order to lessen the deleterious effects of inbreeding on the fruit fly nervous system that are observed in the majority of the inbred DGRP lines (Zwarts et al., 2015), we used F1 hybrid flies (hereafter DGRP hybrids) generated from crosses between males from each of 59 DGRP lines and females from a randomly-chosen standard line, DGRP-83. For brevity, we refer to the DGRP hybrids based on their paternal line. We allowed virgin females of DGRP-83 to mate with males from each of the other 59 lines and lay eggs in food vials with 5 mL of standard food and a sprinkle of live yeast. We maintained consistent rearing densities by removing excess eggs from the vials. We collected experimental DGRP hybrid flies 11 days after egg laying. To avoid the deleterious effects of CO_2 anesthesia (e.g. Bartholomew et al., 2015), we sexed and transferred flies using gentle aspiration.

We analyzed the data with general linear mixed-effects models in R version 3.3.3 (R-Core-Team, 2014) with the package lme4 version 1.1-12 (Bates et al., 2014). For tests of fixed effects, we report Wald χ^2 values generated with the Anova function from the car package version 2.1-4 (Fox and Weisberg, 2011). For random effects, we report p-values calculated as the fraction of parametric bootstrapped likelihood ratio test (LRT) statistics (with 10,000 iterations) that were larger than the observed LRT values, using the package pbkrtest version 0.4-7 (Halekoh and Højsgaard, 2014). To generate 95% confidence intervals on model variance components and heritability estimates, we performed hierarchical non-parametric bootstrapping (with 10,000 iterations). In each iteration, sampling with replacement occurred first at the level of DGRP hybrids, and then samples within DGRP hybrids. This approach also enabled us to verify model estimates for general linear mixed-effects models since the assumption of normally distributed residuals of these models was violated, due to our measure of sociability being bounded. We used custom code for the bootstrapping based on Roles et al. (2016). For tests of significance of correlations between traits, we report results from Spearman's rank correlations, and bootstrapped 95% confidence intervals (with 10,000 iterations) generated with the boot package (Canty and Ripley, 2017). We describe further statistical details in the sections below.

3.3.2 Genetic variation in sociability and correlation between the sexes

Quantifying genetic variation in sociability

We collected DGRP hybrid adults from each of the 59 crosses within 8 hours of eclosion, and transferred a mixed sex group consisting of 5 males and 5 females from the same cross into each vial containing 5 mL of standard food. We left the flies in an environment chamber for 3 days to gain social experience. Approximately 72 hours post-eclosion, at 9:00 AM, we transferred groups of 4 same-sex flies from the same vial into each test arena. The test arenas (Figure 3.1a) were circular petri dishes (35 mm diameter x 10 mm high) with wooden partitions that divided the dish space into 4 quadrants. Each quadrant had a

single food patch (5 mm diameter x 1 mm thick) with a layer of grapefruit/yeast solution (3 g yeast per 100 mL grapefruit juice) on the surface of the food. Flies could move between quadrants through 3 mm holes in the center of each partition. Our preliminary experiments indicated that flies frequently travelled between quadrants.

We aspirated live flies into the arenas through a 3 mm hole in the dish lid, such that the starting arrangement consisted of 1 fly in each quadrant. We placed the dishes into 4 large semi-transparent plastic containers with opaque lids (l x w x h: 51 x 31 x 30 cm), which were humidified at ~75% RH. We left the flies to explore the arenas and acclimatize from 11:30 AM to 2:30 PM. Then every 10 minutes from 2:30 PM to 4:00 PM, an observer blind to DGRP hybrid identity recorded the number of flies in each quadrant of each dish through a thin opening in the box lids. We tested each group of flies only once. We counterbalanced which box the crosses were tested in, and the location within each box across days. Our sample sizes ranged from 10 to 14 arenas per each DGRP hybrid and sex.

We quantified the sociability score of each group of 4 flies in each arena at each time point using the aggregation index. The aggregation index is a standard ecological measure (Krebs, 1999), which we have previously used successfully to quantify social behaviour in fruit fly larvae (Durisko et al., 2014), and is calculated as the variance divided by the mean number of flies in each quadrant. In our protocol, sociability scores could take 5 possible values ranging from 0 to 4 (Figure 3.1b), with 0 representing the least sociable distribution (1 fly per quadrant), and 4 representing the most sociable distribution (all flies in the same quadrant). A value of 1 (mean = variance) represents random distribution, which could only be taken on by averaged observations. We pooled the data over the 1.5 h observation period as there was very little among-DGRP hybrid variation for the temporal effects compared to the overall among-DGRP hybrid effects (about 1/1000th the variation). We constructed a general linear mixed model with pooled sociability scores as the dependent measure, day and box as simple random effects, and sex as both a fixed effect and allowed to vary by the random effect of DGRP hybrid. We constructed reduced models to test each of the simple random effects, and models that reduced DGRP hybrid to a simple random effect or omitted it to test for a genotype by sex interaction and main effect of

genotype, respectively. We also constructed full models separately for each sex with day, box and DGRP hybrid as simple random effects, and reduced models to test for the sex-specific effects of DGRP hybrid, day, and box. We tested for significant correlations between male and female sociability using sex-specific means of each DGRP hybrid, and also using model-generated sex-specific best linear unbiased predictors of the random effects of each DGRP hybrid. Because the correlations of the best linear unbiased predictors were very similar to the correlations of the means of each DGRP hybrid, we only report the correlations of the latter. We used non-parametric bootstrapping to generate estimates and confidence intervals of sex-specific broad-sense heritabilities (H^2) of sociability. H^2 was estimated as $V_G/(V_G + V_E) = 2\sigma_l^2/(2\sigma_l^2 + \sigma_e^2)$, where V_G is genetic variance, V_E is environmental variance, σ_l^2 is the among-DGRP hybrid variance component, and σ_e^2 is the error variance (including both the residual and day variance components) (Falconer and Mackay, 1996; Shorter et al., 2015). We multiplied the among-DGRP hybrid variance component by 2 to account for the shared maternal line of the DGRP hybrids. We also calculated sex-specific values of the coefficient of genetic variation (CV_G), which is a scaled measure of genetic variation that is not environment-specific, and therefore more easily compared to other traits (Houle, 1992). We calculated CV_G as $\sqrt{V_G}/\bar{X} = \sqrt{2\sigma_l^2}/\bar{X}$, where $\bar{X}$ is the sex-specific overall mean sociability score. Note that the DGRP hybrid males all received an X chromosome from the same standard maternal line, DGRP-83. This means that our measures of genetic variation included all chromosomes in the hybrid females but only the autosomal chromosomes in the hybrid males.

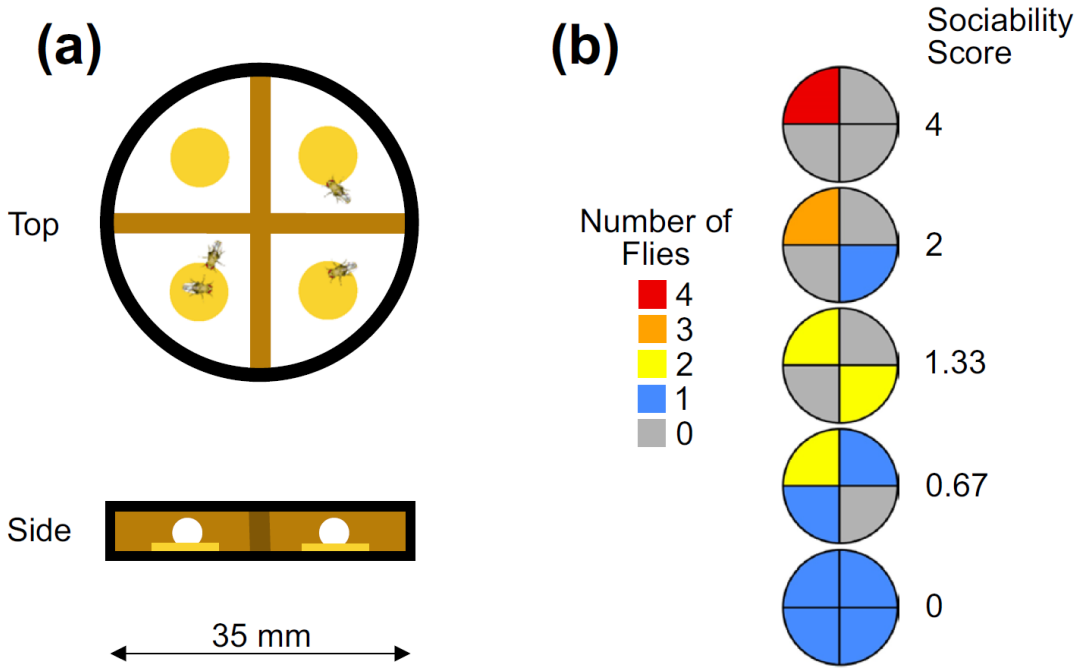

Figure 3.1

(a) Diagram illustrating top and side views of the arenas used for quantifying sociability. Yellow circles (top) or rectangles (side) indicate standard food patch discs, and brown rectangles indicate barriers between quadrants. Holes allowing the flies to move between quadrants are visible in the side view. (b) Diagram illustrating some of the possible arrangements of flies in the sociability arenas and all of the possible values for the sociability score (calculated as variance/mean number of flies in each quadrant), with most to least social arrangements displayed from top to bottom.

3.3.3 Follow-up sociability experiment in a subset of 16 DGRP hybrids

Our initial analyses revealed a weak genetic correlation in sociability between males and females (Figure 3.2c). In order to better characterize the genetic correlation between the sexes, we repeated the sociability test on a subset of 16 DGRP hybrids. We used a randomness generator to choose 4 DGRP hybrids from each quartile of the mean sociability scores in males from the 59-DGRP hybrid assay. The bars of these 16 hybrids are marked with white dots in Fig. 3.2a. We based this choice on the male data due to the larger genetic variation in males compared to females (Figures 3.2a, b). The methods for rearing the hybrids, housing, and testing were similar to the methods for the 59-DGRP hybrid assay above. We assayed a total of 10 arenas per each DGRP hybrid and sex. We analyzed the data using general linear mixed models, and tested for significant male-female correlations as in the assay using the 59 DGRP hybrids.

3.3.4 Behavioural determinants of sociability

To gain insight into the mechanisms that generate the observed genetic variation in sociability score, we conducted two experiments. First, we quantified the activity level of individual flies to assess whether genetic variation in activity is correlated with sociability scores. Second, we video recorded a sub-sample of DGRP hybrids in the sociability test arenas (Figure 3.1) and conducted detailed behavioral analyses of key factors that we expected to influence the sociability scores. These included (i) another measure of activity, the frequency of movement between quadrants, (ii) aggression frequency, and (iii) non-aggressive encounter frequency.

Genetic variation in activity

We assayed 57 of the 59 DGRP hybrids used in the sociability assay for baseline individual activity. Two of the paternal lines, DGRP-757 and DGRP-158, died out between the two experiments. We used the same protocol for rearing, collecting and housing the hybrids

that we used for the sociability assay. Approximately 72 hours post-eclosion, at 9:00 AM, we aspirated a single fly from each mixed-sex housing vial, either male or female depending on the day, into a small snap-cap vial (22 mm diameter x 48 mm long). The lids of the snap cap vials had a small pinhole for ventilation. Each snap-cap vial had a single food patch (5 mm diameter x 1 mm thick) with a layer of grapefruit/yeast solution. We placed the vials into 1 of 2 Drosophila activity monitors (Trikinetics Inc.; software version 3.08). We placed each monitor in an upright position in 1 of 2 opaque plastic containers (l x w x h: 51.5 x 36 x 41 cm) that were humidified at ~75% RH. The vials were held in the monitor slots in a horizontal position, with ~7 mm of clearance between the infrared sensors and the surface of the food patch. We placed an LED lightbulb over a hole in the center of each container lid to illuminate the monitors from above. We left the flies to acclimatize from 11:30 AM to 2:30 PM. From 2:30 PM to 4:00 PM, activity was automatically recorded as the total number of times that each fly crossed the ring of infrared sensors that surrounded each snap-cap vial during the 90 minute test period. We assayed 1 fly from each DGRP hybrid cross per day, alternating testing males and females daily, over 30 days. We counterbalanced which monitor and which position within each monitor the DGRP hybrids were tested in across days. In total, we assayed between 10 to 15 replicates per DGRP hybrid and sex.

We analyzed the data by constructing zero-inflated negative binomial generalized linear mixed models using the package glmmTMB version 0.1.1 (Brooks et al., 2017) because a high proportion of flies (21%) had activity scores of 0. For the conditional model, we included the number of times the fly crossed the infrared sensor as the dependent measure, activity monitor as a fixed effect, day as a simple random effect, and sex as both a fixed effect and varied by the random effect of DGRP hybrid. For the zero-inflation model, we included sex and activity monitor as fixed effects and DGRP hybrid as a simple random effect. We tested for significant correlations between sociability and activity means of each DGRP hybrid, and between sociability and the model-generated best linear unbiased predictors of the random effects of each DGRP hybrid for activity (from both the conditional and zero-inflation models). We found the correlations of the best linear

unbiased predictors to be close to the correlations of means of each DGRP hybrid, so we only report the latter.

Inter-quadrant movement frequency, aggression, and non-aggressive encounters

We conducted video recording during the replicate sociability assay with the 16 DGRP hybrids described above. We focused on males from 8 of the 16 DGRP hybrids, with 2 randomly chosen from each quartile. After introducing the flies into the test arenas, we video recorded them for 1 h using 6th generation Apple iPod Touch devices at 30 frames per second. We focused on the first hour because we assumed that the initial interactions in the arena would be the most important in establishing fly distributions in the arenas and hence their sociability scores. Overall, we video recorded 2 male arenas from each of the 8 selected DGRP hybrids each day for 5 days, for a total of 10 video observations per DGRP hybrid.

Observers blind to DGRP hybrid identity recorded aggressive interactions from minutes 5 – 20, and non-aggressive interactions and boundary crossing from minutes 0 – 60 of each video using BORIS behaviour coding software version 3.50 (Friard and Gamba, 2016). Observers recorded aggressive interactions, which included lunging, wing threat, high-level fencing, charging, holding, boxing and tussling (Chen et al., 2002; Baxter and Dukas, 2017). Because almost all aggressive events were lunges, we quantified aggression as the lunging frequency. Observers recorded non-aggressive encounters using the same criteria that we established in a previous experiment (Anderson et al., 2017), in which we defined these encounters as inspections of one fly by another (e.g. licking or prodding with legs), or the movement of one fly towards another followed by a response from the other fly (e.g. wing fluttering or moving away). Observers recorded boundary crossings as a fly moving from one quadrant to another. We analyzed the data using general linear mixed models as in the 59 DGRP hybrid and replicate sociability assays and included inter-quadrat movement rate, lunging rate, and non-aggressive encounter rate as quantitative predictors.

3.3.5 Genetic variation in the plasticity of sociability

We assayed sociability in males of 16 DGRP hybrids across 4 pre-test social environments. We used the same DGRP hybrids as those in the replicate sociability assay, except for the hybrid with paternal line DGRP-38, which died out between experiments. We replaced this line with a hybrid with paternal line DGRP-843, which we randomly selected from the same quartile as DGRP-38. After sexing the flies, we introduced males of each DGRP hybrid cross into standard food vials with 1 of 4 social environments for the 3-day pre-test period: males housed individually, single males housed with single females, males housed in groups of 4, and mixed sex groups of 4 males and 4 females. Having males with and without females allowed us to test both a natural situation (mixed sex groups) and a situation that controls for male mating status (male only groups). On the morning of the test day, when all flies were about 72 h post eclosion, we transferred males from the same social treatment and DGRP hybrid cross to the test arenas. For the treatments with 4 males in a vial, we transferred groups that were housed together into the same arena. Our sample sizes were either 9 or 10 arenas per each DGRP hybrid and treatment.

We analyzed the data by constructing general linear mixed models as in the other sociability assays, with pooled sociability scores as the dependent measure, number of males (1 vs 4) and female presence (yes vs no) as fixed effects, and with both effects allowed to vary by the random effect of DGRP hybrid (equivalent to random slopes models). We initially included both day and box as simple random effects, but removed them as the variance estimates were very close to zero. We used non-parametric bootstrapping to generate estimates and confidence intervals of the broad-sense heritabilities (H^2) of the plasticity of sociability under the different social environment contexts. H^2 was estimated as $V_G/(V_G + V_E) = 2\sigma^2_{l*t}/ (2\sigma^2_{l*t} + \sigma^2_e)$, where V_G is genetic variance, V_E is environmental variance, $2\sigma^2_{l*t}$ is the DGRP hybrid-by-treatment interaction variance component (treatment being number of males or female presence), and σ^2_e is the error variance (Scheiner and Lyman, 1989). We also calculated coefficients of genetic

variance (CV_G) estimates as $\sqrt{V_G}/\bar{X} = \sqrt{2\sigma^2_{l*t}}/\bar{X}$, where $\bar{X}$ is the overall mean sociability score.

3.4 Results

3.4.1 Genetic variation in sociability and correlation between the sexes

We found significant genetic variation in sociability among the 59 DGRP hybrids in both males (range of mean sociability scores: 0.77 – 2.85; $p < 0.001$, Figure 3.2a) and females (range of mean sociability scores: 1.10 – 2.35; $p < 0.01$, Figure 3.2b). The broad-sense heritability of sociability was 0.24 (95% CI [0.14, 0.35]) for males, and 0.21 (95% CI [0.11, 0.31]) for females. The estimated coefficients of genetic variance (CV_G) were 0.31 (95% CI [0.22, 0.39]) for males and 0.24 (95% CI [0.16, 0.31]) for females. On average, males were more sociable than females (1.81 vs. 1.60 mean sociability scores respectively; Wald $\chi^2_1 = 13.16$, $p < 0.001$) but there was a significant DGRP hybrid-by-sex interaction ($p < 0.01$). Within the male data, there was no significant effect of day ($p = 0.27$) or observation box ($p \approx 1$); within the female data, there was a significant effect of day ($p < 0.01$) but not of observation box ($p = 0.09$).

In the analysis of the 59 DGRP hybrids, we found a weak significant positive genetic correlation between the sexes for sociability ($r_s(57) = 0.28$, $p = 0.03$, 95% CI [0.01, 0.51]; Figure 3.2c). However, in the follow up experiment using a subset of 16 DGRP hybrids, we found no correlation in sociability scores between the sexes ($r_s(14) = 0.037$, $p = 0.89$; 95% CI [-0.55, 0.63]; Figure 3.2d).

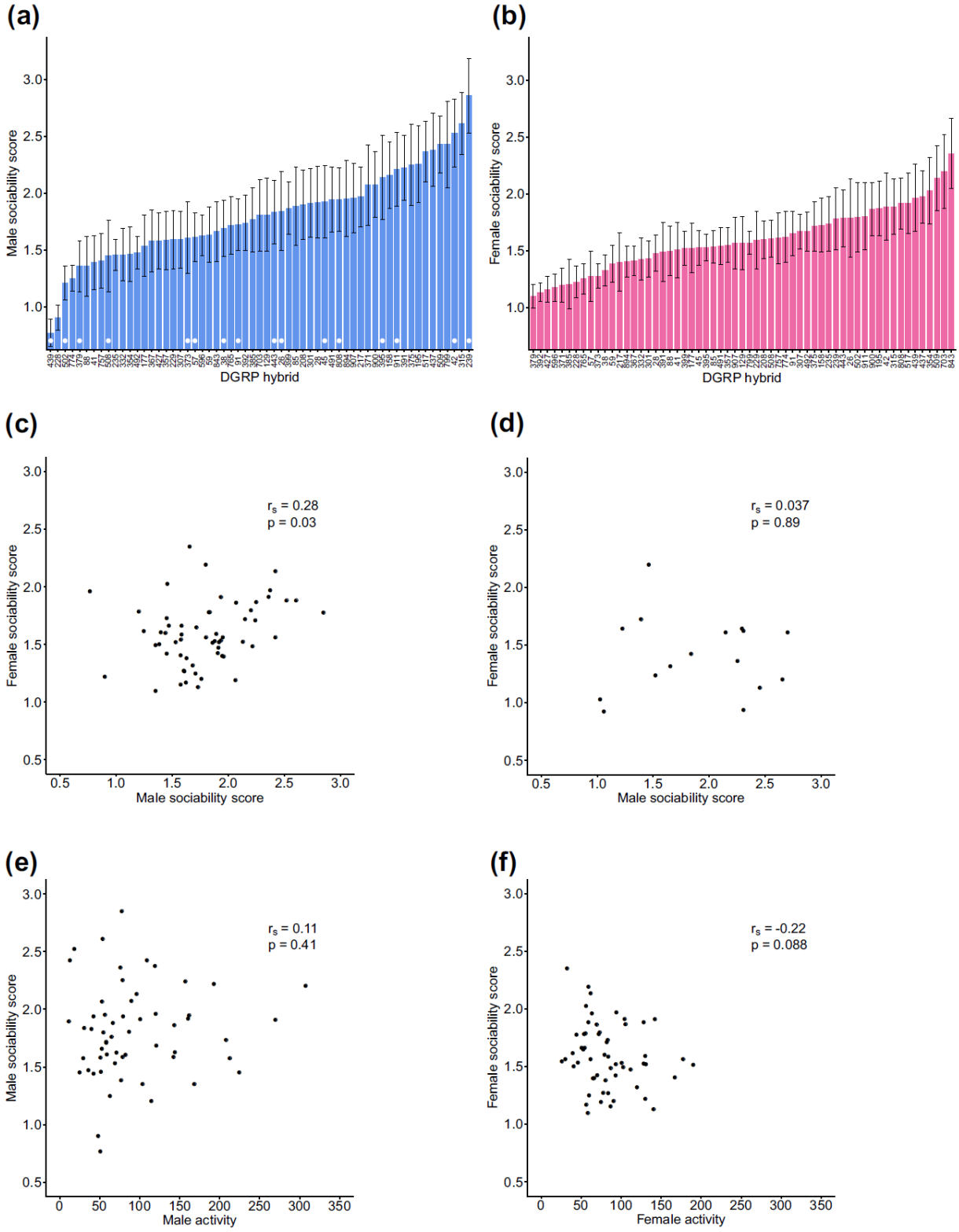
(a)
Male sociability score
DGRP hybrid
(b)
Female sociability score
DGRP hybrid
(c)
Female sociability score
r_s = 0.28
p = 0.03
Male sociability score
(d)
Female sociability score
r_s = 0.037
p = 0.89
Male sociability score
(e)
Male sociability score
r_s = 0.11
p = 0.41
Male activity
(f)
Female sociability score
r_s = -0.22
p = 0.088
Female activity

Figure 3.2

Genetic variation in sociability and correlations in sociability across sexes and between sociability and activity. Mean sociability scores ± 1 standard error of the mean (SEM) of 59 DGRP hybrids are shown in (a) males and (b) females. Bars are ordered along the x axis by increasing mean, and are labeled according to the paternal DGRP line. The bars of the 16 DGRP hybrids used in the replicate sociability assay are marked with white dots in Fig. 3.2a. Genetic correlations between males and females for sociability are shown in (c) the original 59 DGRP hybrid assay and (d) the 16 DGRP hybrid subset assay. Correlations between sociability and activity are shown in (e) males and (f) females. Points in the scatterplots represent means for each DGRP hybrid generated from the raw data.

3.4.2 Behavioural determinants of sociability

Genetic variation in activity

We found no significant genetic correlations between activity and sociability in either males ($r_s(55) = 0.11$, $p = 0.41$, 95% CI [-0.17, 0.37]) or females ($r_s(55) = -0.22$, $p = 0.088$, 95% CI [-0.44, 0.02]; Figures 3.2e,f).

Inter-quadrant movement frequency, aggression, and non-aggressive encounters

We found no significant effects of inter-quadrant movement rate (Wald $\chi^2_1 = 0.035$, $p = 0.85$), lunging rate (Wald $\chi^2_1 = 0.72$, $p = 0.40$) and non-aggressive encounter rate (Wald $\chi^2_1 = 0.31$, $p = 0.58$) during the initial acclimatization period on subsequent sociability in males of 8 DGRP hybrids. We noted that means of non-aggressive encounter rates were correlated with means of lunging frequencies ($r_s(6) = 0.85$, $p = 0.008$). However, taking either encounter or lunging frequency out of the model did not change the effects of the other quantitative predictors. We also noted that 6 of 8 DGRP hybrids had mean lunging rates close to 0 (between 0.1 and 0.9 lunges per 15 minutes), DGRP hybrid-26, which had a mid-level mean sociability score in the subset assay, had a mean lunging rate of 4.1 per 15 minutes, and DGRP hybrid-502, which had the lowest mean sociability score among the 8 video-recorded hybrids, had the highest mean lunging rate (21.3 per 15 minutes).

3.4.3 Genetic variation in the plasticity of sociability

We found a significant effect of the number of males housed together during the pre-trial period on subsequent male sociability, with males housed with other males being more sociable than males housed singly (Wald $\chi^2_1 = 37.52$, $p < 0.001$; Figure 3.3a). However, female presence had no significant effect on subsequent male sociability (Wald $\chi^2_1 = 1.55$, $p = 0.21$; Figure 3.3a). There was significant genetic variation in sociability among males of the 16 DGRP hybrids ($p < 0.001$; Figures 3.3b,c). The interaction between DGRP hybrid and the number of males housed together approached significance ($p = 0.083$; Figure 3.3b),

and the interaction between DGRP hybrid and female presence was significant ($p = 0.038$, Figure 3.3c). The broad-sense heritability of the plasticity of sociability was 0.22 (95% CI [0.04, 0.41]) in the context of number of males housed together, and 0.26 (95% CI [0.07, 0.45]) in the context of female presence. The coefficients of genetic variation (CV_G) of the plasticity of sociability were 0.26 (95% CI [0.10, 0.41]) in the context of number of males housed together, and 0.30 (95% CI [0.14, 0.44]) in the context of female presence.

