GENETIC VARIATION INFLUENCES FRUIT FLY SOCIAL BEHAVIOUR

GENETIC VARIATION AND ITS INFLUENCE

ON *DROSOPHILA* SOCIAL BEHAVIOUR

By

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Abstract

Social interactions can have profound influences on an individual's fitness. As part of a long-term research program on the mechanisms and functions of social behaviour in fruit flies (*Drosophila melanogaster*), we addressed two main questions. First, we asked whether social behaviour is positively correlated between the larval and adult stages. We quantified genetic variation in social behaviour by measuring aggregation among larvae and adults taken from each of 29 inbred, wild-derived lines. We found significant genetic variation in social behaviour in both larvae and adults. While these lines also showed significant genetic variation in baseline locomotor activity, it had no significant influence on social behaviour. We found that neither social behaviour nor activity were correlated between larval and adult flies. This is consistent with the hypothesis that metamorphosis adaptively decouples the expression of genes between distinct life stages in animals with complex life cycles. That is, genetic variation in social behaviour during each life stage may reflect adaptation to the specific ecological settings during that stage. Our second question was whether social behaviour in adult flies was influenced by indirect genetic effects (IGEs), defined as the effect an individual's genotype has on the phenotype of an interacting partner. IGEs can have profound effects on the rate of phenotypic evolution. We found that groups of 6 focal males maintained shorter inter-individual distances among themselves when interacting with 12 males from a line previously identified as highly social than when paired with 12 males from the least social genotype. Overall, our results indicate that heritable genetic variation influences an individual's predisposition to engage in social behaviour as well as its effect on social interactions among other individuals it encounters.

Acknowledgements

First and foremost, I would like to thank my supervisor, Reuven Dukas for all his guidance throughout my graduate school career, as well as the members of my supervisory committee, Paul Andrews and Ana Campos, for their input throughout this project. I would also like to thank my fellow lab members, past and present: Carling Baxter, Kevin Abbott, Andrew Scott, Andrew Nguyen, Shane Golden, Zachary Durisko, Constance Imbault, and everyone else who helped out along the way. Lastly, a special thank you to Mackenzie Churchill, and all of the other graduate students here at McMaster, the past two years have been an incredible experience, and wouldn't have been half as enjoyable if they weren't shared with you.

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General Introduction

When interacting with their environment, animals must constantly inform their behaviour using previously acquired information. Individuals often garner this information through trial-and-error based interactions, which presumably incur some cost to fitness, in terms of time and energy that could otherwise be allocated to growth or reproduction (Dall, Giraldeau, Olsson, McNamara, & Stephens, 2005). In the event that an individual's learned strategies are insufficient, they may alternatively inform their behaviour by utilizing socially available information in the form of either intentional cues provided by conspecifics or inadvertent signaling (Laland, 2004). The evolution of social learning is expected in animals with life history traits that would maximize its benefit, which includes species with overlapping generations, frequent social interactions, and parental care (Dukas, 2010).

The degree to which animals rely on social information is not consistent across species. Despite ample evidence of social learning, and even culture, amongst non-human primates (Whiten, Horner, & de Waal, 2005), some invertebrate species learn solely through individual experience in certain contexts (Lancet & Dukas, 2012). Despite this, insect species provide an ideal, yet idiosyncratic model for the study of social behaviour, as they provide, among taxa, some of the most numerous instances of both the evolution of (Choe & Crespi, 1997), and losses of social behaviour (Wcislo & Danforth, 1997). While studies of sociality in invertebrates have predominantly focused on colonial insects, such as bees or ants, these species represent a highly social minority of the insect class. A growing body of research has indicated that social learning drives behaviour related to

feeding, predator avoidance and reproduction in many non-colonial insect species (Dukas, 2010), though social behaviour can extend broadly to include attraction to conspecifics, as well as the social facilitation of behaviour (Zajonc, 1965).

While many studies quantify direct measures of behaviour (in terms of frequency and magnitude), the overarching theme of this work aimed to quantify the degree of gregariousness exhibited by groups of individuals based on their distribution within constrained regions of space. The existence of group living is considered a fundamental driving force for the evolution of social behaviour (Alexander, 1974), as denser groups would likely engage in more frequent interactions, increasing opportunities to learn from social cues. There is evidence that animals of a variety of species adaptively modulate their group size to achieve an optimal density, which can be contingent on resource or mate availability (Orsdol, Hanby, & Bygott, 1985; Despland, Collett, & Simpson, 2000).