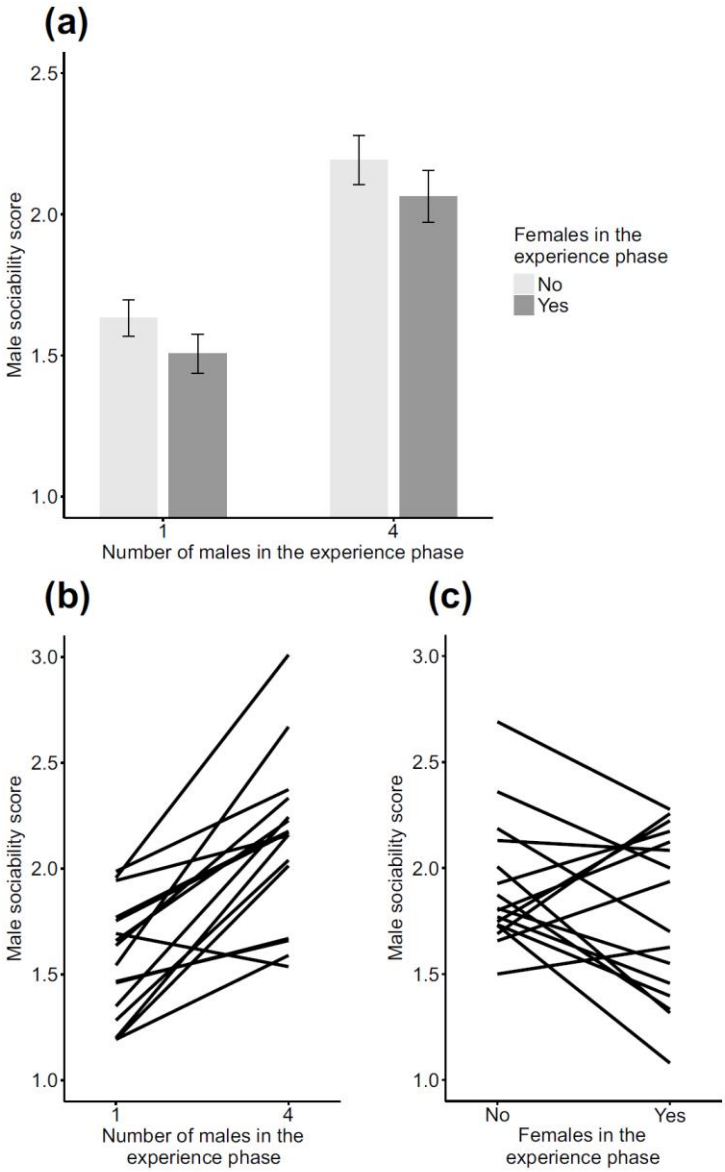

Figure 3.3

Social plasticity in males of 16 DGRP hybrids. All tests involved calculating the sociability scores of groups of 4 males after they had experienced distinct social settings. (a) shows the mean (± 1 SEM) sociability scores averaged across the 16 DGRP hybrids for males previously housed singly with no females, singly with a female, in groups of 4 with no females, and in groups of 4 males + 4 females. (b) and (c) show the mean sociability scores for each of the 16 DGRP hybrids (reaction norm lines) as a function of their previous social experience, (b) alone or in groups of 4 males in the experience phase, and (c) without or with females in the experience phase. Error bars in (b) and (c) are omitted for clarity.

3.5 Discussion

Our major findings were, first, that there was significant genetic variation in sociability in both males and females with broad-sense heritability of 0.24 and 0.21 respectively (Figs. 3.2a,b). Second, there was little genetic correlation in sociability between the sexes (Figs. 3.2c,d). Third, sociability scores were not correlated with activity levels (Fig. 3.2e,f), aggression, or non-aggressive inter-individual interactions. Finally, we found genetic variation in social plasticity among the DGRP hybrids (Fig. 3.3). We discuss these results in turn.

We defined sociability as the tendency to engage in social activities with other individuals and developed a new apparatus to quantify it. In that apparatus, each of four individual flies decided whether to join others, stay with others, deter others from joining, or move to an unoccupied food patch (Fig. 3.1a). A glance at Figs. 3.2a,b indicates first, that flies clearly did not avoid each other as only two DGRP hybrids had a sociability score below 1 (see Fig. 3.1b). Second, most hybrids had a sociability score above the random value of 1. Finally, no hybrids approached the maximum score of 4. Hence we can conclude that fruit flies are moderately sociable. We have reached similar conclusions in two previous studies using distinct fly life stages, lines and protocols. The first project involved larvae that were descendants of wild-caught fruit flies (Durisko et al., 2014) and the other project included larvae and adults of 29 inbred DGRP lines (Anderson et al., 2016). Interestingly, our sociability apparatus is conceptually similar to the two-tube version of the tube co-occupancy test, which was recently developed for quantifying sociability in mice (Figs S2A and 1E in Tuttle et al., 2017). The tube co-occupancy test is supposed to advance research on mouse sociability as it allows for the more realistic direct contact between individuals. This does not occur in the traditional apparatuses, which rely on testing the proximity of a focal mouse to either a mouse or control object placed beyond screens (Tuttle et al., 2017).

As noted in the introduction, there is currently limited information on natural genetic variation in sociability (De Bono and Bargmann, 1998; Saltz, 2011; Anderson et

al., 2016; Ward and Webster, 2016). In humans and other mammals, much of the research effort has focused on candidate genes for autism (Abrahams and Geschwind, 2008; Moy and Nadler, 2008) and for pair bonding (Donaldson and Young, 2008; Walum et al., 2008). In humans, social skills are highly heritable (Viken et al., 1994; Scourfield et al., 1999; Rettew et al., 2008; van den Berg et al., 2016) and variation in a few genes has been linked to measures related to sociability (Skuse et al., 2014; Pearce et al., 2017). Twin studies in humans have provided some estimates of the heritability of social behaviours, such as altruism (Rushton et al., 1986), antisocial behaviour (Mason and Frick, 1994) and reciprocal social behaviour (Constantino and Todd, 2000), and all have been found to be highly heritable. While we found significant genetic variation in sociability among the 59 DGRP hybrids, we cannot yet link that variation to either survival or reproduction. Similarly, we will require further work for linking the variation in sociability among the DGRP hybrids to specific genes and neurobiological pathways. Our estimate of the heritability of sociability (0.24 for males and 0.21 for females) is close to the typical estimate of the heritability of social behaviours, which is around 0.3 (Stirling et al., 2002).

Our data indicated mostly independent regulation of sociability in males and females, in that there was little evidence for a genetic correlation. The most likely explanation for this is that sociability is determined by mechanisms similar to the ones regulating sex specific traits related to maximizing mating opportunities in males and egg laying in females. Interestingly, males' sociability scores were significantly higher than females' (Figs. 3.2a,b) but there was significant DGRP hybrid-by-sex interaction. We still cannot explain this pattern. While we are not aware of data pertaining to genetic correlations in sociability between the sexes, there are some relevant data on aggression. In fruit flies, artificial selection on male-male aggression resulted in a single line in which males were hyperaggressive but there was no change in female-female aggression (Penn et al., 2010). Mouse studies on male-female correlation in aggression are inconclusive, with some studies showing no correlation and others reporting positive correlation between male-male aggression and maternal aggression (Sandnabba, 1996; Gammie et al., 2003). Finally, white throated sparrows (*Zonotrichia albicollis*) have two morphs, which are

determined by an inversion polymorphism on chromosome 2. Both sexes of the white-striped morph show higher levels of some types of aggression than males and females of the tan-striped morph (Thorneycroft, 1966; Thorneycroft, 1975; Thomas et al., 2008; Horton et al., 2014).

We conducted two assays to quantify behavioral correlates of sociability. First, we wished to verify that our sociability scores did not merely reflect genetic variation in levels of activity. For example, if docile flies just stayed where we placed them one per quadrant, we would have classified them as non-sociable (Fig. 3.1). We quantified the activity levels of individual flies so that our measures were not influenced by social interactions. While we found large genetic variation among the DGRP hybrids, it was not correlated with sociability (Figs. 3.2e,f). Our results are consistent with previous analyses using distinct protocols, which showed decoupling of social behavior and activity in larval and adult fruit flies (Anderson et al., 2016). Similarly, measures of activity were not correlated with aggressive behavior in fruit flies (Rohde et al., 2017). Finally, activity and both male-male and female maternal aggression were not genetically correlated in mice (Gammie et al., 2003).

The second assay examining behavioral correlates of sociability involved scoring key behaviors from videos taken during the settlement of flies in the sociability arenas. As expected, our alternate measure of activity, the frequency of inter-quadrant crossing was not correlated with sociability. We found, however, no correlation between sociability and either aggressive or non-aggressive interactions. Superficially, one might expect a negative correlation between sociability and aggression. Mechanistically, what we found was next to no aggression in 6 of the 8 DGRP hybrids examined, suggesting that overt aggression was not the driving force behind the genetic variation in sociability. Ultimately, one might expect a complex interaction between sociability and aggression. The simple reason for this is that the payoff from aggression may be higher in social groups than among solitary individuals. For example, being the dominant member of a social group can provide one with preferential access to resources such as food, shelter and mates. Indeed a phylogenetic analysis of mammals indicated much higher levels of lethal aggression in social than in

solitary species (Fig. 2 in Gómez et al., 2016). Research on humans also indicated no correlation between aggression and sociability (Buss and Perry, 1992). While there is no theoretical foundation for predicting an association between sociability and non-aggressive interactions, our previous work indicated a positive correlation between non-aggressive interactions and inter-individual distance. That is, the lines where individuals were physically closest together had the fewest interactions (Anderson et al., 2017). We intend to quantify the association between our current sociability index, nearest neighbor index and fly interactions in future work.

Finally, in the experiment on social plasticity, we quantified genetic variation in males' sociability in response to two relevant factors, social isolation and exposure to females. Overall, we found significant social plasticity, with males housed in groups being more sociable than males held alone prior to the test (Fig. 3.3a). Being housed with or without females, however, did not significantly affect male sociability (Fig. 3.3a). Our former results are consistent with Simon et al. (2012), who found that social isolation subsequently led to greater inter-fly distance. Studies on fruit fly aggression are also consistent with the conclusion that flies held in isolation are subsequently less sociable than flies held in group as indicated in higher levels of aggression (Hoffmann, 1990; Ueda and Kidokoro, 2002; Wang et al., 2008). Similar results of isolation increasing subsequent aggression are known in many other species (Allee, 1942; Valzelli, 1973).

While the effects of social isolation on sociability are somewhat established, the effects of prior experience with females are not as clear. Unlike us, Simon et al. (2012) reported shorter inter-fly distance in males previously housed with females than in males kept only with males. Simon et al. (2012) measured inter-fly distance in a large arena with 40 flies and no food, so our protocols are rather distinct. The effects of prior experience with females on aggression are similarly conflicting. Yuan et al. (2014) found that males previously housed with females were less aggressive than virgin males. In two experiments using distinct protocols, however, we found no difference in aggression based on prior sexual experience (Baxter and Dukas, 2017). A possible explanation for the different results is genetic variation in social plasticity among the lines used in the different studies.

We found genetic variation in social plasticity, which was marginally significant when we placed males either alone or with 3 other males (Fig. 3.3b), and significant when we housed males with or without females prior to the test (Fig. 3.3c). Most notably, about half the DGRP hybrids had higher sociability scores after being held with than without females, while the other half showed the opposite pattern. We will require further experiments to elucidate the social dynamics during the experience phase that generate the distinct patterns of social plasticity. We will also need additional work to find out the mechanisms underlying social plasticity. The most relevant study on genetic variation in social plasticity compared aggression in males kept in mixed sex groups and in isolated males of 87 inbred fruit fly lines. That study documented significant genotype by social environment interaction (Rohde et al., 2017). Unexpectedly though, many of the lines showed greater aggression after housing in groups than alone (Fig. 2 in Rohde et al., 2017), which is inconsistent with the well replicated, robust effects of social isolation on aggression discussed above (Hoffmann, 1990; Ueda and Kidokoro, 2002; Wang et al., 2008). In humans, natural variation in the gene encoding the neurotransmitter-metabolizing enzyme, monoamine oxidase A (MAOA), has been linked to plasticity in aggression, with only carriers of the low activity allele responding to maltreatment with heightened aggression (Caspi et al., 2002; Gallardo-Pujol et al., 2013). There are few other estimates of the genetic variation in social plasticity in particular or behavioral plasticity in general because estimating variation in the slopes of behavioral reaction norms can be challenging (Araya-Ajoy and Dingemanse, 2017). In the three-spined stickleback (*Gasterosteus aculeatus*), there was limited evidence for population-specific genetic variation in plasticity of a few animal personality traits including sociability in the context of predation risk (Dingemanse et al., 2009) and significant genetic variation in plasticity of exploration behaviour in novel environments (Dingemanse et al., 2012). In a recent study, the heritability of the plasticity of aggression in wild great tits (*Parus major*) was estimated to be 0.266, but this estimate was highly uncertain (Araya-Ajoy and Dingemanse, 2017). Also in great tits, the heritability of the plasticity of egg-laying date was estimated as 0.3 (Nussey

et al., 2005). Our estimate of the heritability of social plasticity in fruit flies (0.21 – 0.24) was similar to these estimates.

In sum, we documented large genetic variation in sociability and some genetic variation in social plasticity in fruit flies. These finding open up exciting opportunities for future work on the mechanisms that underlie that variation as well as the ecological and evolutionary forces that maintain it.

3.6 Acknowledgements

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CHAPTER 4 – EVOLUTION OF SOCIABILITY BY ARTIFICIAL SELECTION

Scott, A. M., Dworkin, I., Dukas, R. (submitted). Evolution of sociability by artificial selection.

4.1 Abstract

There has been extensive research on the ecology and evolution of social life in animals that live in groups. Less attention, however, has been devoted to apparently solitary species even though recent research indicates that they also possess complex social behaviors. To address this knowledge gap, we artificially selected on sociability, defined as the tendency to engage in non-aggressive activities with others, in fruit flies. Our goal was to quantify the factors that determine the level of sociability and the traits correlated with this feature. After 25 generations of selection, the high sociability lineages showed sociability scores about 50% higher than did the low sociability lineages. Experiments using the evolved lineages indicated that there were no differences in mating success between flies from the low and high lineages. Both males and females from the low lineages, however, were more aggressive than males and females from the high lineages. Finally, the evolved lineages maintained their sociability scores after ten generations of relaxed selection, suggesting no costs to maintaining low and high sociability, at least under our settings. Sociability is a complex trait, and we will keep assessing its ecology and evolutionary biology through ongoing genomic work on the evolved lineages.

4.2 Introduction

Social behavior, broadly defined as interactions among conspecifics, has attracted substantial research effort for a long time (Allee 1938; Tinbergen 1953; Wilson 1975; Clutton-Brock 2016; Ward and Webster 2016). Some minimal social activity occurs in most animals as it is typically essential for acquiring mates. In the relatively small

proportion of animals that engage in parental care, individuals may also participate in parent-offspring and sibling interactions. Relatively few animals, however, live in groups, and that fraction of species has been the focus of most studies on social behavior. Notable long term studies on such highly social species include work on the social behavior of ants, wasps and bees (Wilson 1971; Michener 1974; Seeley 2010; Kapheim et al. 2015), social mammals including naked mole rats (*Heterocephalus glaber*) (Jarvis 1981; Sherman et al. 1991; Barker et al. 2021), elephants (*Loxodonta africana*) (Moss et al. 2011) and primates (Goodall 1986; Cheney and Seyfarth 2008; Clutton-Brock 2016), and cooperatively breeding birds (Brown 1987; Koenig and Dickinson 2004).

While the research on animal societies has been illuminating, there has been increased recognition that apparently solitary species engage in persistent social interactions outside the obvious realms of brief encounters in the context of courtship and mating (Caro 1994; Costa 2006). For example, recent work on a classical solitary, territorial mammal, the puma (*Puma concolor*), has indicated that every individual participated in a dense social network, with animals routinely sharing their kills with other individuals (Elbroch and Quigley 2017; Elbroch et al. 2017). The evidence for complex social behaviors in apparently solitary species suggests that we can gain insights about the evolutionary biology of social behavior by focussing on animals traditionally classified as non-social.

A key evolutionary model species, the fruit fly (*Drosophila melanogaster*), had been historically classified as non-social. While hints of fruit flies' social behavior existed for a long time, much of the research on that topic is recent. The discovery that cis vaccenyl acetate (cVA) serves as an aggregation pheromone of fruit flies (Bartelt et al. 1985) implied social attraction, which led to research on its adaptive significance (Wertheim et al. 2002). Further research has documented social synchronization of the circadian clock (Levine et al. 2002), social learning (Sarin and Dukas 2009; Battesti et al. 2012), the formation of social groups (Saltz 2011; Schneider et al. 2012; Simon et al. 2012; Anderson et al. 2016; Scott et al. 2018; Bentzur et al. 2021) and collective response to danger (Ramdya et al. 2015; Ferreira and Moita 2020).

While social behavior includes many features, we focus here on a key trait, sociability, defined as animals' tendency to engage in non-aggressive activities with conspecifics. Such activities may include feeding together, traveling in a group, and communal resting or sleeping. Sociability means that individuals either seek each other, tolerate other members of a group, or often both. Field and laboratory studies indicate that both larval and adult fruit flies show significant sociability, as they prefer to group together at food patches (Durisko et al. 2014; Anderson et al. 2016; Scott et al. 2018; Dukas 2020). In the adults, the broad sense heritability of sociability is about 0.22 (Scott et al. 2018). The heritable variation in sociability opens up exciting opportunities for assessing the evolutionary biology of this trait in a prominent model animal. Specifically, we were interested in quantifying costs and benefits of sociability as well as its genetic correlation with other fitness-relevant traits. To this end, we artificially selected on low and high sociability for 25 generations.

Given the heritable variation in sociability, we predicted that we would succeed in generating diverged low and high sociability lineages. We then focused on four key predictions tested on the evolved lineages. First, we predicted that flies from the low and high lineages would vary in their mating success. We expected lower mating success of males from the high than low lineages as we expected them to be more docile in their interactions with females. For the females, however, we had no *a priori* rationale for a directional prediction. Second, we predicted that flies from the low lineages would be more aggressive than flies from the high lineages. Intuitively, it is sensible to assume that the tendency to share a small food patch with others would be negatively associated with aggression. Nevertheless, the genetic correlation between sociability and aggression may be complex given that aggression is often necessary for establishing dominance in social groups.

Our third prediction implicated unknown likely costs of possessing sociability scores below and above those expressed by the baseline wild population. We thus predicted that ten generations of relaxed selection would lead to convergence in the sociability scores of the low and high lineages. Finally, as noted above, social behavior comprises many

features. While we focused on individuals' tendencies to seek and tolerate others at a small food patch, one can measure other potentially relevant traits. One such trait is the nearest neighbor distance (NND), which indicates how tolerable individuals are to other proximate individuals (Conder 1949; Marler 1956). Given the likely positive association between sociability and NND, we predicted a larger NND in the low than high lineages.

4.3 Methods

4.3.1 Overview of the artificial selection experiment

Establishing starting population and selection and control lineages

We derived all artificial selection lineages from a population of ~600 wild *Drosophila melanogaster* females caught in various locations in and around Hamilton, Ontario in late spring and early summer 2018. We transferred each female into a standard food vial (1L standard food = 90 g sucrose, 75 g cornmeal, 10 g agar, 32 g yeast, and 2 g methyl paraben dissolved in 20 mL ethanol), and we verified the species based on male morphology in F1 progeny. We chose to use a freshly wild caught population over a lab adapted population to maximize ecologically relevant genetic variation available for selection. A caveat with this approach, however, is that lab adaptation occurs simultaneously with artificial selection, potentially reducing the effectiveness of our selection regime.

We mixed 3 F1 males and 3 F1 females from each of these isofemale lines together in 3 large populations. We then amplified these populations over 1-2 generations, generating a large total population size of ~6000 flies, mixed among the 3 populations, and then randomly assigned flies to 12 separate lineages: 4 lineages to be selected for low sociability, 4 lineages to be selected for high sociability, and 4 control lab adaptation and domestication lineages. The control lineages were not involved in the present experiments, and are used as controls in ongoing genomic and gene expression analyses. We housed each lineage in a population cage (20x20x30 cm^3) with standard food bottles for one generation (~150 males and 150 females per cage), with their offspring being the first

generation subjected to artificial selection. Once selection began, we maintained the lineages in vials, as described in the detailed selection methods section below.

Original sociability selection arena

We developed a novel arena capable of both quantifying the sociability of groups of flies and allowing for the selection of flies based on their sociability (Fig. 4.1a). We used polystyrene Petri dishes (90 mm wide x 20 mm high) as the base of each arena, with 1.5 mm thick opaque white polystyrene dividers permanently fused to the inside of the dish. The dividers separated the interior of the arena into 8 equally sized radial compartments that converged on a ~16 mm wide central area in the middle of the dish. Openings ~5 mm wide allowed access to each compartment from the central area.

We built the lids out of square pieces of 3 mm thick acrylic, as the stock Petri dish lids are not sufficiently flat to prevent fly movement over the dividers. We drilled two 16 mm wide holes in each lid (Fig. 4.1b), one located centrally to allow aspirating flies into the central area of the arena, and one off-centre directly above a compartment to allow aspirating out selected flies. We also added small strips of acrylic to the underside of the lid to act as guides which allowed the lid to remain in position while we rotate the lid so that the off-centre hole could be above any compartment. We bolted a small piece of rectangular 3mm thick acrylic to the surface of the lid above the off-centre hole, which acted as a swinging door (Fig. 4.1b). We used 25.4 mm thick foam cylinders as plugs for the central hole in the lid, with 16 mm wide plastic circles hot-glued to the bottom of the foam and coated with a slippery substance (Surfasil, ThermoFisher, Ottawa, ON, Canada) to deter flies from standing on the foam, which was evident during preliminary testing.

Before adding flies to an arena for testing, we added small discs of standard food (7 mm wide x ~2 mm thick) coated with a layer of grapefruit-yeast solution (3 g yeast dissolved in 100 mL grapefruit juice) to the centre of each of the 8 compartments (Fig. 4.1a). We then fit the foam plug so that just the central hole in the lid was plugged, but the entrances to the compartments were fully open and thus allowing free fly movement.

Revised sociability selection arena

Our observations during the first 10 generations of selection suggested that the arenas were too large, allowing individuals to be effectively socially isolated from each other within a single compartment. Hence, starting at generation 11, we switched to smaller arenas while maintaining an identical design (Fig. 4.1). We made each small arena from cut sections of PVC tubing 47.5 mm in diameter and 7 mm high glued to an acrylic base. We used 0.75 mm thick polystyrene dividers, making the gaps between entrances to each chamber ~4 mm and the central area ~12 mm in diameter. The dimensions of the food disc remained the same. We implemented the new arena starting in generation 11 for males and generation 12 for females.

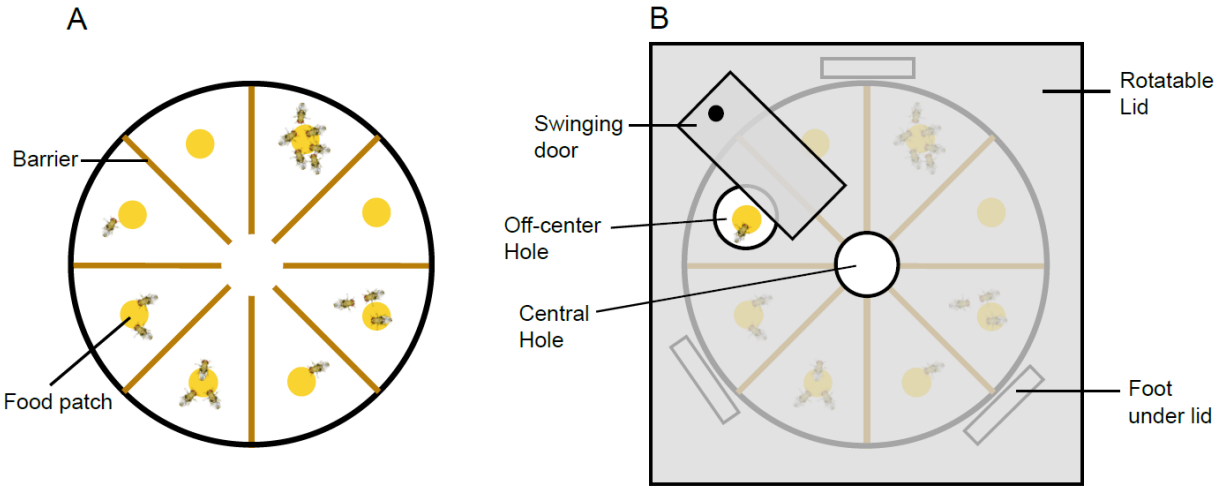

Figure 4.1

The arena used for quantification of and artificial selection on sociability. A) illustrates the arena without the lid, showing the 8 compartments and an example arrangement of 16 flies. B) shows the arena with the lid (note that the lid and swinging door were fully transparent, and opacity in the diagram is only for clarity). A foam plug at the central hole (not shown in the figure) allowed fly movement among the 8 compartments when at the top position, and locked flies within their compartment when in the bottom position.

Overview of artificial selection methods

Overall, each generation, we tested 12 groups of 16 males and 12 groups of 16 females from each of the 8 selection lineages (4 low sociability, 4 high sociability). We selected 4 flies from each group of 16 flies to produce the next generation. In tests involving the low sociability lineages, we chose the least sociable flies. In tests involving the high sociability lineages, we chose the most sociable flies (see detailed methods below). We ran 2 experimental sessions per day over 2 days, with each session including 3 male groups and 3 female groups from each of the 8 lineages.

4.3.2 Detailed artificial selection methods

We housed selected flies for egg-laying in groups of 4 males and 4 females in standard food vials with a sprinkle of live yeast for a total of 3 days, moving flies to a fresh set of vials with yeast after 2 days. We had 12 vials per lineage except for the parents of the first generation under selection, which we housed in population cages with food bottles. After egg-laying, we transferred all the parental flies of a lineage (48 males and 48 females) to a single food bottle with live yeast for egg-laying to generate a backup population for each lineage, kept at 18°C. We stored all egg-laying and housing vials and bottles in an environmental chamber at 25°C and 50% RH, and with a 12:12 light:dark cycle with lights on at 9 AM.

Eleven days after egg laying, we collected newly eclosed virgin flies to be selected for the next generation. Within 8 hours of eclosion, we sexed 192 males and 192 females per lineage with light CO_2 anesthesia. We housed 16 same-sex flies in standard food vials for 96 hours and checked on the test day that the females were virgin.

We performed the sociability selection assay in a room kept at 25°C and 50% RH. We ran 4 sessions of sociability testing and selection over 2 days in order to select 48 males and 48 females per selection lineage to produce the next generation (i.e., 25% truncation from 192 males and 192 females). At 9:30 AM on the first day of testing, we added 8 food discs to each of 48 arenas. At 10:30 AM, we added flies to the arenas using gentle

aspiration. We aspirated groups of 16 same-sex flies from the same holding vial at once into the central area of the arena by squeezing the aspirator between the foam and the plastic edge of the hole. From 11:00 AM – 12:30 PM, we allowed the flies to acclimatize. At 12:30 PM, we blocked the central area of each arena by pushing down the foam plug, sealing the flies into the compartment that they had settled in. At this point we recorded the number of flies in each compartment of each arena. We then selected flies to produce the next generation for each lineage based on the number of flies in each compartment. We removed flies by rotating the lid so that the off-centre hole was above a particular compartment, then rotating the plastic door so that the hole was uncovered, and aspirating the flies out. For the low sociability lineages, we selected 4 flies per arena from compartments with the lowest numbers of flies, unless those numbers were 3 or more, in which case we took flies from other replicate arenas of that session with smaller groups. Similarly, for the high sociability lineages, we selected 4 flies per arena from the compartment(s) with the highest number of flies, unless that number was 3 or less, in which case we took flies from larger groups in replicate arenas. The unselected flies from each arena were discarded. After each of the 4 selection sessions, we ended with 12 males and 12 females selected per lineage. We then placed the selected flies in sex-specific holding vials.

At 2:00 PM, we added flies for the second session to the same 48 dishes, recorded, collected selected flies at 4:00 PM, and placed flies in single sex vials. After the second session, we discarded the food discs and washed the arenas with 10% ethanol, allowing them to dry overnight. The following day, we ran sessions 3 and 4 in the same way. After all 4 sessions were completed at the end of test day 2, we mixed all selected flies within each lineage in a population cage to ensure among-vial gene flow, and then redistributed 4 males and 4 females into fresh food vials with a sprinkle of live yeast for egg laying.

To reduce the effects of genetic drift, we allowed for some flies to ‘migrate’ between corresponding low and high lineages, similar to the strategy used by Turner and Miller (2012). Every other generation between generations 2 and 10, we selected 2 males and 2 females from each lineage to be transferred to a lineage selected in the opposite direction (i.e., on generation 2, Low sociability 1 to High sociability 1 and vice versa for

each set of lineages. The paired lineages rotated on subsequent migration generations). We selected these flies based on the criteria for the lineage that they were 'migrating' to. For example, for flies migrating from a high sociability lineage to a low sociability lineage, we selected flies that were alone in a compartment, or with the fewest number of other flies. We selected on sociability for 25 generations. Subsequently, we quantified the effect of 10 generations of relaxed selection. This period coincided with laboratory restrictions owing to the COVID-19 pandemic.

Quantifying sociability

Every generation, observers blind to selection treatment identity quantified a sociability score for each arena just after we lowered the foam plug using the formula: variance ÷ mean number of flies in each compartment (Scott et al. 2018). A sociability score of 0 indicated uniform fly distribution (2 flies per compartment), a score of 1 implied random distribution, and any score significantly above 1 indicated significant sociability. A theoretical maximum sociability score of 16 could be achieved if all flies formed a single group within one compartment.

We also performed behavioral observations on a subset of arenas immediately after adding flies in generations 9 and 12. We intended to use these observations to gain insight into the interactions among flies at the beginning of the acclimatization period, as sociability scoring took place once these interactions had presumably occurred, and flies had settled in their preferred social arrangement among the compartments. In generation 9, we scanned 16 arenas in each of the 12:30 PM and 4:00 PM sessions for 1 minute across 3 consecutive observation rounds, and in generation 12, we scanned 16 arenas in the morning session in the same way. The observer was blind to selection treatment identity, and the subset of arenas chosen included an equal number of arenas from each sex, treatment, and lineage. The only interactions we observed included low-level aggression (lunging in males, headbutting in females, and fencing in both sexes; Chen et al. 2002; Nilsen et al. 2004) and wing waving, which males use to signal to other males to back off (Paillette et al. 1991). These observations indicated that flies were mostly settled and showed very little

movement within and between compartments after about 30 minutes into the acclimatization period.

Artificial selection data analysis

We analyzed generations 1 to 25 of the artificial selection experiment in a single mixed effects general linear model, fitted using the lmer function from the R (ver. 4.0.4; R Core Team 2021) package lme4 (ver. 1.1-26; Bates et al. 2015). We took the $\log_{10}$ of the sociability scores as the response variable. This transformation allowed us to use a general linear model without violating the assumption of normality of the residuals. Sex, generation, treatment, all their 2- and 3-way interactions, and test session (i.e., 12:30 PM or 4:00 PM observations) were fitted as fixed effects. Both the random intercept of test arena (which corresponds to the location the arena was placed in the test room), and random effects for the intercept and generation varying by lineage nested within treatment, however the random slope included in the latter term was removed to reduce complexity in the random effects in order to fix a singular fit. Model assumptions of normality and homoscedasticity of the residuals were verified by inspecting plots of the results of the simulateResiduals function in the DHARMa package (ver. 0.3.3.0; Hartig 2020). Significance of the fixed effects was assessed using the Anova function from the car package (ver. 3.0-10; Fox and Weisberg 2019), and results of these tests are reported as Wald χ^2 test statistics and associated p values.

We analyzed the effect of relaxed selection by fitting a model of sociability scores from generation 25, which was the last generation with artificial selection, and generation 35. The model was fitted and fixed effects tested in the same form as described above for the Generation 1-25 model, except with no 3-way interaction in the fixed effects, and arena was fitted as a fixed effect instead of a random effect due to model convergence issues.

We analyzed the direct observations of aggressive and social interactions conducted in generations 9 and 12 as the presence or absence of behavior during the 1 minute observation period, using generalized linear mixed effects models with a binomial distribution, fitted using the glmmTMB function in the glmmTMB package (ver. 1.0.2.1;

Brooks et al. 2017). We modelled male and female low-level aggression separately as observations where aggression was present or absent as a function of treatment, generation, observation round, test session (12:30 PM or 4:00 PM), and test arena as fixed effects. We included the random effect of lineage nested within selection treatment. We modelled male social interactions (wing waving) using a separate model specified as above. Significance of the fixed affects was assessed as above.

4.3.3 Experiments on the evolved lineages

Mating success and choice of males and females

In generation 28, we performed 3 experiments to assess mating success of flies from the low and high sociability selection lineages: male mating success with wild females, wild female mate choice between low and high sociability males, and wild male mate choice between low and high sociability females.

Male mating success (forced choice)

We measured the frequency of successful matings of individual males from the low and high sociability selection lineages paired with single females from a control population. Four days before testing, we sexed virgin males from the 8 low and high selection lineages within 8 hours of eclosion and housed them as in the regular selection procedure: 16 individuals per standard food vial. Two days before testing, we sexed virgin females from our standard wild population within 8 hours of eclosion and housed them in vials of ~10 individuals. We used 2-day old females because our previous unpublished data indicated that such young females are reluctant to mate, with only 64% mating within 1 h. Starting at 8:30 AM on test day, we added 1 male and 1 female to each empty test vial, and recorded whether a mating occurred within 1 hour. We tested 40 males per lineage for a total of 320 males.

We analyzed the data with a generalized linear mixed effects model with a binomial distribution using glmer from the lme4 package, and verified that the model assumptions

were not violated with the DHARMa package. We modelled whether the male mated or not as a function of treatment, session, and the random effect of lineage nested within treatment, and tested the fixed effects with the Anova function.

Mate choice under competitive conditions in females and males

In the female mate choice experiment, we measured the mating frequency of males from the low and high sociability selection lineages with single control females when these females were given the choice between 1 low and 1 high sociable male. Such apparent mate choice, however, may be determined by male-male interactions including courtship interference (Baxter et al. 2018). Test males and females were reared and housed as with the male mating success protocol described above. One day before testing, half of the males were dusted with a pink, fluorescent powder to allow for identification during the test. Colouring was counterbalanced among selection treatments and lineages. Starting at 8:30 AM on test day, using new empty vials, we added 1 uncoloured male, then 1 pink male, then the female. Observers blind to fly treatment recorded matings that occurred, and with which male, within 1 hour of the trial start. We performed 70 trials for each of 4 Low vs High sociability competitions (i.e., males from each lineage competed against males from one other lineage of the opposite treatment: Low1 vs High1, Low2 vs High2, Low3 vs High3, Low4 vs High4), for a total of 280 trials.

In the male mate choice experiment, males from the control population were given the choice between 1 female from a low sociability lineage and 1 female from a high sociability lineage. The protocol for this experiment was similar to the female choice version, with the sexes reversed. We performed 70 trials for each of the same 4 High vs Low sociability competitions for a total of 280 trials.

We analyzed the data from each of the two experiments with a generalized linear mixed model with binomial distribution using glmer. We set up the model and tested the fixed effects as with the mating success experiment, but also included fly colour (pink or uncoloured) as a fixed effect, and trial as a random effect to account for the non-independence of outcomes within a trial.

Male-male aggression

We tested male-male aggression in flies from the low and high sociability selection treatments in generation 28 using our established protocol (Baxter and Dukas 2017). We sexed virgin males from the selection lineages within 8 hours of eclosion, and housed them in standard food vials in groups of 16 for 96 hours, as in the artificial selection protocol. Aggression arenas were 35 mm wide by 8 mm tall Petri dishes coated with Surfasil on the walls and underside of the lid to keep flies from walking on these areas. We covered the floor of each dish with a piece of circular filter paper, and placed a food patch (8 mm wide by 1.5 mm thick) topped with a 3 mm ball of thick yeast paste (5g live yeast in 10 mL grapefruit juice) in the center.

At 8:30 AM on the test day, we aspirated 2 males from the same lineage into each arena, and placed 2 arenas under each of 6 tripod-mounted Logitech c920 webcams, and recorded for 15 minutes. We repeated this for 4 consecutive recording sessions per day over 2 days, for a total of 96 trials (12 per lineage, 48 trials each per high and low selection treatments). We had 1 arena with high sociability males and 1 with low sociability males under each camera, and counterbalanced locations across sessions.

Observers blind to fly selection treatment recorded aggression behaviors via BORIS behavior observation software (ver. 7.9.8; Friard and Gamba 2016). We recorded the durations of the following aggressive behaviors to obtain a total duration of aggression for each trial: holding, lunging, boxing, and tussling (Chen et al. 2002; Baxter and Dukas 2017). We also recorded non-physical aggressive displays in the form of wing threat.

We analyzed the data using a generalized linear mixed effects model with a tweedie distribution and log link function, using glmmTMB. The tweedie distribution is ideal for aggression data, which usually have a substantial mass at zero and positive skew (Dunn and Smyth 2005). We modeled the total duration of aggression in each trial as the response variable, selection treatment and test day as fixed effects, and observer, test session, arena, and lineage nested within treatment as random effects. We fit a separate model the same way to look at non-physical wing threat. We verified that the assumptions of the models

were not violated as before with the DHARMa package, and tested the fixed effects with Anova from the car package.

Female-female aggression

We also tested female-female aggression in two lineages from each of the low and high sociability selection treatments in generation 33. We sexed virgin females within 8 hours of eclosion, and housed them in individual food vials for 96 hours. We housed the females in isolation since female-female aggression is relatively rare, and isolation is known to increase aggression in females (Ueda and Kidokoro 2002). One day before testing, we added a male from our standard lab wild population (which was also derived from the same wild caught population as the selection lineages, and maintained in population cages of a few hundred individuals) to each female vial and observed for mating, which is also known to increase female aggression (Bath et al. 2017). After mating, we discarded the males. We used the same aggression arenas and test protocol as described in the male-male aggression experiment, except that videos were recorded for 20 minutes.

We performed 96 trials over 2 days (24 per lineage, 48 per low and high sociability treatment). An observer blind to fly selection treatment recorded aggression behaviors via BORIS software, including head-butting, lunging, and pushing (i.e., 1 female pushing another off the food disc with her front legs) to obtain a total duration of aggression.

We analyzed the female-female aggression data as with the male-male data, except without an observer random effect term as there was only one observer.

Alternative sociability measure: Nearest-neighbor distance

We tested male and female flies from the selection lineages in generation 28 for their level of sociability as measured by the median nearest-neighbor distance of single-sex groups in a homogenous open arena (Anderson et al. 2016). We sexed flies within 8 hours of eclosion, and housed them in same-sex groups of 14 for 72 hours prior to testing. For test arenas, we used 25 mm Petri dishes with 8 mL of standard food (cornmeal omitted for video clarity with automated tracking) covering the bottom, effectively constraining the flies to 2

dimensions. At 9 AM on the test day, we briefly anesthetized the flies with CO_2 and transferred 12 from the same vial into each arena. We allowed the flies 5 hours to acclimatize. We then transferred the arenas in groups of 10 to each of 6 climate-controlled semi-transparent test boxes equipped with overhead webcams. We allowed the flies an additional 30 minutes to acclimatize to the test boxes, at which point they were mostly settled, and then recorded the arenas for 30 minutes.

We performed 2 consecutive recording sessions of 60 arenas per day (~3:00-3:30 PM and ~4:00-4:30 PM) over 2 days for a total of 120 arenas per sex (15 arenas per lineage, 60 per selection treatment). We used the same custom Python script to automate video analysis described in Anderson et al. (2016) which samples frames of video every 30 s and calculates the nearest-neighbor distance of each fly (i.e., for each fly, the distance between its centroid and the centroid of the closest fly), which was then used to calculate the median nearest-neighbor distance for each arena as a measure of sociability.

We analyzed the data with a general linear mixed effects model using the lmer function of the lme4 package, and verified model assumptions were not violated as before. We used the mean of the median nearest neighbor distance of each arena across the duration of the trial to obtain one value for each trial to model as the response variable. We modeled test day, session, treatment, sex, and treatment×sex as fixed effects, and test box, arena, and lineage nested within treatment as random effects. We tested the significance of the fixed effects using the Anova function from the car library.

4.4 Results

4.4.1 Sociability artificial selection

There was a significant effect of our artificial selection regime on the sociability of the low and high sociability treatments, with the lineages starting the experiment at the same sociability level and then diverging (Generation×Treatment interaction: $\chi^2_1 = 48.75$, $p < 0.001$; Fig. 4.2). Males were more sociable than females ($\chi^2_1 = 66.53$, $p < 0.001$; Fig. 4.2).

By the end of the experiment, female flies from the high sociability treatment had, on average, about a 40% higher sociability score compared to the low treatment, and males from the high treatment had about a 54% higher sociability score compared to the low treatment (Main effect of Treatment in Generation 25: $\chi^2_1 = 25.18$, $p < 0.001$; Fig. 4.2).

In our behavioral observations of a subset of arenas in generations 9 and 12, we only recorded a few cases of low-level aggression in a small proportion of the arenas, which occurred at similar frequencies in the low and high lineages (proportion of arenas with aggression, females: low sociability = 0.31, high sociability = 0.19; $\chi^2_1 = 1.78$, $p = 0.18$; males: low sociability = 0.11, high sociability = 0.17; $\chi^2_1 = 0.44$, $p = 0.51$). We also recorded a few cases of social interactions in the form of wing waving among males, which were also not significantly different among selection treatments (proportion of arenas with social interactions, low sociability = 0.25, high sociability = 0.36; $\chi^2_1 = 0.78$, $p = 0.38$).

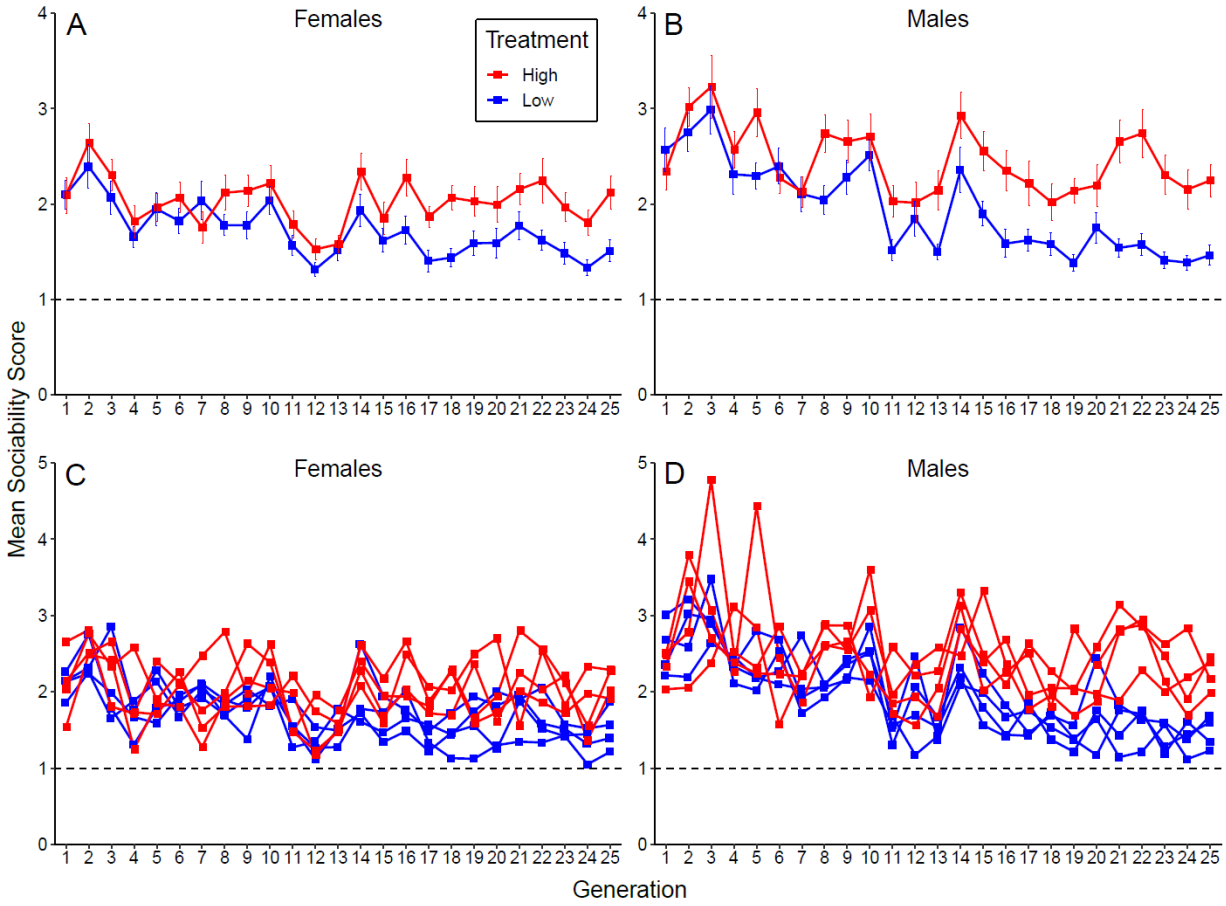

Figure 4.2

Divergence in selection treatments in sociability score over 25 generations. Mean ± S.E.M. sociability scores across all selection lineages for low and high sociability treatments in A) females and B) males. The same data are displayed by replicate lineages (error bars excluded for clarity) in C) females and D) males. Values significantly above 1 (dashed lines) indicate significant sociability.

4.4.2 Mating success

We did not detect a significant effect of selection treatment on individual male mating success with single control females (χ^2_1 = 0.020, p = 0.89; Fig. 4.3a), on male mating frequency with single control females given a choice between 1 low and 1 high sociability male (χ^2_1 = 0.003, p = 0.96; Fig. 4.3b), or on female mating frequency with single control males given the choice between 1 low and 1 high sociability female (χ^2_1 = 0.27, p = 0.60; Fig. 4.3c).

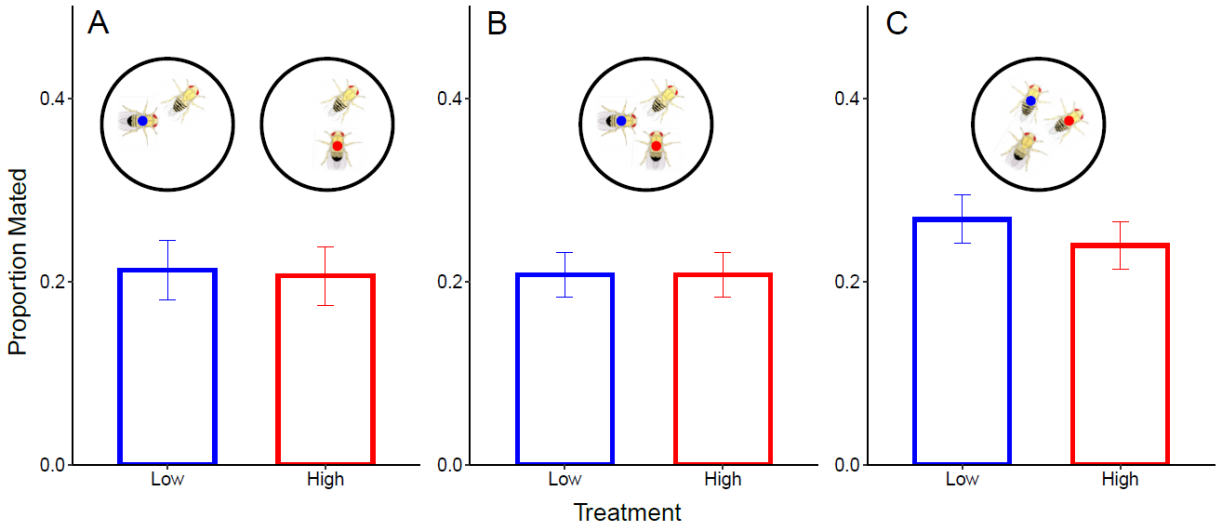

Figure 4.3

Mating success of selected males and females. Males can be identified by the black tip of their abdomen. Flies from the low sociability lineages are marked with blue dots, flies from the high sociability lineages are marked with red dots, and flies from the control population are unmarked. A) Mating success of single males from the selection treatments with single control females. The maximum possible value of each bar is 1. B) Competitive mating success of males from the selection treatments in vials each containing a single control female, 1 low and 1 high sociability male. Here the maximum possible value of both bars combined is 1. C) competitive mating success of females from the selection treatments in vials each containing a single control male, 1 low and 1 high sociability female. The maximum possible value of both bars together is 1. Colored dots on flies in the cartoons are only to distinguish treatments in this figure, and were not applied in the actual experiment. Error bars show ± the standard error of the proportion p, $\sqrt{p(1-p)/n}$. The 95% confidence intervals for the non-significant treatment effects are A) [-0.27, 0.31], B) [-0.34, 0.30], C) [-0.28, 0.46].

4.4.3 Female-female and male-male aggression

Low sociability females were significantly more aggressive than high sociability females (χ^2_1 = 12.20, p < 0.001; Fig. 4.4a). Similarly, low sociability males were significantly more aggressive than high sociability males (χ^2_1 = 4.05, p = 0.044; Fig. 4.4b). We did not, however, observe a significant difference in time spent performing wing threat between selection treatments (mean ± S.E.M, low sociability = 0.35 ± 0.18 s/min; high sociability = 0.39 ± 0.12 s/min; χ^2_1 = 0.005, p = 0.95).

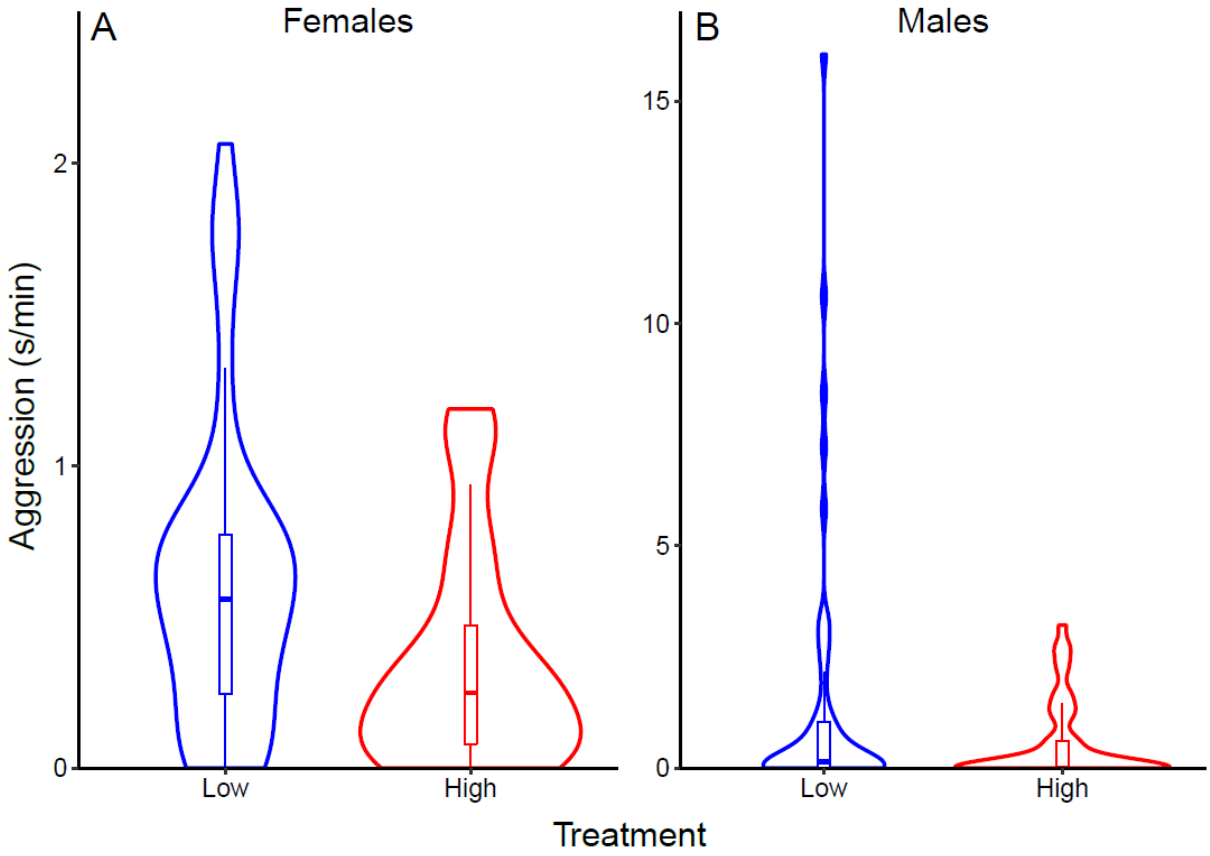

Figure 4.4

Aggression frequency in A) females and B) males from the selection treatments after 25 generations of selection. Inner box plots show median, inter-quartile range, and whiskers up to 1.5×I.Q.R. Outer violin plots show the shape of the distribution of the data.