Recently, there has been an increased interest in developing simple, tractable model systems within which to study the neurogenetic basis of social behaviour. The fruit fly (*Drosophila melanogaster*) is chief among such efforts, which, due to its simple nervous system, tractability as a lab organism, and long standing availability of genetic tools, allows complex behaviours to be studied at the level of individual genes and neurons.

Chapter 1: Correlation in Behaviour between Life Stages

<u>Introduction</u>

Social Behaviour in Larvae

Fruit fly larvae, much like their adult counterparts, are highly amenable to genetic manipulation, yet possess a much simpler nervous system and behavioural repertoire. Additionally, fruit fly larvae are known to exhibit a broad array of social behaviours. Larvae have been shown to exhibit attraction to conspecifics, and bias their food preference towards foods previously experienced in the presence of foraging conspecifics over those experienced alone (Durisko & Dukas, 2013). The attraction of individuals towards groups of foraging larvae has been suggested to be caused by a number of cues, primarily, to volatiles produced by the *Drosophila* commensal bacteria *Lactobacillus* (*L. brevis and L. plantarum*; Venu, Durisko, Xu, & Dukas, 2014). Additionally, the larval fatty acids (Z)-5-tetradecenoic acid and (Z)-7-tetradecenoic acid have been shown to act as an attractive cue to other foraging conspecifics. Silencing either R58F10 neurons or the expression of *pickpocket23* (*ppk23*) and *pickpocket29* ion channels effective blocks response to these molecules, and reduces the strength of social attraction exhibited by the larvae (Mast, De Moraes, Alborn, Lavis, & Stern, 2014).

The propensity for groups of larvae to aggregate is prone to change throughout development, peaking during the late 2nd and early 3rd instars (approximately 50-60 hours post hatch; Durisko, Kemp, Mubasher, & Dukas, 2014), a time point which corresponds with peak burrowing behaviour. From an ecological standpoint, burrowing may serve several purposes, including the maintenance of regular body temperatures and sufficiently

high environmental humidity (Gibbs, Perkins, & Markow, 2003). Additionally, burrowing may aid in the avoidance of larval parasitoid species that rely on ovipositor searching strategies (repeated probing of substrate) rather than vibrotactic cues (Carton & David, 1985; Carton & Sokolowski, 1992). Parasitoids can act as a major selective force upon their *Drosophila* hosts; field studies have suggested that larval parasitism rates can approach 100% in natural populations, with survivorship as low as 6.5% in vulnerable *D. melanogaster* populations (Carton, 1986). Given as little as 5 generations of artificial selection for parasitoid resistance, a tenfold increase in parasitoid encapsulation (and subsequent survival) has been observed (Kraaijeveld & Godfray, 1997). Though this comes at the cost of increased mortality under competition due to reallocation of resources towards hematocyte production (Kraaijeveld, Limentani, & Godfray, 2001).

Larger groups of foraging larvae have been shown to actively condition aspects of their microenvironment, both inhibiting the growth of moulds (Rohlfs, 2005) and regulating the density and diversity of yeast species growing in their microenvironment (Stamps, Yang, Morales, & Boundy-Mills, 2012). The influence of larvae on their microenvironment is suggested to underlie an Allee effect in *Drosophila*, whereby egg to adult survival increases with population density to a point, beyond which costs associated with larval crowding become too high (Rohlfs & Hoffmeister, 2003). Under these conditions of high density and low resource availability, there is an increased risk of larval cannibalism (Vijendravarma, Narasimha, & Kawecki, 2013), as well as a temporal accumulation of toxic nitrogenous waste products such as ammonia (NH_4) and urea (Borash, Gibbs, Joshi, & Mueller, 1998). Consequently, even intermediate larval density (4 eggs per mL of

standard media) is associated with measureable declines in egg to adult survival, and eclosing adult body mass (Durisko & Dukas, 2013). Following several generations of selection under conditions where environmental NH_4 is present at high concentrations, larval populations can evolve heightened tolerance for toxic metabolites, exhibiting significantly increased fitness under crowded conditions (Borash, Teotonio, Rose, & Mueller, 2000). Despite the presence of numerous fitness tradeoffs associated with forming larval aggregations, the overwhelming preference of females to oviposit on mediums occupied by conspecific eggs and larvae (del Solar & Palomino, 1966; Durisko, Anderson, & Dukas, 2014) suggests that, over evolutionary time, group living has been advantageous for larvae. Furthermore, the degree to which *D. pseudoobscura* aggregate their eggs has been shown to change with successive generations of artificial selection, suggesting a genetic basis (Del Solar, 1968), which may also influence similar social behaviours exhibited by larvae.