4.4.4 Relaxed selection

We did not observe a significant effect of 10 generations of relaxed selection after stopping selection with generation 25 (Generation×Treatment interaction: $\chi^2_1 = 1.02$, $p = 0.31$; Fig. 4.5). In generation 35, the significant effect of selection treatment remained ($\chi^2_1 = 30.25$, $p < 0.001$; Fig. 4.5).

4.4.5 Alternative sociability measure: Nearest-neighbor distance

We did not detect a significant main effect of selection treatment on nearest-neighbor distance ($\chi^2_1 = 0.06$, $p = 0.81$; Fig. 4.6). Overall, males had smaller nearest-neighbor distances than females ($\chi^2_1 = 19.22$, $p < 0.001$; Fig. 4.6), and the treatment-by-sex interaction approached significance ($\chi^2_1 = 3.28$, $p = 0.070$; Fig. 4.6).

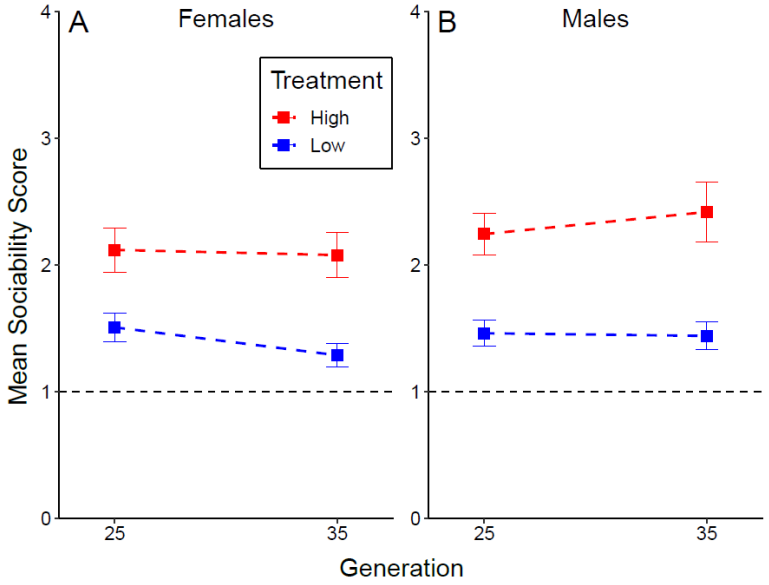

Figure 4.5

Mean ± S.E.M. sociability scores at the end of 25 generations of selection, and after 10 generations of relaxed selection in A) females and B) males. Values significantly above 1 (dashed lines) indicate significant sociability.

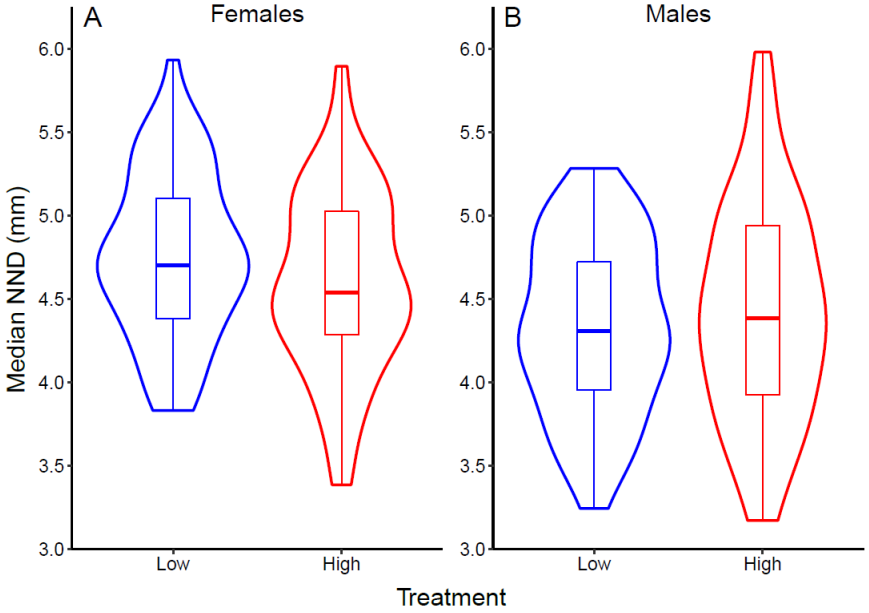

Figure 4.6

The median nearest-neighbor distances in A) females and B) males, after 25 generations of selection. Inner box plots show median, inter-quartile range, and whiskers down and up to 1.5×I.Q.R. Outer violin plots depict the data distribution. Confidence intervals for the non-significant treatment effect (95% C.I.: [-0.12, 0.15]).

4.5 Discussion

Our key findings were first, that we were able to generate significant divergence in sociability scores between the selection treatments over 25 generations of artificial selection in both females and males (Fig. 4.2). This resulted in relatively 40% higher sociability scores in high sociability females, and relatively 54% higher sociability scores in high sociability males. Second, flies from the low and high lineages had similar mating success (Fig. 4.3). Third, low sociability females and males had higher levels of intrasexual aggression compared to their high sociability counterparts (Fig. 4.4). Fourth, the low and high sociability lineages did not converge even after 10 generations of relaxed selection (Fig. 4.5). Finally, the low and high sociability lineages did not differ in their nearest-neighbor distance (Fig. 4.6). We will discuss each of these findings in turn.

By successfully evolving via artificial selection lineages of low and high sociability in a highly tractable model system, we pave the way for further investigations on the ecology and evolution of a central phenotypic trait that structures behavior and determines fitness in numerous species including humans. For example, long-term field observations on savanna and chacma baboons (*Papio cynocephalus* and *P. hamadryas ursinus*) indicated that females with stronger and more stable social bonds lived longer and had higher infant survival rates (Silk et al. 2003, 2010). In another well studied system, many species of fish move in tight groups typically referred to as schools. Field observations, which were followed up by controlled laboratory studies, indicated that Trinidad guppies (*Poecilia reticulat*) from distinct populations that vary in predation risk show heritable variation in school size, with guppies from high predation pools having larger and more cohesive groups as well as higher survival rates when exposed to predators (Seghers 1974; Magurran et al. 1992; O'Steen et al. 2002; Huizinga et al. 2009). Recently, Kotrschal et al. (2020) artificially selected for three generations on guppies' group polarization, which is the tendency of school members to align with each other's directional movement. This led to significant increases in polarization and cohesiveness in females. Finally, humans show heritable variation in sociability and there is a strong positive correlation between the

quality of social relationships and both health and life expectancy (House et al. 1988; Holt-Lunstad et al. 2010; Day et al. 2018; Abdellaoui et al. 2019).

While high levels of sociability positively affect fitness in some species, they could have negative effects in others. For this reason, we predicted that our evolved high sociability lineages would show some decrements in performance. Specifically, we expected sociable males to have lower mating success because we assumed that they might be less aggressive in pursuing reluctant females. However, we found no differences in mating success between males from the low and high lineages under both no choice and choice experiments (Fig. 4.3). Similarly, females from the low and high lineages had similar mating success (Fig. 4.3). Apparently, selection on sociability affects neither courtship behavior nor attractiveness to the other sex.

Unlike the sexual features, selection on sociability led to a correlated change in aggression (Fig. 4.4). One can then argue that, although we quantified sociability, we actually selected on aggression. We should note, however, that our direct observations on flies just after we set up the sociability arenas during the artificial selection stage indicated low frequencies of only low-level aggression. This was not surprising because we housed all flies in groups of 16 same-sex individuals from sexing through testing, and such group settings are associated with low levels of aggression (Wang et al. 2008). Furthermore, in an earlier work quantifying genetic variation in sociability, we found that genotypes that varied widely in sociability did not show significant variation in aggression (Scott et al. 2018). Nevertheless, our current results suggest a negative correlation between sociability and aggression, which we intend to explore further in our ongoing genomic work on the evolved sociability lineages.

One may argue that it is obvious that flies that prefer to be in groups would be less aggressive. Following this logic, we also expected that sociable flies would show shorter nearest neighbor distance when tested in small arenas designed to quantify this alternative measure of social behavior (Simon et al. 2012; Anderson et al. 2017). Surprisingly, however, our low and high sociability lineages did not differ in their nearest neighbor distances (Fig. 4.6). This result illustrates that social behavior is a complex trait and that

apparently related social features may have distinct genetic bases. Somehow the cues, signals and mechanisms that determine individuals' tendency to form groups differ from the ones that affect nearest neighbor distances. That is, regardless of individuals' tendencies to seek and tolerate others at the same food patch, they seem to have a similar preferred minimum inter-individual distance when compelled to share a single patch. Although it sounds counterintuitive, inter-individual distance has been well studied in a variety of social animals, in which individuals simultaneously balance their social attraction to as well as minimum distance from others (Hall 1966; Sorokowska et al. 2017). For example, in black headed gulls (*Larus ridibundus*), members of the flock maintain distance through a combination of avoidance and mild threat (Conder 1949). Our recent genetic work indeed indicates distinct genetic effects on nearest neighbor distance and sociability (Figs 2a,b vs. Figs 4a,b in Yost et al. 2020), and we intend to further characterize the sociability phenotype in our ongoing genomics work.

While we measured a few parameters in the evolved lineages, there may have been other correlated traits that have changed with sociability. Because we selected on sociability scores lower and higher than the likely optimal sociability levels in the baseline population, we expected some fitness costs associated either with sociability or other correlated traits that would lead to convergence of the low and high lineages towards the initial sociability scores. Such convergence under relaxed selection is rather common. For example, artificial selection on phototaxis in *D. pseudoobscura* led to rapid divergence of the negative and positive selection lineages followed by quick convergence under relaxed selection (Dobzhansky and Spassky 1969). In our case, however, we found no convergence under relaxed selection (Fig. 4.5). Apparently, there are no costs associated with possessing below and above the sociability scores of wild fruit flies under the specific parameters of our protocol. Nevertheless, such costs may exist in both natural settings and population cages in the laboratory. For example, costs of high sociability could include increased larval competition if females lay more eggs on a portion of the available food patches (Atkinson 1979; Grimaldi and Jaenike 1984; Durisko and Dukas 2013; Golden and Dukas 2014). Our

protocol, however, did not allow for this to happen because we collected eggs for the next generation only when flies were in low density vials with ample live yeast and media.

Our current and previous work, as well as research in other laboratories indicate that fruit flies have rich social life. Importantly, wild fruit flies spontaneously form social groups under controlled natural settings (Dukas 2020). They show heritable variation in sociability (Figs 2,5; Scott et al. 2018) as well as related social traits (Wice and Saltz 2021). Fruit flies, however, also engage in aggressive encounters within their naturally occurring social groups (Dukas 2020) and show heritable variation in such aggression (Hoffmann and Cacoyianni 1989; Dierick and Greenspan 2006; Edwards et al. 2006). Fruit flies are socially influenced by each other (Levine et al. 2002), socially learn relevant information about egg laying substrates (Sarin and Dukas 2009; Battesti et al. 2012), and their collective behavior enhances their responses to hazards (Ramdya et al. 2015; Ferreira and Moita 2020). We failed, however, to identify costs associated with the evolved sociability values, which were lower or higher than those in the initial wild population, and will keep pursuing this topic in future work.

Overall, we succeeded in generating via artificial selection fly lineages that show low and high sociability and to employ the evolved lineages for addressing relevant questions about the evolutionary biology of sociability. We found that variation in sociability is not associated with either attractiveness or competitive ability in a mating context, that sociability is genetically negatively correlated with intrasexual aggression, but that it is not positively correlated with flies' preferences for inter-individual distance. Finally, there were no other costs to the evolved lower and higher levels of sociability as ten generations of relaxed selection did not lead to convergence of the selected low and high sociability lineages. As expected, sociability is a complex trait, which we will keep studying through our ongoing genomics and gene expression work on the evolved sociability lineages.

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CHAPTER 5 – THE GENETIC BASIS OF VARIATION IN SEXUAL AGGRESSION: EVOLUTION VERSUS PLASTICITY

Scott, A. M., Baxter, C. M., Yan, J. L., Dworkin, I., Dukas, R. (in prep) The genetic basis of variation in sexual aggression: evolution versus plasticity.

5.1 Abstract

Male sexual aggression towards females is a form of sexual conflict that can result in increased fitness for males through forced copulations or coercive matings at the cost of female lifetime fitness. Little is known about the genetic factors that influence an male's success in engaging in sexual aggression. We used fruit flies (*Drosophila melanogaster*) as a model system to uncover the genetic underpinnings of variation in forced copulation, both due to standing variation in a wild population, and due to plastic changes associated with variation in social experience. We used RNAseq methods to analyze head tissue whole-transcriptome differential expression associated with evolved changes in forced copulation from lineages previously selected for high and low forced copulation rate (Dukas et al. 2020), and in flies with varying forced copulation rates due to social experience. We identified hundreds of genes associated with evolved and plastic variation in forced copulation, however only a small proportion (27 genes) showed significant differential expression due to both modes of variation. We confirmed this trend of low concordance in gene expression effects across broader sets of genes significant in either the evolved or plastic analyses. We identified enriched gene ontology terms associated with the significant genes, including neuropeptide hormone activity and serotonin receptor activity. Of 7 genes chosen for RNAi knockdown validation tests, knockdowns of 4 genes showed the expected effect on forced copulation behaviours. Taken together, our results provide important information about the apparently independent genetic architectures that underlies natural variation in sexual aggression due to evolution and plasticity.

5.2 Introduction

There are many diverse strategies that males and females use to increase their fitness, some of which may not align with the ideal fitness outcomes for their sexual partners. This fitness misalignment generates sexual conflict, which has been a subject of thorough research by evolutionary biologists (Arnqvist and Rowe 2005; Chapman 2006; Fricke et al. 2010). Such sexual conflict can be relatively inconspicuous, for example on a molecular scale after copulation has occurred, where male seminal proteins can have a marked influence on female behaviour in favour of the male's fitness and at a cost to the female's (Chapman et al. 1995; Wigby and Chapman 2005). On the other hand, sexual conflict can be quite obvious, as in the case of male sexual strategies that involve sexual aggression, such as forced copulation with females, which result in not only a potentially sub-optimal mate that the female is unable to reject, but also physical harm that may reduce the female's lifetime fitness. Examples of such male forced copulation of females have been observed in waterfowl (McKinney et al. 1983; McKinney and Evarts 1998), wolf spiders (*Schizocosa ocreata*) (Johns et al. 2009), and Lake Eyre dragons (*Ctenophorus maculosus*) (McLean et al. 2016; Olsson 2017). Sexually aggressive behaviours may represent an alternative mating strategy employed by males that would otherwise be outmatched by other males vying for females, or rejected by females themselves. For example, male sailfin mollies (*Poecilia latipinna*) use either a courtship or sneaker strategy depending on their genotype or social environment, with the sneaker strategy employing forced insemination without female cooperation (Farr et al. 1986; Fraser et al. 2014).

Sexual aggression may act as an important target of sexual selection, and understanding the genetic underpinnings that contribute to its variation in populations can give us a better picture of how these behaviours evolve, how variation in such behaviours can persist, and how this variation may be associated with environmental variation. Recently, fruit flies (*Drosophila melanogaster*) have been used as a model for understanding variation in sexual aggression. Fruit fly sexual aggression, in the form of male forced copulation of recently eclosed teneral females, was first observed in wild

populations in the field (Markow 2000). Teneral females have a soft cuticle and folded wings, and are unable to prevent forced intromission or escape from persistent males. Forced copulation of teneral females is beneficial for males since they are able to sire offspring, but is detrimental to females due to negative effects on survival and reproduction (Seeley and Dukas 2011; Dukas and Jongsma 2012). There is clear variation in male tendency to force copulate that can be attributed to both genetic and environmental variation. Assays of forced copulation rate in isogenic lines of fruit flies have shown that its broad-sense heritability is about 0.16 (Baxter et al. 2019), and variation present in wild populations is sufficient for rapid divergence in forced copulation rate via artificial selection (Dukas et al. 2020). A similar difference in forced copulation rates has been observed in flies from the same genetic background that have been exposed to different social environments prior to exposure to teneral females. Males housed with no females forcibly mate at a higher rate than males who have been housed with virgin females prior to testing (Baxter and Dukas 2017).

Having access to a genetically tractable model system that shows both genotypic variation and variation due to social plasticity in sexual aggression gives us an excellent opportunity to investigate the similarity in the genetic mechanisms underlying these two modes of variation. The similarity of the changes in gene expression due to genotypic variation and plasticity may have important ramifications for trait evolution. For example, shared mechanisms of plastic and genotypic effects on a trait may indicate the facilitation of adaptive evolution through genetic assimilation (Waddington 1942; Scheiner and Levis 2021). Evidence for the co-option of genetic mechanisms that underlie plasticity facilitating adaptive evolution has been observed in zooplankton (*Daphnia melanica*) adaptation to introduced predators (Scoville and Pfrender 2010), in aggression in honey bees (*Apis mellifera*) (Alaux et al. 2009), and in sailfin molly male reproductive tactics (Fraser et al. 2014). It is also possible that plasticity may hinder adaptive evolution; for example, in guppies (*Poecilia reticulata*), the direction of plasticity in gene expression for anti-predatory genes is opposite to evolutionary gene expression effects in a transplanted populations (Ghalambor et al. 2015). Examining whether effects on sexual aggression gene

expression due to plasticity and evolution are concordant or discordant could open the door to examining whether these effects may indicate the facilitation or hindrance of adaptive evolution of sexual aggression. Looking at the degree of overlap in genetic underpinnings of genotypic and plastic effects on a trait can also focus attention on key genes whose expression modification are necessary in both mechanisms of variation. For example, in fruit flies, just a single gene (*Cyp6a20*) was found to influence both evolutionary (Dierick and Greenspan 2006) and plastic effects on male-male aggression (Wang et al. 2008), indicating that it may be particularly important in modification of aggressive behaviour over genes that may only influence one of those mechanisms of variation.

In the present study, we used fruit flies as a model system for genotypic and plastic variation in sexual aggression (specifically male forced copulation rate) to ask several questions: 1) which genes show differential expression due to evolved differences in forced copulation rate, socially plastic differences in forced copulation rate, or both; 2) which ontological terms are overrepresented in these sets of differentially expressed genes; 3) to what degree are gene expression changes similar (in terms of direction and magnitude) due to evolution and plasticity; and 4) do flies with knocked down expression for candidate genes identified as important for forced copulation variation show the expected effects on forced copulation rate? To answer these questions, we used lineages of evolved flies artificially selected specifically for high and low forced copulation rate (Dukas et al. 2020), and used an established protocol to generate flies with high and low forced copulation rate due to prior social experience (Baxter and Dukas 2017). We then performed whole-transcriptome RNAseq on samples of head tissue from these males, followed by differential gene expression analysis between males with low and high forced copulation rates. Finally, we performed tests to validate chosen candidate genes using RNA interference knockdown lines.

5.3 Methods

5.3.1 Modification of forced copulation rate due to artificial selection and plasticity

We have previously generated lineages of flies diverged in male forced copulation (FC) rate as a result of 20 generations of artificial selection (Dukas et al. 2020). A similar difference in FC rate can also be generated by varying the social environment males experience prior to exposure to teneral females (Baxter and Dukas 2017). We first wished to verify this plastic effect on male FC rate as well as on potential determinants of FC success: pursuit of and mounting attempts on teneral females.

Plastic effects of social experience on forced copulation and its determinants

To test the effect of prior experience on FC rate and its determinants, we first sexed male flies under light CO_2 anesthesia within 8 h of eclosion from a lab population of *D. melanogaster* (wild-caught in 2018). These males were housed individually in vials with 5 mL standard food for 3 days (1L standard food = 90 g sucrose, 75 g cornmeal, 10 g agar, 32 g yeast, and 2 g methyl paraben dissolved in 20 mL ethanol). We gave males in the experienced treatment a new 3-day old virgin female each day starting when the males were 1-day old without removing previous females, while males in the isolated treatment were left alone. We then tested the males when they were 4 days old, at which point the experienced males had been given 3 virgin females (Fig. 3a in Baxter and Dukas 2017). Test arenas consisted of 35 mm Petri dishes coated with Surfasil (Thermo Fisher, Ottawa, ON, Canada) on the walls and ceiling to keep flies on the bottom, with a circle of filter paper covering the bottom, and a thin food disc (5 mm diameter by 1.5 mm thick) with a small (1 mm) drop of yeast paste (1 part yeast:2 parts grapefruit juice) placed in the middle. Starting at 8:00 AM, we placed single isolated or experienced males with single teneral females from the same lab population into each test arena, and placed 2 arenas under each of 5 Logitech C920 webcams. We recorded the arenas for 30 minutes, and then continued to manually scan for matings for 2 hours after setting up. We tested 10 males (5 from each experience treatment) per test session, and performed 3 test sessions per day over 3 consecutive days for a total N = 90 (45 per treatment). Mating data were recorded for all 3

days, but videos were only recorded for the first 2 days (n for pursuit and mounting measurements = 30 per treatment).

Observers blind to treatment used BORIS behaviour observation software (v. 7.9.8, Friard and Gamba 2016) to record durations that males spent pursuing and mounting teneral females. We defined pursuit as males following the teneral female with or without (usually with) singing, which was visible as wing vibration. We defined mounting as the male clearly arching his abdomen under and toward the teneral female, usually while grabbing onto the female, though this was not necessary. We analyzed the mating data with a generalized linear mixed model using the glmmTMB package (v. 1.0.2.1, Brooks et al. 2017) in R (v. 4.1.0, R Core Team 2021) with a binomial distribution, including treatment as a fixed effect, and random intercepts of test day, session, and arena. The random intercept of session was changed to a fixed effect to resolve model convergence issues. We similarly fit separate models for mounting duration and pursuit duration, which included video observer and Day as fixed effects (as these variables had only 2 levels). The pursuit data had large proportion of observations with 0 behaviour observed, and a right skew, so we fit these models with using a Tweedie distribution, which fits these types of data well (Dunn and Smyth 2005). Model assumptions were checked with the simulateResiduals function from the DHARMa package (v. 0.4.1, Hartig 2020). We tested the significance of the fixed effects in these models using the Anova function (car package, v. 3.0-10, Fox and Weisberg 2019) and report Wald χ^2 and associated p-values.

5.3.2 Fly collection for gene expression analysis

We collected male fly head tissue samples for RNA sequencing from both the FC artificial selection lineages, and from flies with varying social experience prior to exposure to teneral females. We collected males from the artificial selection lineages generated by Dukas et al. (2020) in generation 21, after 20 generations of artificial selection. We matched the morning timing and environmental conditions at collection to those used by Dukas et al. (2020) when flies would be tested for mating rate with teneral females, and included both

experiment-matched and non-matched conditions. We gave three-day-old experiment-matched males a single teneral female via mouth aspiration in a standard food vial with a foam plug lowered to 1.5 cm above the food to increase interactions and allowed them to interact for 10 minutes following first pursuit before snap freezing the males in liquid N_2. We wished to prevent the males from actually forcibly copulating, as mating would produce confounding effects on male gene expression, and keeping the interaction duration to 10 minutes achieved this. In the non-experiment-matched condition, 3-day-old males were not given a teneral female during the 10-minute experience phase but were otherwise handled exactly as in the experiment-matched condition, including receiving a 'sham' aspiration to simulate adding a teneral female with a mouth aspirator. Including a non-experiment matched treatment allowed us to determine any differential expression effects among the selection treatments specifically in the presence of teneral females, which would presumably make them important in influencing FC outcome. Each sample prepared for extraction included 15 males of the same lineage and treatment combination collected during the same session. We collected 3 samples per lineage and treatment combination, at the level of maintenance vial (i.e., the artificial selection lineages are each maintained in 12 food vials, which were split into 3 groups of 4 vials for the purposes of collecting 3 vial-level replicates). In total, we collected 90 males from each of the 9 lineages (3 low FC, 3 control, and 3 high FC), 45 of which were experiment-matched and 45 non-experiment matched in 3 vial-level replicates. All samples of one replicate lineage from each selection treatment were collected per morning over 3 consecutive mornings. After snap freezing, all 54 samples were stored at -80°C until heads were removed, and RNA was extracted.

Samples of male flies with diverged FC rate due to social plasticity were collected in a similar manner. The lab population used, as well as rearing, timing, handling, and environmental conditions were matched to the experimental conditions described in the previous section, and we also included experiment-matched (males exposed to a teneral female) and non-experiment matched (males not exposed to a teneral female) conditions. Sample collection was performed as previously described for the artificial selection lineages, with 90 males being collected for each experience treatment (isolated or socially

experienced), 45 of each being experiment-matched, and 45 non-experiment-matched. Three replicates for each treatment combination were collected from each of 3 sets of population rearing bottles, for a total of 24 samples.

5.3.3 RNA extraction and sequencing

We homogenized head tissues in 1.5 mL Eppendorf tubes using small metal beads and the NextAdvance Bullet Blender (NextAdvance, Troy, NY, USA). We extracted total RNA from heads using the Invitrogen MagMAX Total RNA Isolation Kit (Thermo Fisher, Ottawa, ON, Canada) following kit specifications. This kit uses TRIzol (TRI reagent), followed by binding to magnetic beads to isolated RNA. We checked sample purity using a NanoDrop (ND 1000, Thermo Fisher) spectrophotometer and quantified concentrations with an Invitrogen Qubit RNA HS Assay Kit (Thermo Fisher) and DeNovix fluorometer (DeNovix, Wilmington, DE, USA). We then sent samples to the Génome Québec sequencing centre (Centre d'expertise et de services, Génome Québec, Montréal, QC, Canada) for library preparation and sequencing. Samples were then further assessed for quality and quantity of RNA using a Bioanalyser 2100 (Agilent, Santa Clara, CA, USA). Libraries were prepared using NEB mRNA stranded Library preparation (using NEBNext dual multiplex oligos), and sequenced using a single lane Illumina NovaSeq 6000 S4 system (Illumina, San Diego, CA, USA), using 100 bp paired-end sequencing technology. One sample from the plasticity set (socially experienced, experiment-matched) did not have usable RNA, and was therefore not used in further analyses (although the 2 remaining replicates with this treatment combination were used). Samples had between 22.4 million and 75.8 million reads, with an average of 36.6 million reads. We checked sample RNA quality, per-sequence GC content, duplication content, and adapter content using FastQC (v. 0.11.9, Andrews 2019). The mean per-base Phred quality score of reads for all samples was > 35. We trimmed adapters using trimmomatic (v. 0.36, Bolger et al. 2014), with leading and trailing both set to “3”, and with settings “MAXINFO:20:0.2”. We then generated an index file based on the Flybase *D. melanogaster* transcriptome (v. r6.34) for

use with Salmon (v. 1.1.0, Patro et al. 2017) to quasi-map RNAseq reads and generate count files of transcripts for each sample.

5.3.4 Differential expression analysis

We imported count data into R using the tximport package (v. 1.16.1, Soneson et al. 2015), which automatically summed counts to the gene level using the Flybase transcript-to-gene file (v. 03/2020), such that counts for 13758 genes were obtained. We computed offsets for the counts for use with downstream GLMs based on effective library sizes and transcript length, and we also filtered out lowly expressed genes (fewer than 5 counts). We then used two different differential expression (DE) analysis packages for use with our two types of data: NEBULA (v. 1.1.7, He et al. 2021) which allows for the use of negative binomial generalized linear mixed effects modelling, which is necessary for the artificial selection data as these data include replicate lineages that need to be modelled as a random effect, and edgeR (v. 3.34, Robinson et al. 2010) which allows for negative binomial generalized linear modelling for use with the plasticity data. The plasticity data were not analyzed with NEBULA as flies were obtained from a single population, rather than from replicate lineages, and NEBULA requires a single random effect to be specified.

For the artificial selection count data, we fit a model of the form: count ~ selection treatment + teneral presence (i.e., experiment-matched, or not) + treatment:teneral presence. NEBULA allows for a single random effect, which we included as replicate lineage. We originally included the effects of test day and vial-level replicate in the model; however, the resulting model coefficients were not estimable, and an extremely high condition number was produced. Therefore, we omitted day and vial-level replicate from the model, but did verify the estimates obtained from NEBULA by manually fitting models with glmmTMB. We fit GLMMs using glmmTMB for the top 200 genes obtained from the NEBULA main effect of selection treatment, which were of the form: count ~ treatment + teneral presence + treatment:teneral presence, with random effects specified as: (1 | Day) + (1 + teneral presence | lineage/vial replicate). We included normalization factors calculated

with the voom function (limma package, v. 3.48.0, Ritchie et al. 2015) as offsets. We ran a reduced model if the above specification produced inestimable coefficients: the same fixed effects, but with just the random intercept of lineage. The estimates from the NEBULA models and glmmTMB models were highly correlated ($r = 0.8$ [95% C.I.: 0.74, 0.85], $p < 0.0001$), so NEBULA estimates were used going forward in the analysis. We tested for DE genes first in the interaction between selection treatment and teneral presence, however this revealed no significant DE genes. We then tested for DE genes in the high and low selection treatment contrast, and all further analyses were based on these results.

NEBULA does not have a built-in method for shrinking estimates to account for high biological variation, especially in genes with low mean expression. Therefore, we used the apeglm function from the apeglm package (v. 1.14.0, Zhu et al. 2019), which employs an empirical Bayes approach to shrink the estimates generated from NEBULA. We then obtained DE genes for the treatment contrast as above. We report both the results from the unshrunken and shrunken estimates for the artificial selection.

For the plasticity data set, we analyzed the data using edgeR, which has a built-in empirical Bayes method to squeeze gene-wise dispersions toward a global dispersion trend. We fit a model of the form: count ~ treatment + teneral presence + treatment:teneral presence + day + vial replicate. As with the artificial selection, the initial analysis of the interaction term revealed no significant DE genes. Subsequent analyses are based on the significant DE genes from the main effect of treatment (isolated vs. socially experienced). As the number of DE genes obtained was still relatively low, we also included the significant DE genes based on the isolated vs. experienced contrast within the group exposed to teneral females. We verified the accuracy of the edgeR estimates by comparing them to estimates obtained from identical models fit with limma-voom, and observed a high correlation between the estimates produced by these two methods ($r = 0.985$, [95% C.I.: 0.980, 0.989], $p < 0.0001$).

Gene ontology analysis

We performed gene ontology analysis on the sets of significant DE genes generated from the artificial selection and plasticity analyses, as well as the set of genes present in both (the overlap set). We used the gene ontology term list (v. 05.2021) and the gene-GO association list (v. 2.1) from Flybase, and the R package topGO (v. 2.44.0, Alexa and Rahnenführer 2016) to identify enriched GO terms in our sets of significant DE genes. We required GO terms to have at least 5 annotated genes to be included, and we used Fisher's exact test to test for significant enrichment. The p-values obtained here are not adjusted for multiple comparisons, which topGO does not perform by default, and a number of reasons are suggested by the package developers about why these corrections are not preferrable for gene ontology analyses (see Alexa and Rahnenführer 2016). We also used topGO to graph the relationships of significant GO terms (Figs. S5.1, S5.2, S5.3).

5.3.5 Comparison of directions and magnitudes of DE estimates due to artificial selection and plasticity

To get a better view of the overall degree of similarity in gene expression effects due to artificial selection and plasticity, we performed a vector correlation and magnitude analysis of: 1) the DE effects in the set overlapping DE genes in each of the artificial selection and plasticity experiments, and 2) the DE effects in the broader set of genes significant in one experiment and the corresponding set in the other experiment (e.g., the DE effects in the significant genes in the plasticity Isolated-Experienced contrast, and the effects in the corresponding set in the artificial selection High-Low FC treatment contrast) regardless of significance in the latter experiment. This is analogous to the analysis performed in Zinna et al. (2018), and it allowed us to get a broader view of the similarity of the direction and magnitude of effects among the two mechanisms of FC behaviour change without simply using a more lenient false-positive rate, and specifically ask whether the DE effects due to plasticity also show correlated effects in those genes due to selection, and vice versa.

We calculated the vector correlations as $r_{VC} = \frac{|\boldsymbol{a}\cdot\boldsymbol{b}|}{||\boldsymbol{a}||\times||\boldsymbol{b}||}$ where $\boldsymbol{a}$ and $\boldsymbol{b}$ are vectors containing $\log_2$ fold changes (i.e., the estimates) obtained from relevant model contrasts

(Zinna et al. 2018). For example, the estimates obtained for the set of significant DE genes in the plasticity comparison, and the estimates for the same set of genes in the artificial selection comparison. Vector correlation values close to 1 indicate a high concordance in the direction of the effects in the two comparisons for that set of genes, while values close to 0 indicate low concordance. We also calculated the value α for each of these vector comparisons as $\alpha = \frac{||\boldsymbol{a}||}{||\boldsymbol{b}||}$ which is the ratio of the magnitudes (L2 norms) of the vectors (Kuruvilla et al. 2002; Zinna et al. 2018), giving an estimate of the relative difference in the magnitude of DE effects between the two comparisons. Values close to 1 indicate a similar magnitude of DE effects in the two vectors, while values less than 1 indicate higher magnitudes in $\boldsymbol{b}$, and values greater than 1 indicate higher values in $\boldsymbol{a}$. In all of our analyses, the vector of estimates from the plasticity analysis was the numerator.

We compared our observed r_{VC} and α values to empirical distributions of 10,000 such values generated by resampling estimates from the entire set of genes (including the set of significant DE genes) as in Zinna et al. (2018). As described in that study, this approach is not a comparison to null expectations, and is instead a comparison of how extreme the observed values are to values obtained from vectors of the same length containing random estimates from the full set of genes. We considered observed values outside of the 95% C.I.s generated from this sampling to be extreme.

5.3.6 Candidate gene choice and validation

We chose 5 genes from the set of overlapped DE genes significant in both the artificial selection and plasticity analyses as candidates for further validation of their effects on FC rate and pursuit of teneral females. These 5 genes were selected based on the following criteria: having the highest logFC estimates, concordant direction of effects in the artificial selection and plasticity analyses, and availability of mutants for candidate validation. This ruled out *lectin-28C* (RNAi lines not readily available) and *CG14025* (DE effects in opposite direction) (Fig. 5.4). We also selected 1 non-overlapping gene from each of the

artificial selection and plasticity significant DE gene lists for validation based on the same criteria (Figs S5.4, S5.5 respectively).

We used RNAi knockdown lines crossed to a general nervous system GAL-4 to specifically knock down gene expression of our chosen candidate genes and observe the effects on FC rate and teneral pursuit. RNAi lines from the TRiP collection (Zirin et al. 2020) and the general nervous system GAL-4, elav-GAL4, were obtained from the Bloomington *Drosophila* Stock Centre (BDSC; see Table 5.1 for genotypes). We generated 3 crosses for each candidate gene: TRiP-RNAi/elav-GAL-4, TRiP-RNAi/CyO (for simplicity, we refer to as TRiP-RNAi/+) and TRiP-control/elav-GAL-4 (+/elav-GAL-4). Conveniently, as the elav-GAL-4 line is maintained over a CyO balancer, crosses to this line generate the experimental cross TRiP-RNAi/elav-GAL-4 and the control TRiP-RNAi/+ cross in the same set of offspring. We do note that flies with CyO have curly wings, however we believe this has a negligible effect on FC rate as the ability to sing properly does not influence teneral female receptibility, since teneral females do not "accept" any matings. Teneral females were reared from a lab population wild-caught in 2020.

We tested all 3 crosses for each gene concurrently, with testing for each gene spread over 2 consecutive days with an equal number of each cross tested per day. We sexed males under light CO_2 anesthesia within 8 hours of eclosion and housed them individually in vials with 5 mL standard food for 4 days before testing. Starting at 8:00 AM we added a single teneral female to each male vial and lowered the vial plug to ~1 cm above the food to constrain the space and encourage interaction. We set up the vials for observation in vial racks in groups of 10, with all 10 being of the same genotype. We randomized the order racks were set up and counterbalanced the order between test days. An observer blind to genotype scanned all vials every 5 minutes for matings, and scanned a subset of vials every 10 minutes to record whether males were pursuing teneral females. Trials lasted until a forced copulation occurred, or 2 hrs had elapsed. We aimed for 600 trials per gene (200 per cross), with a subset of ~240 of these (~80 per cross) also scanned for pursuit. Total sample sizes for each gene were as follows (with the subset scanned for pursuit in parentheses): *CG14153* – 540 (230), *Drsl4* – 580 (154), *GstZ1* – 574 (243), *Nepl18* – 479 (222), *verm* –

577 (180), *Lsp2* – 210 (143), *Nazo* – 90 (87). Note that for the pursuit analyses, trials were excluded if there was a mating before the first pursuit scan, as in these cases there was no pursuit data. Sample sizes among genes varied due to teneral female availability, which was low in testing *Lsp2* and *Nazo* crosses. Sample sizes of crosses within each gene were nearly the same, ± <5%.

We analyzed the mating data by fitting generalized linear mixed effects models for each gene using the glmmTMB function and a binomial distribution, with the model specified as: mating (y/n) ~ Genotype + Day + (1 | Vial rack). We also modelled the pursuit data using a binomial GLMM, and included whether a trial ended in a mating as an explanatory variable, as well as an observation-level variable (representing the time of the observation during the trial), and a trial ID as a random effect to account for repeated measures. These models took the form: pursuit (y/n) ~ Mated + Genotype + Day + Rack + Observation + (1 + Observation | Trial_ID). We checked model assumptions using the simulateResiduals function from the DHARMa package. We performed 2 contrasts: the first between the experimental genotype (RNAi/GAL-4) and the mean of the two control genotypes, and a second contrast between the two control genotypes. We computed the generalized inverse of these custom contrasts to get a contrast matrix, and hard coded this into the Genotype variable, to obtain z and p values directly from the model summary after fitting.

Line	**Genotype**
RNAi-*CG14153*	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMJ22317}attP40
RNAi-*Drsl4*	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMC04568}attP40
RNAi-*GstZ1*	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS05870}attP2
RNAi-*Nepl18*	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMJ23000}attP40
RNAi-*verm*	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMC04570}attP40
RNAi-*Lsp2*	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMC04820}attP40
RNAi-*Nazo*	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS02717}attP40
elav-GAL-4	P{w[+mC]=GAL4-elav.L}2/CyO
TRiP control – attP2	y[1] v[1]; P{y[+t7.7]=CaryP}attP2
TRiP control – attP40	y[1] v[1]; P{y[+t7.7]=CaryP}attP40

Table 5.1.

Genotypes used to generate crosses for candidate gene validation behavioural tests.

5.4 Results

5.4.1 Generation of flies with high and low FC success via artificial selection and environmental variation (plasticity)

We previously generated lineages of flies with significantly diverged forced copulation (FC) rate using artificial selection (Dukas et al. 2020). These males had on average a 0.15 FC rate in the low selection lineages versus a 0.3 FC rate in the high lineages (Fig. 5.1A, generation 20). In the present study, we were able to generate males with low and high FC rate by modifying the social environment experienced prior to exposure to teneral females. Isolated males had a higher, marginally significant FC rate compared to socially experienced males ($\chi^2_1 = 3.02$, $p = 0.08$, Fig. 5.1B). A closer analysis of male behaviours that typically precede forced copulation revealed that isolated males also had significantly higher rates of pursuit of teneral females ($\chi^2_1 = 64.2$, $p < 0.001$, Fig. 5.1C) and mounting attempts ($\chi^2_1 = 31.4$, $p < 0.001$, Fig. 5.1D) compared to sexually experienced males.

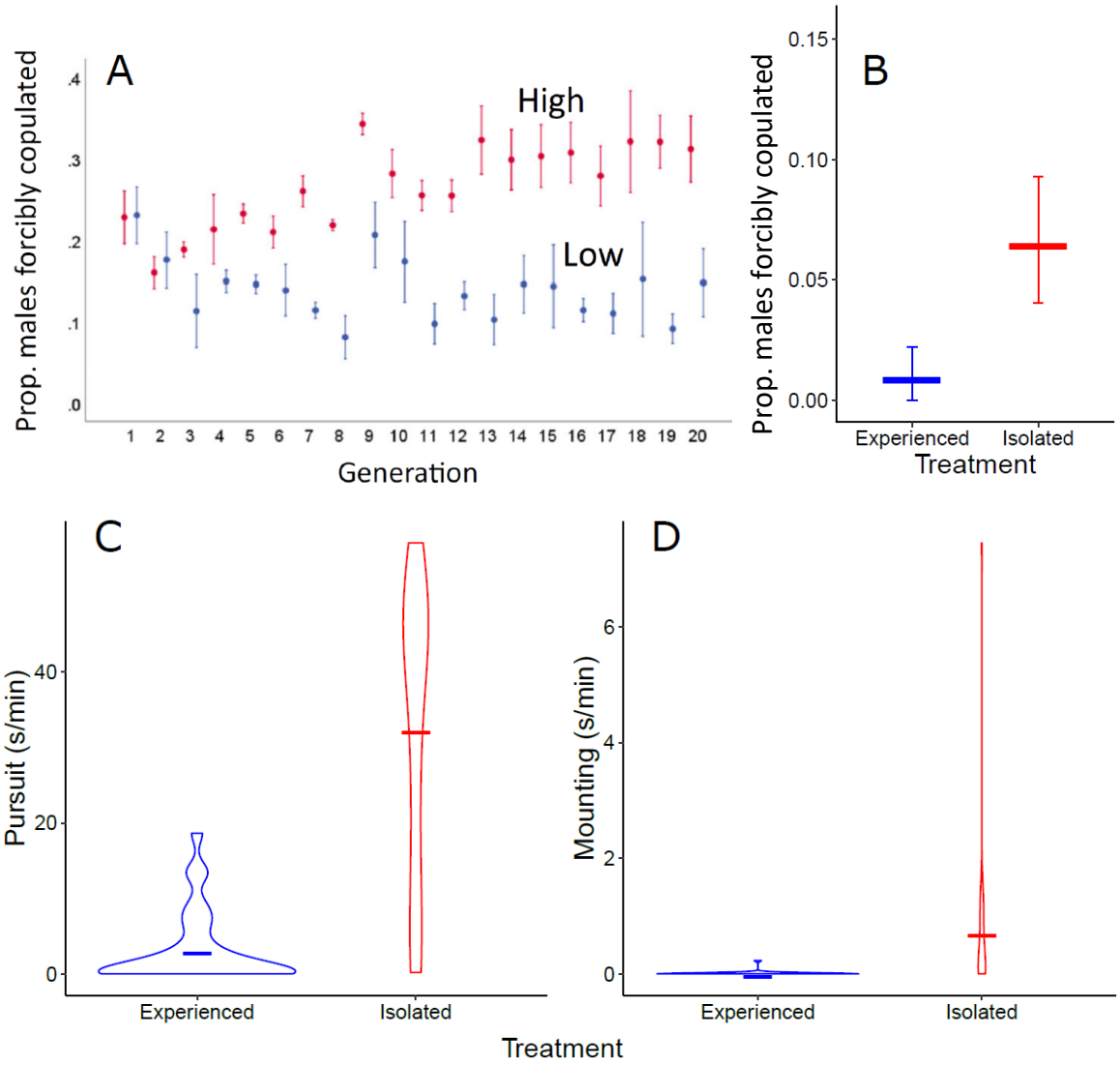

Figure 5.1

Divergence in male forced copulation rate via artificial selection over 20 generations (from: Dukas et al. (2020)). Offspring of flies from generation 20 were snap frozen for RNA sequencing in this study. B) Divergence in forced copulation rate of males from the same population as a result of prior social experience. This plasticity effect was also seen in presumed behavioural determinants of forced copulation: C) male pursuit of teneral females, and D) male mounting of teneral females.

5.4.2 Gene expression in evolutionary diverged and plastically diverged males

The contrast between low and high selection treatments revealed 903 significant DE genes using unshrunken estimates (Fig. 5.2A, D), and 209 significant DE genes using shrunken estimates (Fig. 5.2B, E). This reduction in number of significant genes is expected as highly significant genes with low average total counts are discounted in the shrunken analysis. Eighty-two genes were significant in both analyses of the unshrunken and shrunken estimates. The main effect of treatment in the plasticity analysis revealed 375 genes with significant DE between experienced and isolated males (Fig. 5.2C, F). A small proportion of significant DE genes in either the artificial selection analysis (~0.02-0.05) or plasticity analysis (~0.03-0.05) were significant in both analyses (Fig. 5.3A, B). In total, 27 genes were significantly DE in both artificial selection and plasticity (Fig. 5.3A, B; Fig. 5.4).

We performed gene ontology (GO) analyses to identify ontological terms that are overrepresented in our samples of significant DE genes (for terms with at least 5 total annotated genes). Fourteen terms were significantly overrepresented among significant DE genes in the artificial selection analysis, 35 terms were overrepresented in the plasticity analysis, and 5 terms were overrepresented in the 27 genes significant in both analyses (Table 5.2). Of particular note, in the overlapping genes set and plasticity set, neuropeptide hormone activity and general hormone activity were significantly enriched, and in the artificial selection set, serotonin receptor activity was enriched. GO graphs showing the relationship among significantly enriched terms are in Figs. S5.1A, B, and C.

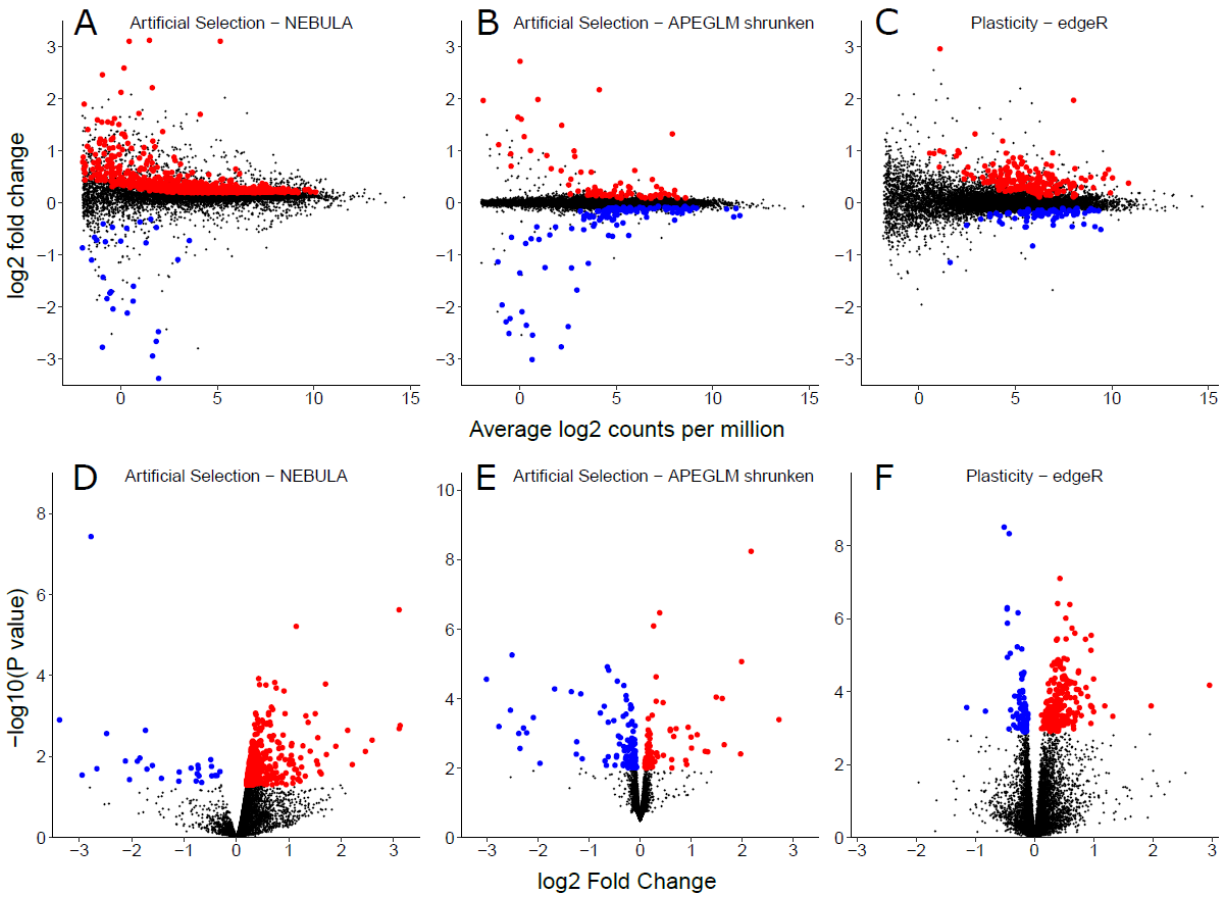

Figure 5.2

Differential expression of genes in flies with diverged forced copulation tendency. The top row are MA plots showing the log2 fold changes as a function of mean log2 counts per million of A) High/Low FC lineages from the artificial selection, B) High/Low lineages using shrunken estimates, and C) Isolated/Experienced plasticity treatments. The bottom row are volcano plots showing the -log10 p-values from the above contrasts as a function of log2 fold changes (as in corresponding plots above) for D) artificial selection, E) artificial selection using shrunken estimates, and F) plasticity. Red dots indicate genes with significant upregulation in the high FC or isolated groups, and blue dots indicate genes with significant upregulation in the low FC or experienced groups.