Since social interactions in larvae inherently take place upon food substrates, it is possible that genetic variation in physiological processes related to foraging and metabolism may subsequently influence choice of social environment (Dall, Houston, & McNamara, 2004). Variation in the *foraging* (*for*) gene, a naturally occurring polymorphism related to food governed locomotion in larvae (Sokolowski, 1980; de Belle & Sokolowski, 1987), has been shown to also influence the use of socially available information in adult flies when engaging in a spatial navigation task (Foucaud, Philippe, Moreno, & Mery, 2013), though the contribution of the *for* gene to larval social behaviour remains unclear. Given that continuous genetic variation explains a large degree of the

differences in larval foraging behaviour (Reed et al., 2010), it is likely larval social behaviour exhibits a similar pattern of polygenic inheritance.

Social Behaviour in Adult Flies

As adults, social groups are formed through strong social attraction of individuals towards the combined presence of food and the male pheromone cis-Vaccenyl Acetate (cVA; Bartelt, Schaner, & Jackson, 1985; Brieger & Butterworth, 1970). Similar to their larval counterparts, volatiles emitted by commensal *lactobacillus* species have also been shown to facilitate attraction of adults towards sites occupied by larvae (Venu et al., 2014). Fruit flies are known to exhibit social learning in several contexts, including female mate choice (Mery et al., 2009), oviposition site choice (Sarin & Dukas, 2009; Durisko, Anderson, et al., 2014; Battesti, Moreno, Joly, & Mery, 2012), and aggressive outcomes in males (Yurkovic, Wang, Basu, & Kravitz, 2006). Aggressive behaviour in fruit flies is predominantly studied in males, where conflict occurs over food patches, often in the presence of females, which may provide potential mating opportunities (Hoffmann, 1987). Female aggression has been shown to emerge through competition over access to a limited resource (yeast paste), though female aggressive bouts are less frequent and utilize a behavioural repertoire distinct from that of males (Nilsen, Chan, Huber, & Kravitz, 2004). Presumably, aggression in fruit flies is associated with a substantial energetic cost, in addition to any potential risks. For these reasons aggressive interactions are less frequent in the absence of females, upon poor quality food sites, and within territories that are too large to easily monopolize (Hoffmann & Cacoyianni, 1990).

Within constrained regions of space, interactions between individual flies have been demonstrated to be significantly non-random, with groups of individuals engaging in distinct approach behaviours and social grooming (Schneider, Dickinson, & Levine, 2012). These grooming behaviours consist of physical contact between the forelegs of one fly and its interacting partner, which can allow for the exchange of relevant information through the gustatory receptors present on the forelegs. This information can be used to guide male mate choice (Everaerts, Lacaille, & Ferveur, 2010), as well as food preference. When naïve focal flies are left to interact with demonstrator flies trained to prefer a specific type of food, the change in focal food preference is proportional to the frequency of their interactions with the demonstrators in the time leading up to testing (Battesti et al., 2015).

Dense aggregations upon a food site likely represent an initial perception of quality by founding individuals, as adults are sensitive to sucrose and yeast concentrations in their food (Edgecomb, Harth, & Schneiderman, 1994; Golden & Dukas, 2014). In nature, there is typically an upper limit to the degree of aggregation that may occur on a single patch, as physical interaction between flies does elicit a dispersal response among adults (Wertheim, Allemand, Vet, & Dicke, 2006; Ramdya et al., 2015; Etienne, Wertheim, Hemerik, Schneider, & Powell, 2002). It is apparent that social groups of adult flies are maintained by a delicate balance of attractive and repulsive cues, where the optima may reflect current mating status, environmental experience, dominance, or food availability. Naturally occurring genetic variation has been shown to influence traits relating to aggression (Edwards et al., 2009), dispersal (Edelsparre, Vesterberg, Lim, Anwari, & Fitzpatrick, 2014), and resistance to environmental stressors (Hoang, 2001), all of which should

influence the fitness tradeoffs present when living in a social group. Adaptation to environmental factors and subsequent changes in these traits may also influence an individual's tendency to aggregate upon food substrates. Despite a growing body of research assessing how sensory systems and neuronal signaling influence social behaviour (Simon et al., 2012; Schneider et al., 2012; Burg, Langan, & Nash, 2013), few studies have examined the role of naturally occurring genetic variation on fruit fly social behaviour. Perhaps the best example of this demonstrated that focal males from different wild type backgrounds showed significantly different propensities to associate with food patches occupied by conspecifics rather than empty sites (Saltz, 2011).

Correlation between Life Stages

In addition to providing an excellent model system with which to quantify social behaviour in both the adult and larval life stages, fruit flies allow us to easily examine the degree of correlation in social behaviour throughout a complex life cycle. Complex life cycles comprise of those in which the life history of an individual can be separated into two or more stages separated by ontogenetic changes in morphology, physiology and behaviour (Wilbur, 1980). These stages allow individuals to occupy distinct ecological niches, whereby abundance is limited by different factors at each stage. Often these stages serve specialized purposes which may include feeding, growth, dispersal, and/or reproduction. The maintenance of distinct life stages is likely favorable, as it allows for differential activation of genes during each stage (Haldane, 1932; Moran, 1994). It has been implied that adaptive decoupling of genes may be evolutionarily unstable, as it would allow the most productive stage to be over-expressed (Istock, 1967), however, more recent accounts

suggest that these stages may be maintained under balancing selection (Ebenman, 1992); should one life stage dominate over the other, an abundance of resources will accumulate in the neglected niche, increasing the potential fitness of the under-expressed life stage.

Studies on physiological traits, or single genes have offered contradicting evidence of this adaptive decoupling hypothesis. For example, expression of the immune gene *Diptericin* was found to positively correlate between larval and adult fruit flies (Fellous & Lazzaro, 2011), while artificial selection of heat shock resistance in *D. buzzatii* was found to evolve separately for adults and larvae (Loeschcke & Krebs, 1996). Using gene expression profiling, studies of marine tunicates (*Ciona intestinalis*) have suggested that 12.5% of genes are expressed at consistent levels throughout the entire life span, with an additional 20% of genes showing similar expression between just the larval and adult stages (Azumi et al., 2007). The extent to which adaptive decoupling influences behaviour is unclear however. It is possible that the complex genetic architecture underlying behaviour may simply be resistant to decoupling, since an initial disruption of genes underlying behaviour may be maladaptive to the life stage during which it contributes positively to fitness (Aguirre, Blows, & Marshall, 2014; Arnold, 1990). A second hypothesis is that it may actually be adaptive to retain similar behavioural phenotypes following metamorphosis. This is particularly relevant assuming environments and related selection pressures and remain similar across life stages. For example, if an environment's larval density is indicative of the subsequent adult densities, then one might expected a positive correlation in genes mediating behaviours that are density dependent. While physiological measures provide mixed evidence for the adaptive decoupling hypothesis, limited

behavioural evidence in fruit flies suggests behaviour may be positively correlated. The presence of the *for^R* allele (rover) is associated with increased foraging related movement as larvae, as well as higher dispersal rates as adults (Edelsparre et al., 2014). The two *for* alleles are maintained by negative frequency dependent selection (Fitzpatrick, Feder, Rowe, & Sokolowski, 2007), where both larval morphs experience greater fitness when rare. These alleles do not exist at equal frequencies however, as rovers show greater survivorship than sitters (*for^S*) under crowded conditions (Sokolowski, Pereira, & Hughes, 1997).

Rationale

Given the degree of environmental variance encountered across the cosmopolitan range of *D. melanogaster*, it is apparent that there should be observed differences in the propensity for flies to aggregate, which may represent adaptation to a number of ecological factors, such as predation, host plant species, and density. To investigate the influence of continuous genetic variation on fruit fly social behaviour, we modified existing protocols (Durisko, Kemp, et al., 2014; Simon et al., 2012) to quantify social behaviour based on the distribution of individuals in 2-dimensional space. We performed assays with 29 isofemale lines belonging to the *Drosophila melanogaster* Genetic Reference Panel (DGRP; Mackay et al., 2012). In addition to being inbred to near homozygosity, these lines are fully sequenced, providing a strong foundation for studying the genetic architecture underlying social behaviour. We predicted that a significant degree of observed variation in social behaviour, both as adults and larvae, could be attributed to genetic differences between these lines. To verify that social behaviour was not driven solely by differences in baseline

levels of activity, which may influence encounter rate between individuals and dispersal tendencies, we assayed individuals from each line for locomotor behaviour as well. This provided us with a second measure with which to investigate whether behavioural traits are genetically correlated, or decoupled between stages. Due to recent evidence that the endosymbiotic bacterium *Wolbachia*, which infects approximately half of all DGRP lines (Huang et al., 2014), reduces aggressive behaviour in male flies (Rohrscheib et al., 2015), we examined whether our *Wolbachia* infected lines exhibited behavioural differences in terms of both social behaviour and activity.