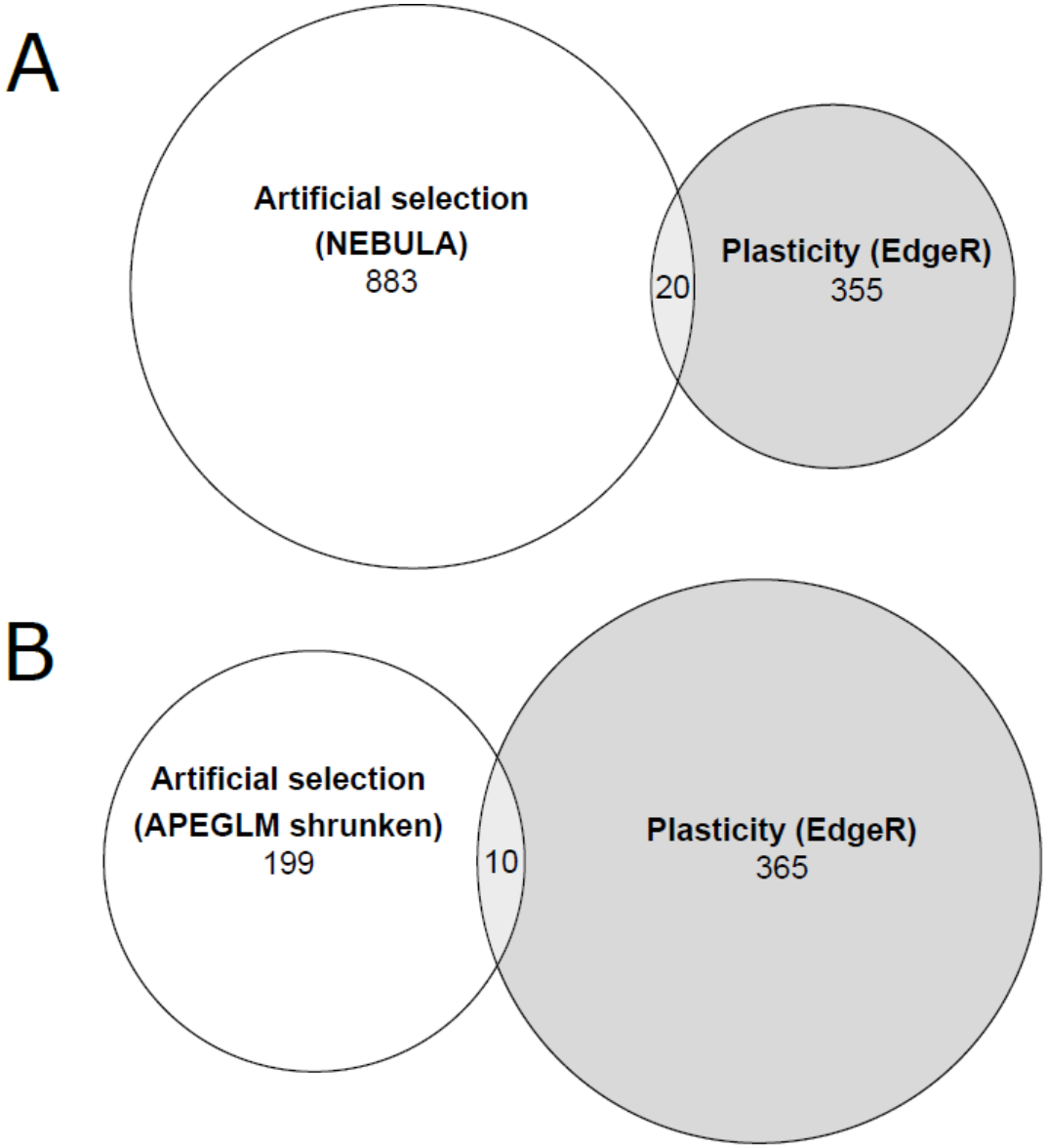

Figure 5.3

Genes with significant differential expression due to artificial selection and plasticity, and those significant in both. A) Venn diagram of the significant artificial selection DE genes, significant plasticity genes, and overlap. B) Venn diagram as in A) except using shrunken estimates for determining significant artificial selection genes.

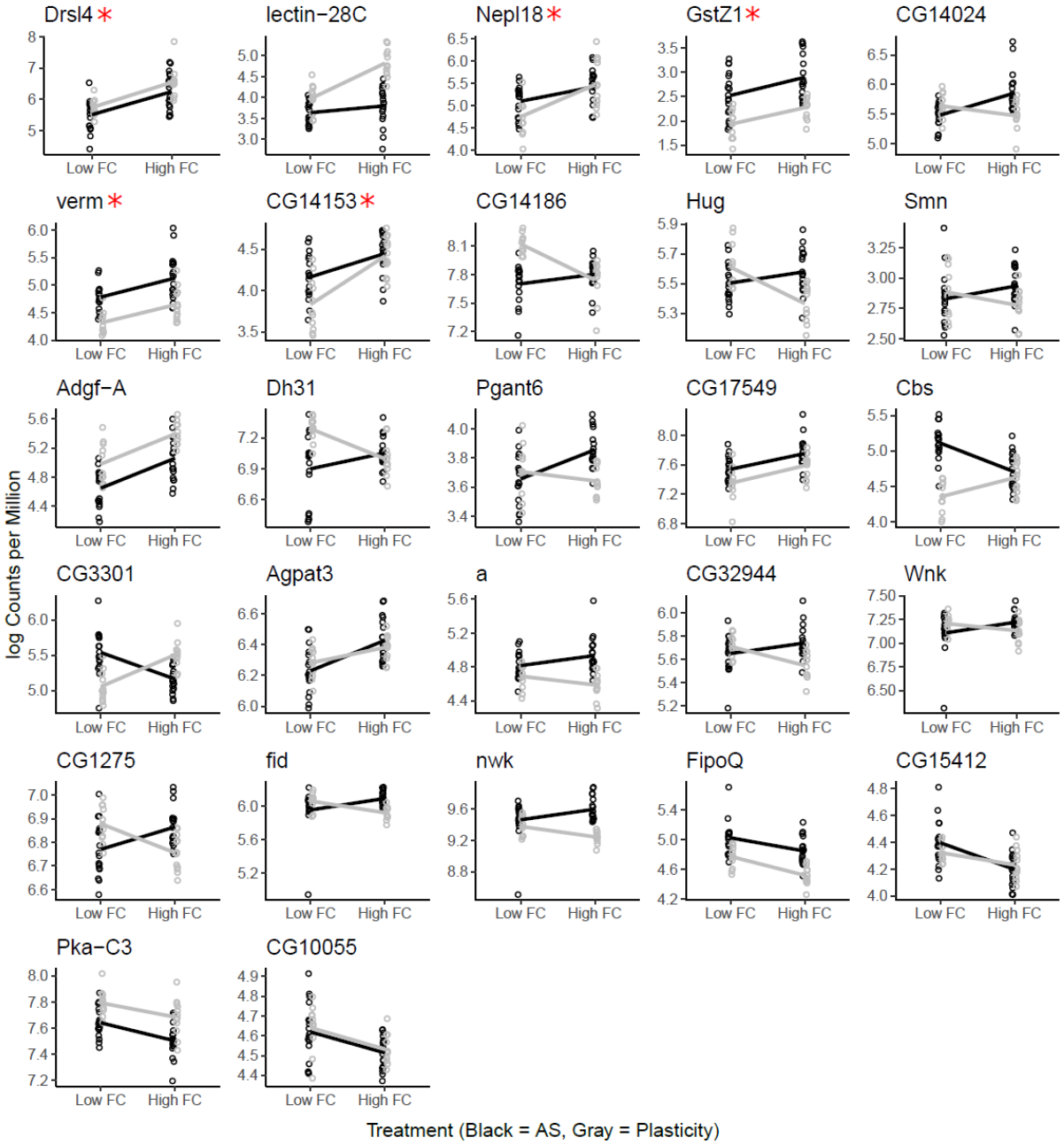

Figure 5.4

The 27 genes that show significant differential expression in the artificial selection experiment (black lines/dots) and plasticity experiment (gray lines/dots). "Low FC" corresponds to both the low FC lineages (AS) and socially experienced (plasticity) treatments; "High FC" refers to the high FC lineages (AS) and socially isolated treatments (plasticity). The genes are ordered (left-right, top-bottom) by decreasing average log2 fold change values for the two experiments. Red asterisks indicate genes that we chose for follow-up candidate gene validation.

Top GO groups from analysis of 27 overlapping genes:

GO #	Gene ontology term	P value
GO:0005184	Neuropeptide hormone activity	0.0025
GO:0005179	Hormone activity	0.0043
GO:0016740	Transferase activity	0.0043
GO:0004674	Protein serine/threonine kinase activity	0.0067
GO:0001664	G protein-coupled receptor binding	0.0081

Top GO groups from analysis of AS top genes:

GO #	Gene ontology term	P value
GO:0038187	pattern recognition receptor activity	0.00097
GO:0042805	actinin binding	0.00097
GO:0051393	alpha-actinin binding	0.00097
GO:0004930	G protein-coupled receptor activity	0.00438
GO:0038023	signaling receptor activity	0.00635
GO:0060089	molecular transducer activity	0.00635
GO:0004860	protein kinase inhibitor activity	0.00645
GO:0004993	G protein-coupled serotonin receptor act...	0.00645
GO:0019210	kinase inhibitor activity	0.00645
GO:0099589	serotonin receptor activity	0.00645
GO:0004448	isocitrate dehydrogenase activity	0.007
GO:0016886	ligase activity, forming phosphoric este...	0.007
GO:0051371	muscle alpha-actinin binding	0.007
GO:0030247	polysaccharide binding	0.00997

Top GO groups from analysis of plasticity top genes:

GO #	Gene ontology term	P value
GO:0016491	oxidoreductase activity	1.40E-07
GO:0003824	catalytic activity	1.50E-07
GO:0005184	neuropeptide hormone activity	8.70E-07
GO:0071855	neuropeptide receptor binding	3.50E-06
GO:0005179	hormone activity	9.60E-06
GO:0030546	signaling receptor activator activity	9.70E-06
GO:0030545	receptor regulator activity	1.40E-05
GO:0017171	serine hydrolase activity	1.90E-05
GO:0033764	steroid dehydrogenase activity, acting o...	2.20E-05
GO:0001664	G protein-coupled receptor binding	2.40E-05
GO:0016229	steroid dehydrogenase activity	4.50E-05
GO:0048018	receptor ligand activity	0.00011
GO:0004303	estradiol 17-beta-dehydrogenase activity	0.00021

GO:0016614	oxidoreductase activity, acting on CH-OH...	0.00027
GO:0030297	transmembrane receptor protein tyrosine ...	0.00048
GO:0016878	acid-thiol ligase activity	0.00086
GO:0016616	oxidoreductase activity, acting on the C...	0.0009
GO:0008236	serine-type peptidase activity	0.0012
GO:0004252	serine-type endopeptidase activity	0.00144
GO:0030296	protein tyrosine kinase activator activi...	0.00159
GO:0008374	O-acyltransferase activity	0.00163
GO:0030295	protein kinase activator activity	0.00198
GO:0019209	kinase activator activity	0.00238
GO:0016411	acylglycerol O-acyltransferase activity	0.00248
GO:0005102	signaling receptor binding	0.00301
GO:0016289	CoA hydrolase activity	0.00362
GO:0015645	fatty acid ligase activity	0.00379
GO:0016405	CoA-ligase activity	0.00379
GO:0004175	endopeptidase activity	0.0044
GO:0016877	ligase activity, forming carbon-sulfur b...	0.00456
GO:0016903	oxidoreductase activity, acting on the a...	0.0048
GO:0016620	oxidoreductase activity, acting on the a...	0.00527
GO:0050660	flavin adenine dinucleotide binding	0.00781
GO:0005342	organic acid transmembrane transporter a...	0.00946
GO:0046943	carboxylic acid transmembrane transporte...	0.00946

Table 5.2.

Significantly enriched gene ontology terms in the sets of genes significant in both the artificial selection and plasticity analyses (27 overlapping genes), and each of the artificial selection and plasticity analyses separately.

5.4.3 Comparison of direction and magnitude of gene expression between artificial selection and plasticity

Overall, we observed a low degree of similarity in the directions of DE among the 27 overlapping genes (Fig. 5.5A, red bar), demonstrated by an observed vector correlation value that is not more extreme than the 95% confidence interval (C.I.) generated by vector correlations of randomly sampled effects across all genes. Similarly, when looking at the entire set of significant DE artificial selection genes, or the set of significant DE plasticity genes, and the corresponding DE effects in the other experiment (i.e., plasticity and artificial selection respectively), the observed vector correlations are low and not outside of the 95% C.I. generated from random sampling of all genes (Fig. 5.5B black and grey bars; S3A, B). These results were similar when using the set of significant artificial selection genes determined with shrunken estimates (Fig. S5.2A), and when we controlled for a potential algorithmic effect due to using different analysis methods for the artificial selection and plasticity (Fig. S5.3A).

The magnitude of DE effects in the 27 overlap genes tended to be higher for plasticity compared to artificial selection, although this was again not more extreme than the 95% C.I. generated from random sampling (Fig. 5.5B, red bar). Overall, the set of significant artificial selection genes had higher magnitudes of DE effects in the artificial selection experiment, and the set of significant plasticity genes had higher magnitudes of DE effects in the plasticity experiment, compared to the corresponding magnitudes of DE effects in the plasticity and artificial selection experiments, respectively (Fig. 5.5B black and grey bars; S3C, D). This is unsurprising as there is little overlap of significant DE genes and low correlation of effects due to selection versus plasticity (Figs. 5.4, 5.5A). This magnitude difference was more extreme than that expected under our random sampling for the significant plasticity set, but not in the significant artificial selection set. When using shrunken artificial selection estimates (Fig. S5.2B), and controlling for analysis method (Fig. S5.2D), the results were generally similar. In this case, however, we also found a

larger magnitude than expected from random sampling in the artificial selection effects for the significant artificial selection genes.

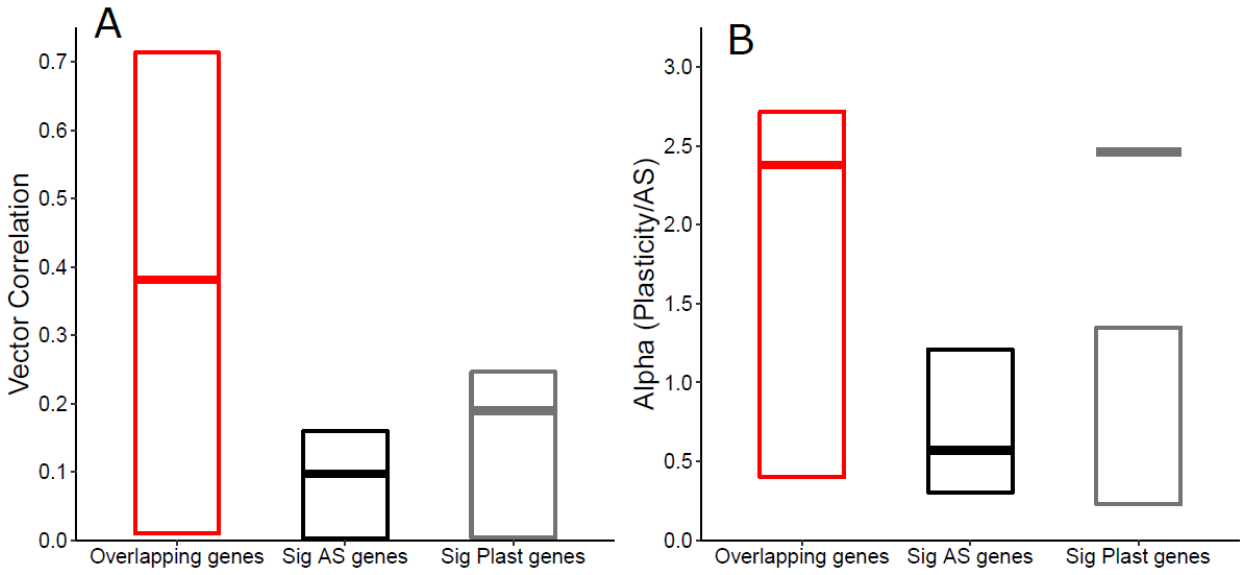

Figure 5.5

Similarity in direction and magnitude of DE estimates between artificial selection and plasticity. A) The observed vector correlation values (thick horizontal lines) between vectors of estimates (log2 fold changes) obtained from the artificial selection and plasticity analyses for 3 sets of genes: the 27 genes significant in both (red), all the genes significant in artificial selection analysis and the corresponding estimates for those genes in the plasticity (black), and all the genes significant in the plasticity analysis and the corresponding estimates for those genes in the artificial selection (grey). B) The observed ratio of vector magnitudes (plasticity/artificial selection), or alphas, for the same vector comparisons. Rectangles represent 95% C.I.s generated from empirical resampling of estimates from all genes.

5.4.4 Candidate gene choice and validation for genes contributing to variation in FC tendency due to artificial selection and/or plasticity

The 27 genes showing significant DE due to both artificial selection and plasticity (Figs 5.3, 4) were our starting point for choosing candidate genes for further analysis. We chose 5 genes from this list (Fig. 5.4, red asterisks) based on a few criteria (see Methods). We also selected 1 non-overlapping gene from each of the significant artificial selection and plasticity lists for validation (Figs. S5.4, S5.5 respectively; red asterisks). We wished to validate the effects of these 7 genes (5 overlap, 1 artificial selection, 1 plasticity) using RNA interference (RNAi) knockdown constructs crossed to a general brain targeted GAL4. All 7 genes showed higher expression in the treatments with higher FC (High selection and isolated), so we expected the gene knockdown effect to manifest as a reduction in FC rate and pursuit of teneral females in RNAi/GAL4 crosses compared to RNAi and GAL4 controls.

Overall, 4 of the 7 genes showed evidence of an effect in the predicted direction in at least one of: FC rate (Fig. 5.6A, B) and pursuit of teneral females (Fig. 5.6C, D). In the overlap set, knockdown of 2 of the 5 genes had the expected effect in FC rate (Fig. 5.6A), and 3 of the 5 genes had the expected effect in pursuit (Fig. 5.6C). In the experiment-specific set, only knockdown of the artificial selection gene (*Nazo*) produced an effect in the expected direction in FC rate (Fig. 5.6B), and in pursuit (Fig. 5.6D). *GstZ1*, *verm*, and *Nazo* showed a reduction of both FC rate and pursuit in the knockdown compared to controls (although the *Nazo* FC rate comparison was not significant due to a lower sample size). The *Nepl18* knockdown showed a reduction in pursuit, but not FC rate. *CG14153* and *Drsl4* knockdowns showed the reverse pattern in FC rate (higher in knockdown vs controls), but were not different from controls in pursuit.

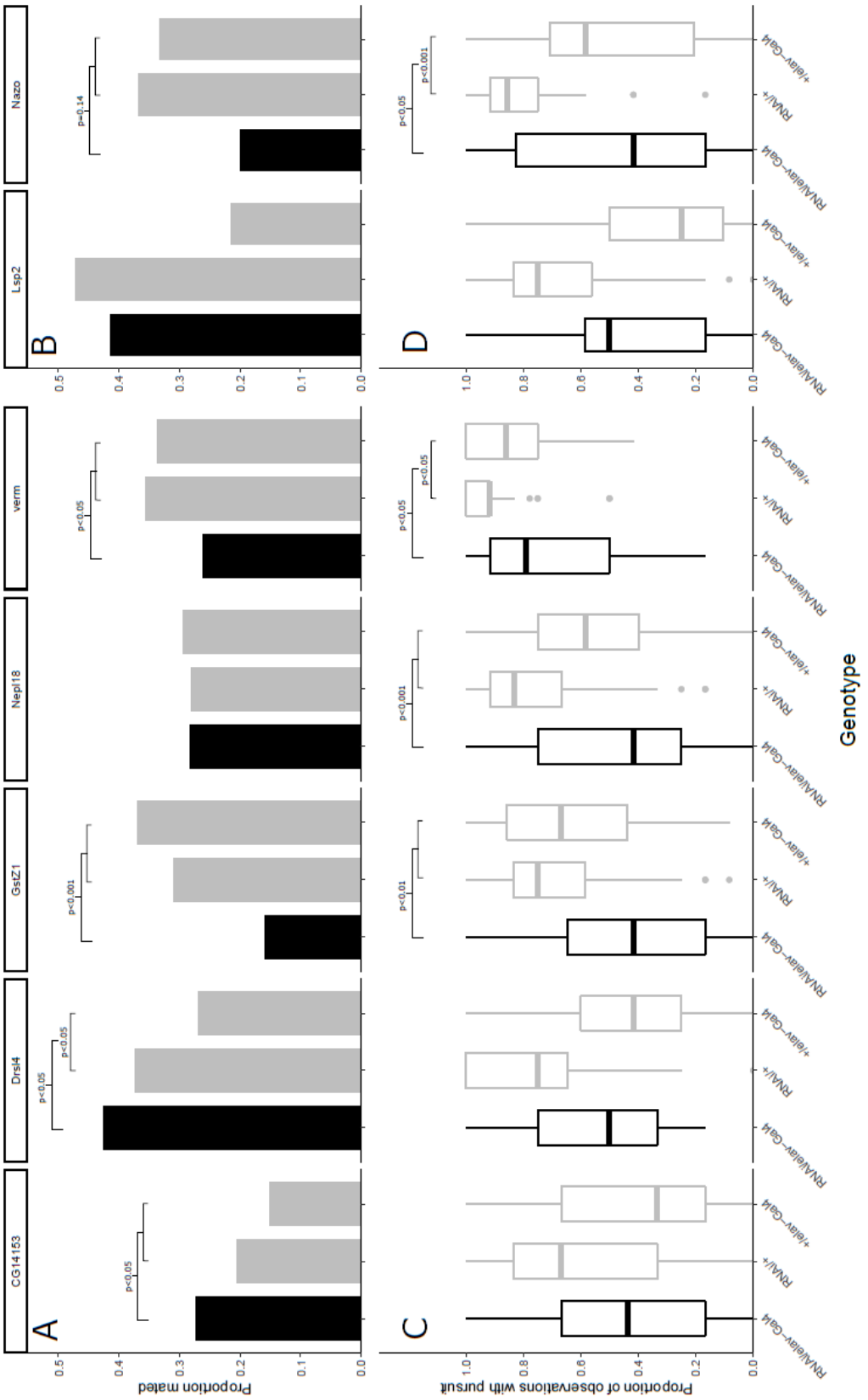
A
B
C
D
CG14153
Drsl4
GstZ1
Nep18
verm
Lsp2
Nazo
Proportion mated
Proportion of observations with pursuit
p<0.05
p<0.001
p<0.01
p=0.14
RNAi/elav-Gal4
RNAi/+
+/elav-Gal4
Genotype

Figure 5.6.

Functional validation of candidate genes that may contribute to variation of FC rate due to artificial selection and plasticity. Males from knockdown crosses (black) and two control crosses (grey) were measured for rate of forced copulation (top row) and proportion of observations with pursuit of teneral females (bottom row). Seven genes were tested for effects on these behaviours in knockdown crosses: 5 that showed significant DE due to both artificial selection and plasticity (A, C), and two that were significant in one of those experiments (B, D; plasticity gene = *Lsp2*, artificial selection gene = *Nazo*). Significant contrasts between the knockdown cross and the mean of the two controls is shown above each plot. A significant contrast between the two controls is also shown if the knockdown-control contrast is significant.

5.5 Discussion

Our main findings were: 1) Variation in forced copulation rate generated by evolution or plasticity (Fig. 5.1) was associated with the significant differential expression (DE) of around 903 and 375 genes respectively (Figs 5.2, 5.3), 2) only 27 of these genes showed significant DE in both artificial selection and plasticity (Fig. 5.4), 3) significant DE genes in plasticity and these 27 overlapping genes showed enrichment in neuropeptide hormone and general hormone activity gene ontology (GO) categories (Table 5.2), 4) the direction of gene expression effects in the set of significant DE genes for artificial selection were not correlated with the corresponding direction of effects in plasticity, or vice verse (Fig. 5.5), and 5) 4 of 7 candidate genes showed the predicted effects of gene knockdown on forced copulation rate and pursuit of teneral females (Fig. 5.6). We discuss each of these findings in turn, and suggest avenues for future research.

Overall, we identified a relatively large number of significant DE genes associated with diverged forced copulation rate (Fig. 5.2). This is in agreement of the growing body of literature showing that behavioural variation is associated with the DE of a large number of genes (Dierick and Greenspan 2006; Gammie et al. 2007; Immonen et al. 2017; Shultzaberger et al. 2019), rather than a few genes with large effects. Of these genes, however, only 2-5%, or 27 total genes, showed significant DE due to both artificial selection and plasticity (Fig. 5.3, 5.4). This is in contrast to a few other findings comparing gene expression effects of trait variation due to evolution and plasticity, which have found a high degree of overlap between evolved and plastic effects on mating strategies in sailfin mollies (*Poecilia latipinna*) (Fraser et al. 2014), and on temperature effects in graylings (*Thymallus thymallus*) (Mäkinen et al. 2016). However, in one other study of the evolved and plastic effects on gene expression in a fruit fly behaviour (male-male aggression), only a single gene was found to be significantly DE in both (Wang et al. 2008). The scarcity of data preclude us from generalizations at this point.

In the set of 27 genes significant in both evolved and plastic variation in forced copulation rate, and in the set of significant plasticity genes, our gene ontology analyses

revealed that a few ontological categories related to neuropeptide hormone activity, and neuropeptide receptor binding, were significantly overrepresented (Table 5.2). Neuropeptides are known to be involved in the regulation of many insect behaviours, from mating behaviour to feeding (Schoofs et al. 2017). They have also been shown to be important regulators of sexual behaviour in mammals. Neuropeptide manipulations in female prairie voles (*Microtus ochrohaster*) early in life are associated with changes in later life sexual behaviour, and neural responses to social stimuli (Cushing et al. 2005; Kramer et al. 2006). It is possible that neuropeptide hormone regulation in response to prior social environments facilitates a plastic shift in mating behaviours, including forced copulation tendency, and that this plastic mechanism may have been partly co-opted in producing genotypic variation via evolution. In addition, serotonin receptor activity was significantly enriched in the set of genes with significant evolved DE, indicating that the serotonergic system, or upstream regulators of it, may have been a target of the forced copulation artificial selection regime. Serotonin is known to have a wide array of effects on sexual behaviour, including sexual motivation and arousal in rodents, and its involvement in sexual behaviour has diverged in males and females (Angoa-Pérez and Kuhn 2015).

We further investigated the overall degree of similarity in gene expression effects between evolved and plastic changes in forced copulation to see if there existed a broader pattern of concordant changes in expression that was not captured simply by looking at the overlapping significant DE genes. Using a vector correlation and magnitude analysis, we found that the small degree of overlap between evolved and plastic effects, represented by only 27 genes significant in both, extended to the broader sets of genes significant in either the artificial selection or plasticity analyses. In other words, genes that were significant in one analysis (i.e., in artificial selection or plasticity) did not tend to show a correlated (even if not significantly DE) effect in the other analysis (Fig. 5.5). This may indicate the relative independence of the mechanisms underlying variation in forced copulation tendency for genotypic variation, and plastic variation. Future studies could look more directly at the behavioural differences in evolved versus plastic effects on forced copulation to disentangle these potentially different mechanisms.

We then focused our attention on the 27 overlapping genes that may be part of a shared mechanism of forced copulation regulation that contributes to both evolved and plastic differences. Of 5 genes chosen for follow-up tests with RNAi knockdown crosses, we were able to validate the effects of *GstZ1*, *Nepl18* and *verm* on forced copulation rate and/or pursuit of teneral females using fly crosses containing gene specific knockdowns (Fig. 5.6A, C). We were also able to validate the effect of *Nazo*, which had one of the largest DE magnitudes in the list of significant DE genes in the artificially selected flies list (Fig. 5.6B, D). *GstZ1*, orthologous to human GSTZ1, is involved in enabling glutathione transferase activity (Saisawang et al. 2012), with no obvious direct link to behaviour. *Nepl18* is orthologous to mammalian Neprilysin, an endopeptidase which degrades amyloid beta, the buildup of which is a hallmark of Alzheimer's disease. Neprilysin-deficient mice have been shown to have neuronal degradation and weakened learning ability (Madani et al. 2006). The potential memory-related effects of *Nepl18* in fruit fly pursuit of teneral females is intriguing, though unclear, and requires further investigation. *verm*, also known as *hlm*, is expressed in the trachea and in photoreceptors, and the knockdown of this gene during fly development results in reduced visual acuity and poor optomotor response (McKay et al. 2008). The visual system has been shown to be important for successful male courtship (Markow 1987), and it is possible that variation in visual acuity may be an important distinction between males that do and do not forcibly mate. Finally, *Nazo*, which was significantly DE in just the artificial selection experiment, is involved in neuromuscular processes and has been shown to result in reduced climbing ability in flies with knocked-down expression (Iuso et al. 2014). The DE observed among selection treatments for *Nazo* may therefore indicate the selection for alleles associated with physical ability to overcome teneral female resistance in the high FC males. Physical traits such as size and ornamentation have been shown to affect male ability to sexually coerce females (Crean and Gilburn 1998; Perry and Rowe 2012), however, no effect of selection was observed on the physical traits of sex comb number or body size in our artificially selected fruit fly lineages (Dukas et al. 2020). We have not, however, quantified more subtle physical characteristics such as strength. We do note that, since we expected the effects of

the gene knockdowns to result in lower FC and pursuit rates compared to controls, it is possible that our positive results could be the consequence of an overall reduction in fly rigor, and so further tests on these flies' mating and courting rates with mature virgin females is required to rule out this explanation of the results.

Overall, we report here that the evolution of male forced copulation success via artificial selection, and the effects on forced copulation success from the social environment, are each associated with the differential expression of hundreds of genes. However, the degree of overlap in differential expression between evolved and plastic differences is minimal and only includes a small subset of potentially key genes. Further investigation into the functions and mechanisms of these overlapping genes, as well as genes that only show differential expression in either evolution or plasticity, will be important in our understanding of the genetic architecture that is necessary for both evolved and plastic changes in forced copulation success, and in our understanding of the genetic architecture that differentiates these two types of variation.

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CHAPTER 6 – DISCUSSION

6.1 Overview

Social behaviours are complex quantitative traits that are, like other complex traits, influenced by a variety of environmental and genetic effects. However, unlike non-social behaviours and traits, social behaviours are also influenced by the genotypes of conspecifics mediated through the social environment as indirect genetic effects (IGEs). I've shown this to be the case for sociability, and that behavioural interactions mediate this effect (Chapter 2). Further, there is genetic variation not only in social behaviours themselves but also in the degree and direction of plasticity (Chapter 3). I've quantified these genetic, environmental, and gene-by-environment influences on variation in sociability. Such genetic variation in these social behaviours in natural populations is sufficient for a rapid response to artificial selection for the extremes of these behaviours (Chapter 4). I've shown that in sociability, this response to selection is not correlated with measures of social aggregation, or measures of fitness, but is negatively correlated with intrasexual aggression, revealing the potential for a shared genetic underpinning, or a behavioural mechanistic underpinning for aggression in determining subsequent sociability, or both. Finally, I've shown that in intersexual aggression (i.e., male forced copulation of females), the response to artificial selection, and the effects due to variation in the social environment, are each correlated with the differential expression of hundreds of genes; however, these genetic effects do not overlap between evolved and plastic effects on behaviour, except for a few potentially core genes (Chapter 5).

In the next two sections I'll discuss two important takeaways from this research as a whole. First, what this research has provided for our overall understanding of the genetic and behavioural underpinnings of sociability variation. Second, I'll discuss the relationship that we can glean between "friendly" and antagonistic social behaviours in general, leading into discussion about the genetic underpinnings of sexual aggression. I will then describe some fruitful avenues for future directions for this research followed by concluding points.

6.2 The genetic and behavioural underpinnings of variation in sociability

Overall, our results provide insight into both the behavioural and genetic underpinnings of variation in sociability, and into the genetic underpinnings of plastic and genotypic variation in sexual aggression. For my research on sociability, while we do not yet have genetic sequencing data that point to the identity of the genetic variants or gene expression modifications that influences phenotypic variation, we do now have a better quantitative genetic understanding of this variation, and an understanding of the mechanisms by which this genetic variation can influence others.

In Chapter 2 I presented data on the mediating effect of social interactions on the influence among flies in a group on resultant sociability phenotypes, representing some of the first data on the behavioural mechanisms that underlie indirect genetic effects (IGEs; Moore et al. 1997). It is still unclear exactly how social encounters among flies may affect group cohesion, as these encounters were not overtly aggressive and appear to be more of a mutual "acknowledgement" of each group member's presence. Additionally, it is certainly possible that important subtle social signals or variation in pheromonal composition that were not captured by our encounter rate measure were driving this effect. Pheromones such as *cis*-vaccenyl acetate and a variety of cuticular hydrocarbons are known to have an effect on social aggregation in flies (Bartelt et al. 1985; Duménil et al. 2016; Rajpurohit et al. 2017). Nevertheless, the influence of group members' genotypes on other group members' phenotypes through the social environment may have profound effects on the rate of sociability evolution (Moore et al. 1997; Wolf et al. 1998; Wilson et al. 2009). For example, a positive covariance between the direct and indirect genetic effects on a trait would result in a positive feedback mechanism in a response to selection. Since the direct and indirect genetic effects on sociability appear to have positive covariance (that is, highly sociable individuals appear to affect the social environment in a way that increases the sociability of others, and vice versa), selection for high sociability, for example, would result in not only an increase in the frequency of "high sociability" alleles, but also a social

environment that will be more likely to affect sociability phenotypes to become more sociable. Although there is great potential for these effects to increase (or attenuate) the effects of selection, such evolutionary consequences of IGEs have not yet been empirically investigated.

While there is great potential for research on the indirect genetic effects on sociability to improve our understanding of the mechanisms that influence its variation and evolution, I decided that, in order to get to the direct genetic effects (that is, the genes expressed in an individual that affect its phenotype), I needed to control for such indirect effects. The *Drosophila* Genetic Reference Panel of clonal lines (Mackay et al. 2012) that together represent natural variation, gave us the opportunity to control the genotypes that interact with one another, and quantify traditional quantitative genetic parameters such as direct genetic and environmental variation as well as heritability. In Chapter 3, I presented such data for sociability in male and female flies, showing a low-moderate (~0.2) broad-sense heritability of sociability. From this, we can start to produce a picture of the degree to which genetic variation influences phenotypic variation in sociability. In addition, the lack of a genetic correlation between males and females further suggests an independence in these genetic mechanisms between the sexes. This could reflect, for example, a difference in the motivations for being sociable between the sexes, with male sociability being driven by motivation to find females, and female sociability being driven by motivation to find food and suitable egg laying substrate. In addition, we found evidence that variation in sociability is not correlated with variation in activity, supporting a mechanistic independence in these traits as well, and ruling out the possibility that our measure of sociability was simply an artifact of activity variation. One could imagine, for example, that high activity would keep animals from remaining together at a food patch, or make animals more likely to move between food patches, lowering sociability (as is seen in the zebra finch, *Taeniopygia guttata*; McCowan and Griffith 2015), or potentially making it more variable and preventing highly sociable groups from persisting. On the other hand, this result is in support of our lab's earlier findings of a lack of genetic correlation between social behaviour and activity (Anderson et al. 2016).

Given that we were able to estimate a significant heritability for sociability in males and females, my next step was to link this observed phenotypic variation to known genetic variation present in the DGRP. This is simplified by the fact that the DGRP lines are clonal, their genotypes have been sequenced, and the data are freely available (Mackay et al. 2012). However, at our multiple-comparison adjusted significance level, our genome-wide association analysis did not reveal any genetic variants significantly associated with variation in sociability (unpublished data). While disappointing, this result is not entirely unexpected, and is actually informative regarding the underlying structure of the relevant genetic architecture and the size of the effects of individual variants on variation in sociability. Quantitative traits influenced by many genes each with a small effect on the phenotype require a great degree of statistical power to confidently draw associations with those genes, as the corrections for multiple comparisons employed in genome-wide association studies sacrifice statistical power to increase reliability (Rietveld et al. 2014; Marigorta et al. 2018). Many animal behaviours have been shown to fall into such a category of traits (Flint 2003; Kendler and Greenspan 2006), and the argument has been made that virtually all human behaviours are underpinned by many genes of small effect (Chabris et al. 2015). Given our inability to confidently find gene-phenotype associations with 59 DGRP lines, this likely applies to sociability as well.

The significant heritability of sociability in the DGRP lines indicated that we should be able to artificially select on high and low sociability in a wild-caught population, giving us the opportunity to not only potentially improve our ability to detect genetic variation associated with variation in sociability (Manolio et al. 2009; Baldwin-Brown et al. 2014), but also to investigate the behavioural underpinnings and fitness consequences of variation in sociability. One option for us was to use the DGRP themselves to form an outbred population, a method that is often used to generate starting populations for artificial selection analyses (Mackay et al. 2005; Turner and Miller 2012; Garlapow et al. 2017). However, since we were interested in all standing variation contributing to sociability, including variation that may have been purged during the inbreeding process, we used a freshly wild-caught population. While upcoming genetic work on the artificial selection

lineages will soon produce important genomic and gene expression data hopefully linking sociability variation to genetic variation (discussed further in future directions, 6.4), the data presented in Chapter 4 has already shed light on the behavioural mechanisms associated with variation in sociability.

First, the lack of a correlated response to selection in another measure of "sociability" (i.e., the nearest-neighbor distance measure on a large homogenous food patch) points to an intriguing independence in the mechanisms underlying sociability (as I define it in section 1.2) compared with general social aggregation or social space. If a correlation existed between these behaviours, we would expect a correlated response to selection since nearest-neighbor preferences have ample genetic variation that could allow for evolutionary change (Anderson et al. 2016). Nearest-neighbor distances of groups of flies in homogenous arenas have frequently been used to measure social aggregation in fruit flies (e.g., Anderson et al. 2016, Chapter 2 of this thesis), and similar methods have been used to quantify fly social space (e.g., Simon et al. 2012a, Brenman-Suttner et al. 2018) and social interactions (Schneider and Levine 2014). The lack of any correlated response to selection provides evidence for the independence of sociability and the social behaviours measured in the above studies, either through lack of shared genetic underpinnings (lack of pleiotropy), shared underpinnings resulting from pleiotropic effects that cancel out resulting in no visible correlation, or independence of behavioural mechanisms driving these traits. From a behavioural perspective, this may indicate that decisions about whether to feed alone or in groups (or the behavioural mechanisms, such as aggression, that lead to individuals feeding in a sociable or non-sociable way) are different from the decisions or behavioural mechanisms underlying general social space preference. This is important, as sociability measures and nearest-neighbor measures are often assumed to be measuring the same thing (e.g., in Chapter 2, we classify nearest neighbor aggregation as sociability).

Second, we found a negatively correlated response to selection in intrasexual aggression, which, points to the shared genetic underpinnings of these traits, and the potential mechanistic influence of aggression on subsequent sociability. The association between sociability and aggression is not well understood, however we have shown

previously that prior social experience has similar plastic effects on aggression (Baxter and Dukas 2017) and sociability (Chapter 3), such that socially deprived males are both more aggressive and less sociable than socially experienced flies. The result in Chapter 4 points to the possibility that there is either a shared genetic underpinning between aggression and sociability, or that aggression is involved mechanistically in generating variation in sociability. In the next section I will discuss this link between sociability and aggression, as well as among different forms of aggression, and how these links may be informed by our work on the genetic underpinnings of variation in sexual aggression.

6.3 The relationship between sociability and aggression, and the genetic underpinnings of variation in sexual aggression

In collaboration with others in our lab, one of our long-term goals has been to determine the link between non-aggressive (friendly) interactions (i.e., sociability) and aggressive interactions (i.e., intrasexual and intersexual aggression). Our main research questions included: are these behaviours genetically correlated? Do they rely on the same underlying mechanisms driven by the same or similar suites of genes (e.g., pleiotropy in aggression and sociability genes)? It has been shown that many behaviours are affected by pleiotropic genes that also affect other behavioural and non-behavioural phenotypes (Anholt and Mackay 2004). However, one study that looked directly at the relationship between sociability and aggression in sticklebacks (*Gasterosteus aculeatus*) found a negative correlation between these traits in some natural populations, yet ruled out the contribution of pleiotropic genetic architecture underlying both behaviours (Lacasse and Aubin-Horth 2014). Our final question is, on a behavioural level, does an individual's aggression phenotype influence its sociability phenotype or vice versa? For example, one could imagine that, in order to display a highly aggressive phenotype, one needs to first be likely to group with others (i.e., be sociable). On the other hand, aggressive interactions may be more likely to disperse conspecifics from the aggressor, reducing the ability of those individuals to then engage in friendly interactions. This is a plausible explanation for the

correlated response to selection on sociability in aggression, as discussed in the previous section.

Aggression has been shown to have profound effects on subsequent social behaviour, through the establishment of dominance hierarchies among conspecifics (Chase and Seitz 2011). While the role, if any, of dominance hierarchies in not well understood in flies, it is still plausible that variation in prior aggression in a group has causal outcomes in subsequent sociability. For example, a low sociable arrangement of flies among several food patches may be the product of prior aggressive interactions by those flies in securing the patches for themselves. It has been previously shown that giving flies environments with distinct food patches can lead to the evolution of increased resource defence (Hoffmann 1988). It is possible that we have selected for flies with greater resource defense capabilities in the low sociability lineages by choosing those flies that had secured a food patch from other flies through aggression. Additionally, a high sociable arrangement may be facilitated by a lack of prior aggressive interactions among flies.

While intrasexual aggression and intersexual aggression (which here I call “sexual aggression”, e.g., forced copulation) are not the same, it is possible that they are correlated, given they share similar features. Both types of aggression use potentially damaging physical force to obtain mates or secure resources, and there is a similarity in the motivations in male-male aggression and male-female aggression: removing barriers to obtain matings. In male-male aggression, such barriers are in the form of male competitors, and in sexual aggression, the barrier is female unwillingness to mate. Such behavioural and genetic studies on these different forms of aggression, and sociability, can help elucidate the underlying relationship among these behaviours, and the evolutionary consequences of selection through correlated responses.

Work on the genetic basis of variation in sociability using the evolved lineages described in Chapter 4 was delayed as a result of the COVID-19 pandemic, and will be conducted by upcoming graduate students. However, in Chapter 5, I reported results on the genetic underpinnings of both evolved and plastic variation in sexual aggression, which was not delayed since extraction of genetic material was completed, and sequencing data

was received before the pandemic. Previous work from our lab has delineated the fitness effects and behavioural correlates of variation in male sexual aggression (forced copulation) via artificial selection (Dukas et al. 2020), and the work presented in Chapter 5 compliments it by adding information about the gene expression changes that underlie this variation, as well as plastic variation due to differences in social experience (Baxter and Dukas 2017). Interestingly, the gene expression changes that underlie these two types of behavioural variation appear to be overall quite different, potentially reflecting a difference in the mechanisms through which these changes in sexual aggression are achieved. For example, given the sets of significant genes in each experiment, plastic effects on forced copulation tendency may reflect changes in the underlying motivational state of males, while evolved differences in forced copulation tendency may reflect changes in physical ability, for example due to changes in neuromuscular-related gene expression (Iuso et al. 2014), as reported in Chapter 5. This lack of correlation in gene expression effects between plasticity and selection is at odds with the few recent studies that have found concordance in evolved vs. plastic comparisons of gene expression in animal behaviours (Alaux et al. 2009; Fraser et al. 2014), and gives us an opportunity to investigate this intriguing independence of mechanisms of variation in sexual aggression.

Given the negative correlation between sociability and intrasexual aggression reported in Chapter 4, and the fact that similar effects of the social environment are seen in both sociability (Chapter 3) and sexual aggression (Chapter 4, Baxter and Dukas 2017), the candidate genes and ontogenetic categories that we have identified in Chapter 5 may be a good starting point for upcoming work on the evolved gene expression effects in sociability. In particular, this will be useful to address questions related to the similarity in the genetic underpinnings of aggression and sociability.

6.4 Future directions

While the research presented here provides a good picture of the quantitative genetic underpinnings and the behavioural and fitness correlates of sociability, and the gene

expression effects of variation in sexual aggression due to evolution and plasticity, there are clearly a number of fruitful avenues to continue these lines of research.

First, while we identified the role of social interactions (i.e., encounters among conspecifics) in mediating indirect genetic effects (IGEs) on sociability (Chapter 2), it is unclear exactly how social encounters affect sociability. For example, fruit flies have been shown to produce and respond to pheromones and odourant cues that can increase social aggregation (Bartelt et al. 1985) and induce social avoidance (Suh et al. 2004), and these encounters may involve the exchange of similar pheromonal cues not detectable in our assay. Alternatively, since these social encounters tended to decrease the sociability of groups, it is possible that we have missed some subtle aggressive behaviours as part of these interactions, given the negatively correlated response in aggression to selection on sociability observed in Chapter 4. For example, encounters that involved flies coming together in close proximity, stopping, and one abruptly leaving while the other stands its ground may involve some type of subtle aggression, analogous to a non-physical "wing threat" (Certel and Kravitz 2012). Such interactions have been acknowledged as subtle aggression, though not quantified, in one study of female-female aggression in flies (Ueda and Kidokoro 2002). A closer analysis of the interactions, for example quantifying interactions that are qualitatively distinct (e.g., interactions that are more "mutual" with both flies in the interacting pair remaining together or leaving, versus more one-sided interactions as described above) may give us some better insight into the behavioural mechanisms at play in sociability IGEs. Such thorough analyses may be more feasible now with technological innovations in fly tracking software, and in automated behavioural observations using machine learning, which has recently been used to automatically annotate subtle fly behaviours, such as ovipositor extrusions and licking during courtship (Mezzera et al. 2020).

In Chapter 3, I reported the apparent independence of sociability between the sexes, revealed by the lack of a correlation between male and female sociability scores in the clonal lines. This points to an intriguing mechanistic difference in the determinants of male and female sociability. Even though males and females were tested under the same

environmental conditions, there still is a difference in the motivations for males and females to be sociable. For example, in males this may be driven by motivation to mate, and even though no females were present, other males could be an indicator of nearby females via local enhancement (Webster and Laland 2013). For females, this may be driven by motivation to find suitable feeding and egg laying sites, as female decision making is driven by future foraging costs for larval progeny (Schwartz et al. 2012), which can also be influenced by social cues of other egg-laying females (Sarin and Dukas 2009). For now, these interpretations are just speculation, and further experiments aimed at separating the underlying mechanistic and motivational differences in sociability between males and females will be required to elucidate this independence. In addition, in Chapter 3, I reported the effect of prior social environments (isolation versus socially enriched) on subsequent sociability, and how this effect varies among genotypes. Such effects of isolation on subsequent social behaviour and well-being in general, and the variation in susceptibility across individuals, is of high importance in current human research on loneliness and its biological determinants (Day et al. 2018; Bzdok and Dunbar 2020), especially as people face increased social isolation due to the COVID-19 pandemic. Analyzing the differential gene expression in flies with varying social experience faced with choices about joining others or not could give insight into the genetic architecture that underlies this social plasticity effect on sociability, and this could also be informative for understanding similar effects in humans through orthologous genetic mechanisms. While the genetic variation in this plasticity reported in Chapter 3 was not sufficient to confidently determine genetic variants associated with it, this variation is significant, and a future experiment with greater power (e.g., a larger sample of DGRP lines) should be able to identify potentially causal variants.

Analysis of the forthcoming genetic data obtained from sequencing evolved lineages of flies with diverged sociability (Chapter 4) is one of the most exciting and promising future directions of the work presented in this thesis. While we (Chapter 3; Anderson et al. 2016) and others (Saltz 2011; Saltz and Foley 2011) have quantified genetic variation in sociability, so far no studies have been able to link this variation to actual causal

genomic variants or differential gene expression, likely owing to the difficulty of performing such large scale assays or selection experiments on social behaviour, and the sensitivity of these behaviours to subtle environmental variation. These exciting prospects could also aid in current efforts in using fruit flies as a genetic model for better understanding atypical social behaviour in humans (Greenblatt and Spradling 2018; Yost et al. 2020), specifically in identifying any causal analogous candidate genes in flies that may have segregating variation present in natural populations. There is also forthcoming whole-genome sequencing data on the evolved forced copulation lineages (Dukas et al. 2020) that will complement the gene expression data presented in Chapter 5, as this will allow for the identification of the actual genomic signals of selection. Overall, extensions from the work presented here will result in promising novel genetic data for the underpinnings of variation in both sociability and sexual aggression arriving in the near future.

6.5 Conclusions

Taken together, the work presented here provides insight into the mechanisms, both behavioural and genetic, that give rise to variation in social behaviours. For sociability, we have identified quantitative genetic parameters, genetic correlations among the sexes and with other traits including activity, aggression, social aggregation, and directly fitness-relevant traits. For sexual aggression, we have identified and verified candidate genes that show differential expression due to both evolved and plastic changes in sexual aggression tendency. For each of these projects, there are several clear future directions, and for some, genetic work has already begun. Finally, the data presented here exemplify the versatility and practicality of *Drosophila melanogaster*, a “simple” model system, for understanding the behavioural and genetic underpinnings of complex social behaviours.

6.6 References

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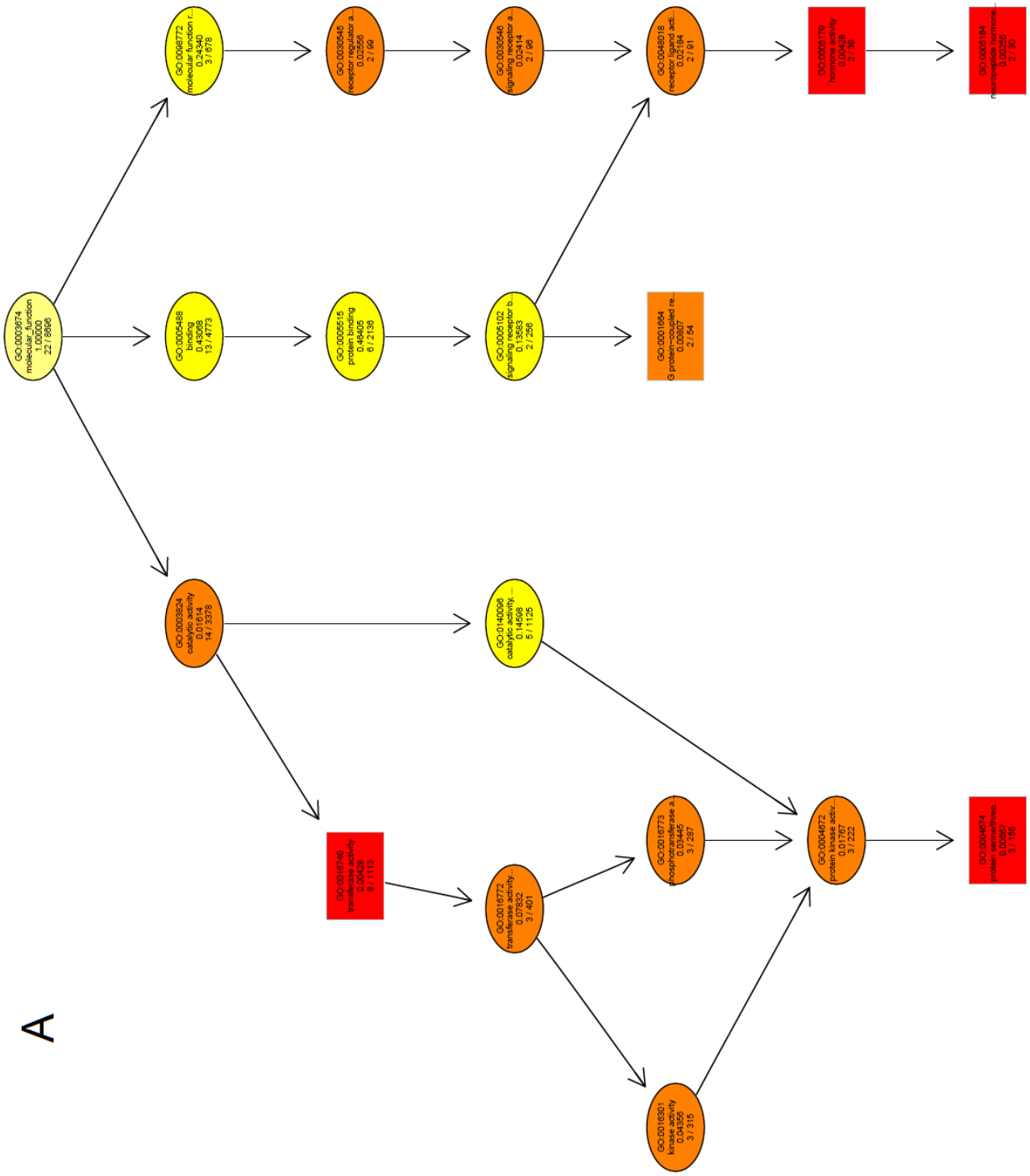
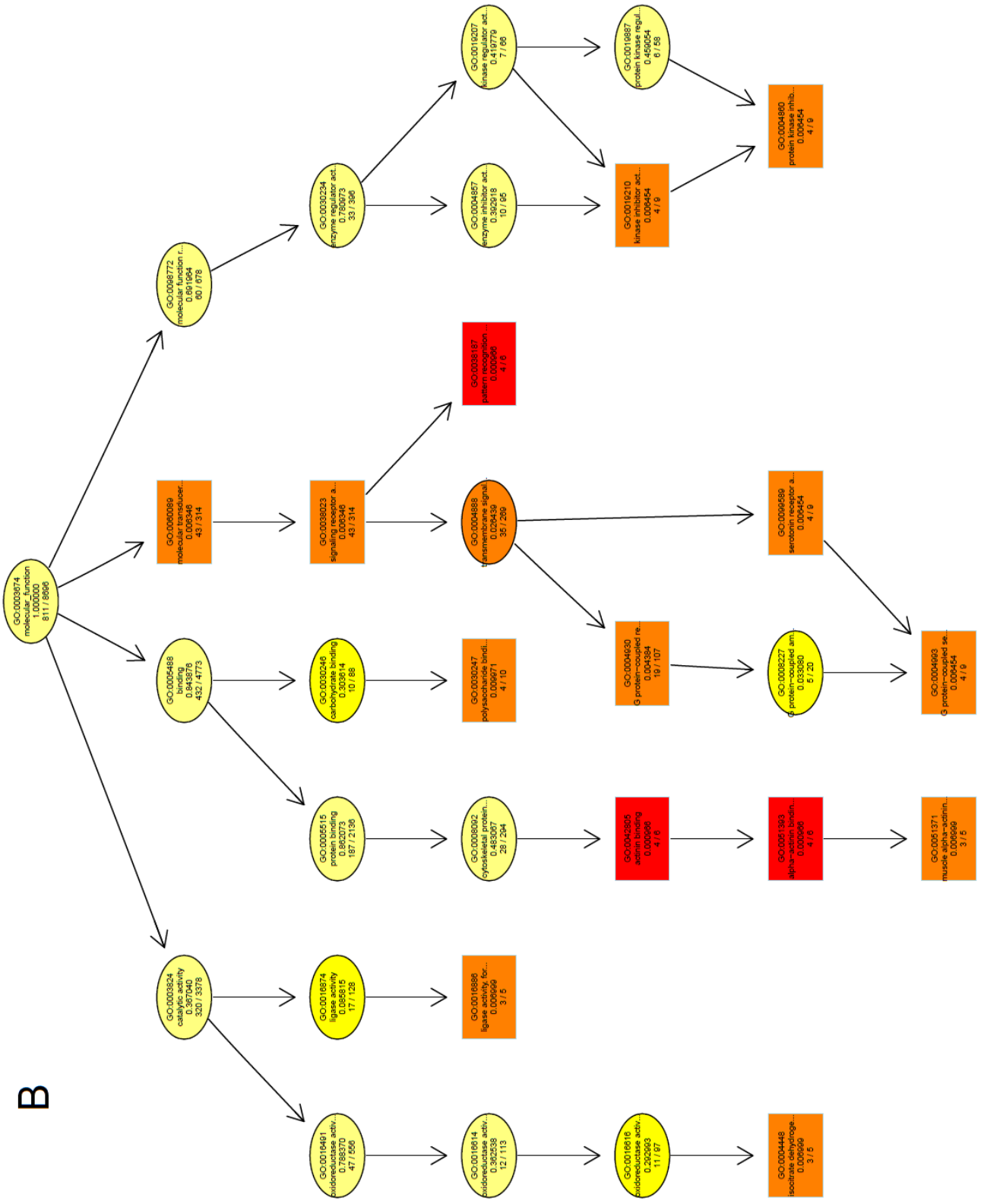
APPENDIX

Supplementary figures for Chapter 5

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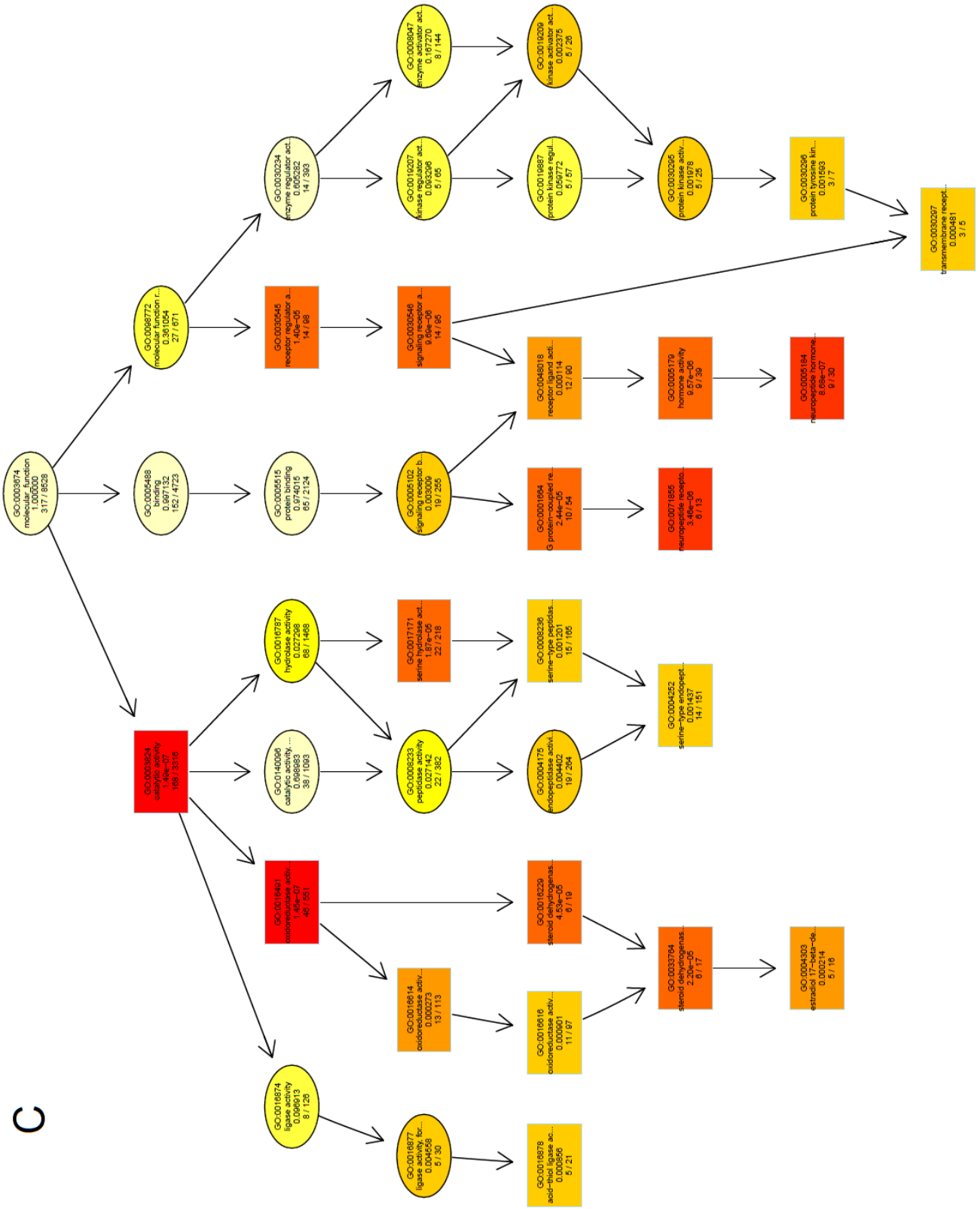
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Figure S5.1

Hierarchical gene ontology (GO) graphs showing parent GO terms at the top, connecting to child GO terms below, for each of A) significant DE genes in both the artificial selection and plasticity analyses, B) significant DE genes in only the artificial selection analysis and C) significant DE genes in only the plasticity analysis. Significance of the GO terms is represented by colour (yellow = larger p-value, to dark red = smaller p-value), and shape (squares = significantly enriched terms, circles = not significantly enriched terms). Inside each node, the text refers to GO identifier, GO term, p-value, and number of genes in the set enriched in that term out of the total number associated with that term.

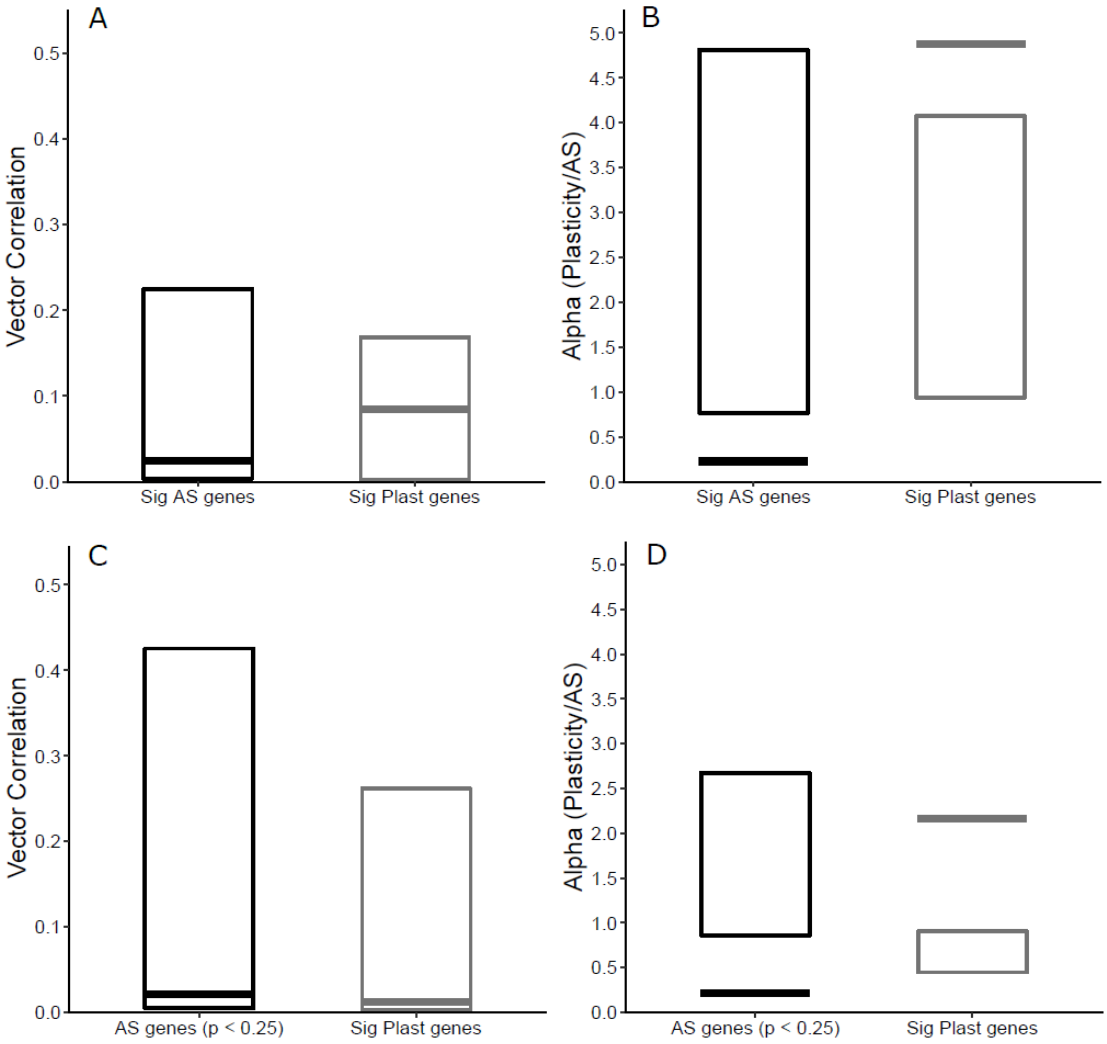

Figure S5.2.

Similarity in direction and magnitude of DE estimates between artificial selection and plasticity. Artificial selection estimates either shrunken using APEGLM, or obtained using the same software (edgeR) as the plasticity analysis from the artificial selection analysis. A), C) The observed vector correlation values (thick horizontal lines) between vectors of estimates (log2 fold changes) obtained from the artificial selection and plasticity analyses for 2 sets of genes: all the genes significant in artificial selection analysis and the corresponding estimates for those genes in the plasticity (black), and all the genes significant in the plasticity analysis and the corresponding estimates for those genes in the artificial selection (grey). B), D) The observed ratio of vector magnitudes (plasticity/artificial selection), or alphas, for the same vector comparisons. Rectangles represent 95% C.I.s generated from empirical resampling of estimates from all genes. Estimates for the artificial selection either A), B) shrunken using APEGLM, or C), D) obtained using edgeR.

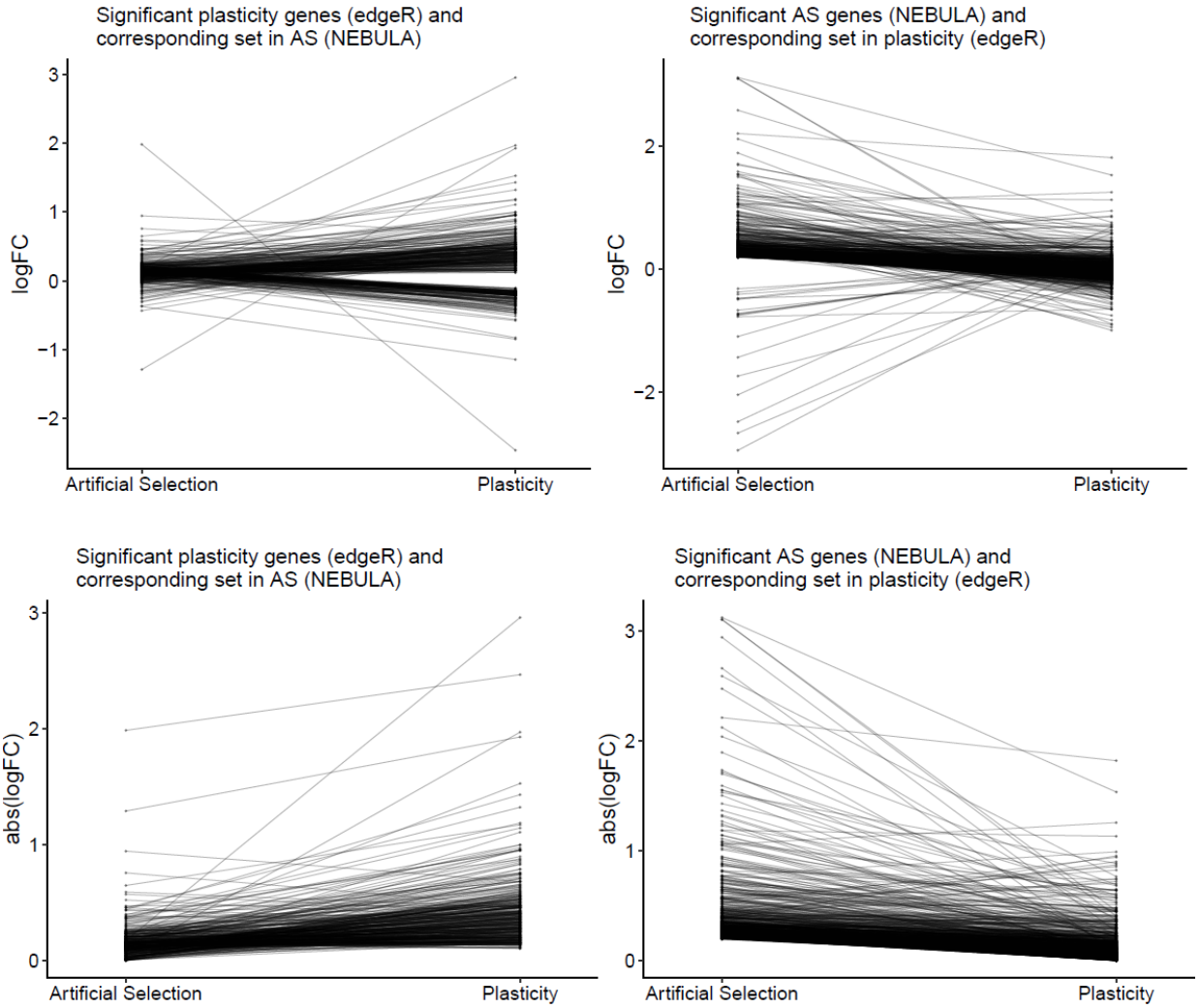

Figure S5.3

Similarity in direction and magnitude of DE estimates between artificial selection and plasticity. Reaction norm plots comparing the logFC (top row) or absolute logFC values (bottom row) from the artificial selection analysis to the plasticity analysis. Each black line represents a single gene. Two sets of genes are compared: the set of genes significant in the plasticity analysis (left column) and the set significant in the artificial selection analysis (right column).

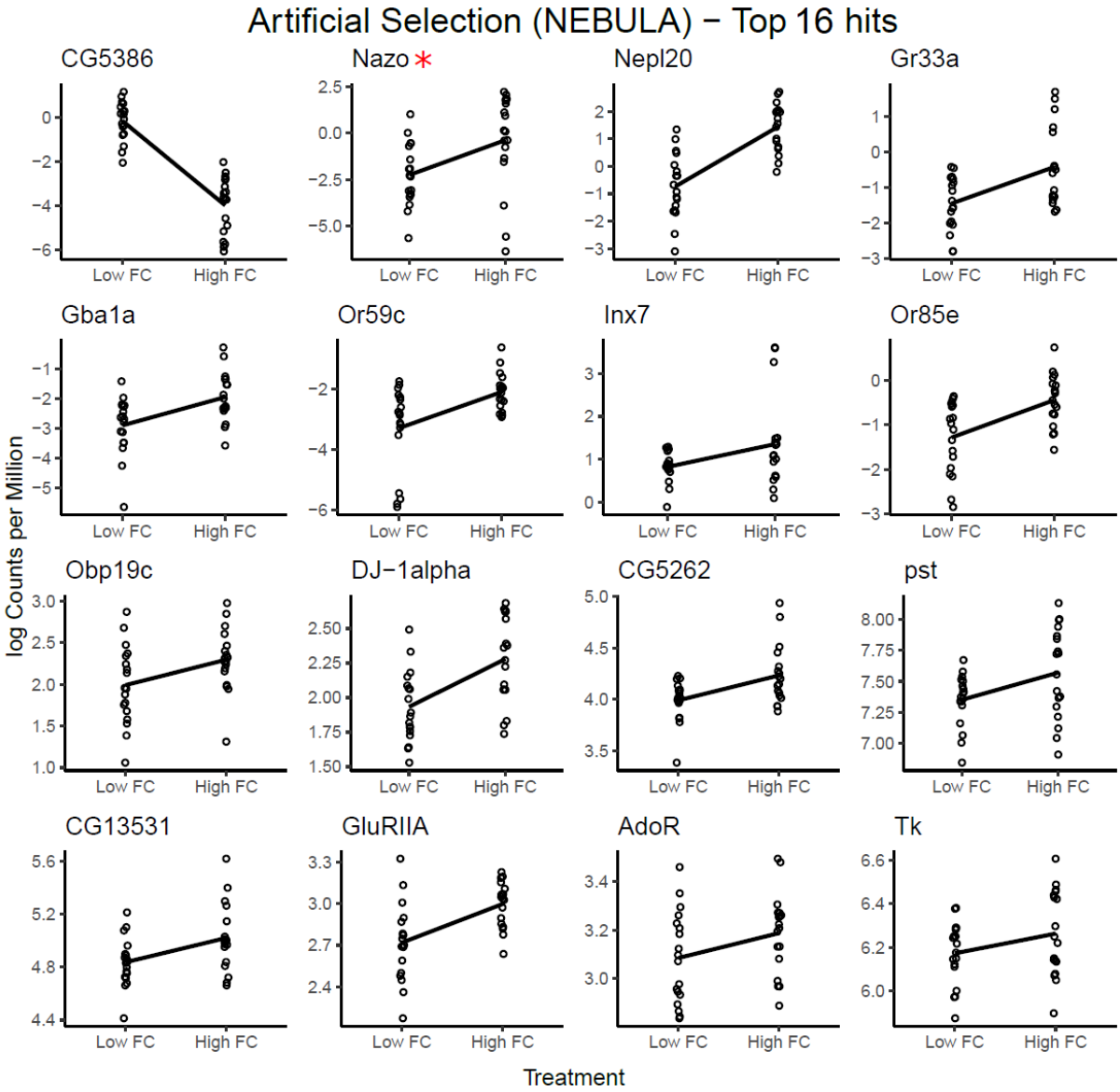

Figure S5.4

The top 16 artificial selection significant DE genes ordered left-right (top-bottom) by decreasing logFC values. *Nazo* (red asterisk) was chosen for further candidate validation (Fig. 5.6).

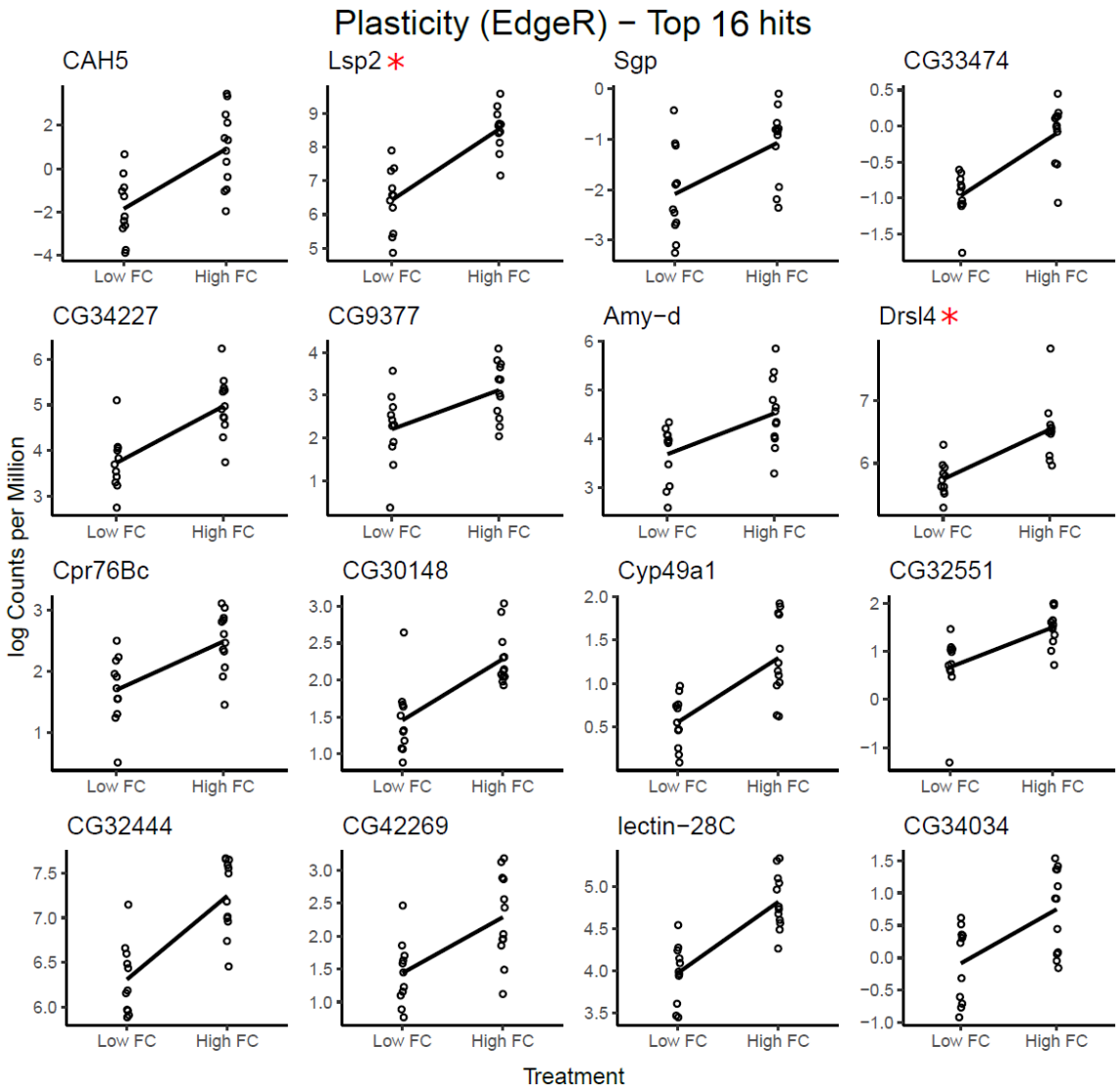

Figure S5.5

The top 16 significant plasticity DE genes ordered left-right (top-bottom) by decreasing logFC values. *Lsp2* (red asterisk) was chosen for further candidate validation (Fig. 5.6), and *Drsl4* (red asterisk) was also present in the overlap set significant in both the artificial selection and plasticity analyses (Fig. 5.4), and was also chosen for candidate validation (Fig. 5.6).