General Methods

We maintained 29 DGRP lines at low densities in vials containing 5 ml of standard food (one liter of which contained 90 g sucrose, 32 g yeast, 75 g cornmeal, 16 g agar, and 2 g methyl paraben) at 25°C and 60% relative humidity on a 12:12 light cycle with lights off at 10 pm. The DGRP flies are isofemale lines established from wild-type gravid females from a Raleigh, North Caroline farmer's market in 2003, and inbred by 20 generations of full sibling mating, followed by random mating (Mackay et al., 2012). We conducted egg laying for experimental adults in vials containing standard food and a sprinkle of live yeast. We removed excess eggs from the surface of all egg laying vials to maintain similar densities across all vials.

All video analysis was conducted in Python 2.7 (Python Software Foundation, 2015) using OpenCV 2.4.11 (Open Source Computer Vision Library, 2015). We analyzed all data in R (Version 3.2.1; R Core Team, 2014) using general linear mixed-effect models (R-package lme4; Bates, Maechler, Bolker, & Walker, 2015) and parametric bootstrapping

(with 2000 iterations) to test all relevant random effects (R-package pbkrtest; Halekoh & Højsgaard, 2014). We report Wald χ^2 for all fixed effects, and bootstrapped P values for all tested random effects. Linear regressions were performed to test for correlations between all measures. The *Wolbachia* infection status of our lines was taken from Huang et al. (2014; supplementary material), who had found that 12 of the 29 lines we tested carried the bacterium.

Larval Behaviour

Experiment 1.1: Larval Social Behaviour

Methods

For our larval experiments, we reared parental adult flies on an altered light cycle with lights off at 1 pm, which placed peak egg laying at midday. We collected eggs for experimental larvae in food vials without added live yeast between 1 pm and 3 pm to minimize any hatching asynchronies. The day after collecting eggs for experimental larvae, at 1 pm, we transferred groups of 12 recently hatched larvae from each line to the center of 35mm petri dishes containing standard food, colored blue to improve visibility. We stored dishes in complete darkness at 25°C and high (>75%) humidity for the duration of the experiment. As a compromise between reducing temporal variation and potentially interfering with larval behaviour, we observed the larvae under red light at two time points corresponding to the late 2nd instar (44 and 52 hours post hatching).

During observations, we overlaid a transparent 0.1 cm^2 grid across the top of the dish, and marked the locations of the larvae on 1:1 scale grid paper. We then scanned the observation sheets, and obtained the Cartesian coordinates of larvae using ImageJ

(Schneider, Rasband, & Eliceiri, 2012). We used the distances between larvae to calculate a nearest neighbor index for each dish. The nearest neighbor index is a measure of distribution in space, defined by the ratio between mean observed nearest neighbor distance and that expected by random chance at the given density. Nearest neighbor indices range from 0, where all points occupy the same region in space, to 2.1491, representing a perfectly uniform distribution (Clark & Evans 1954; Krebs, 1999).

Preliminary analyses indicated that nearest neighbor indices were normally distributed. With a few exceptions owing to insufficient number of hatchlings, we tested 30 dishes of larvae, up to 15 per day, from each genotype. We excluded from our analysis dishes in which we found fewer than 8 larvae during either of our observations due to either mortality, escape from the dish, or burrowing. In total we tested 851 groups of larvae across 10 days. Our model included nearest neighbor index as a dependent measure, *Wolbachia* infection status as a fixed effect, and genotype, day, and dish as random effects, with time of observation as a repeated measure.

To assess the repeatability of our protocol in quantifying social behaviour, we performed a second test on larvae from the 6 genotypes observed to have the lowest mean nearest neighbor indices and the six genotypes with the highest mean nearest neighbor indices during our first screening. Using larvae reared from a subsequent generation of adults, we tested up to 25 dishes of from each of the 12 lines, for a total of 281 dishes over 2 days. We compared the nearest neighbor indices of these lines across screenings using a general linear mixed effects model, which included group (high or low social) as a fixed effect, genotype, day and dish as random effects, and time of observation as a repeated

measure. We predicted that larvae from the 6 lines previously identified as being more social during the first screening would once again show lower mean nearest neighbor indices than the 6 lines previously identified as being less social.

Results

Larval nearest neighbor indices varied significantly by larval genotype (range of mean nearest neighbor indices: 0.88 to 1.20; P<0.001; Fig. 1) with no significant effect of time of observation (Wald χ^2_1=1.88, P=0.17), nor *Wolbachia* infection status (Wald χ^2_1=0.01, P=0.92). When comparing nearest neighbor indices between the first and second screenings, the 6 lines initially identified as more social once again had significantly lower nearest neighbor indices than the 6 lines initially identified as less social (Wald χ^2_1=14.26, p<0.001; Fig. 2). When pooling the data obtained from the first and second screenings, the range of mean nearest neighbor indices was reduced due to an observed regression towards the mean, though the significance of genotype (P<0.001) and all other factors remained unchanged.
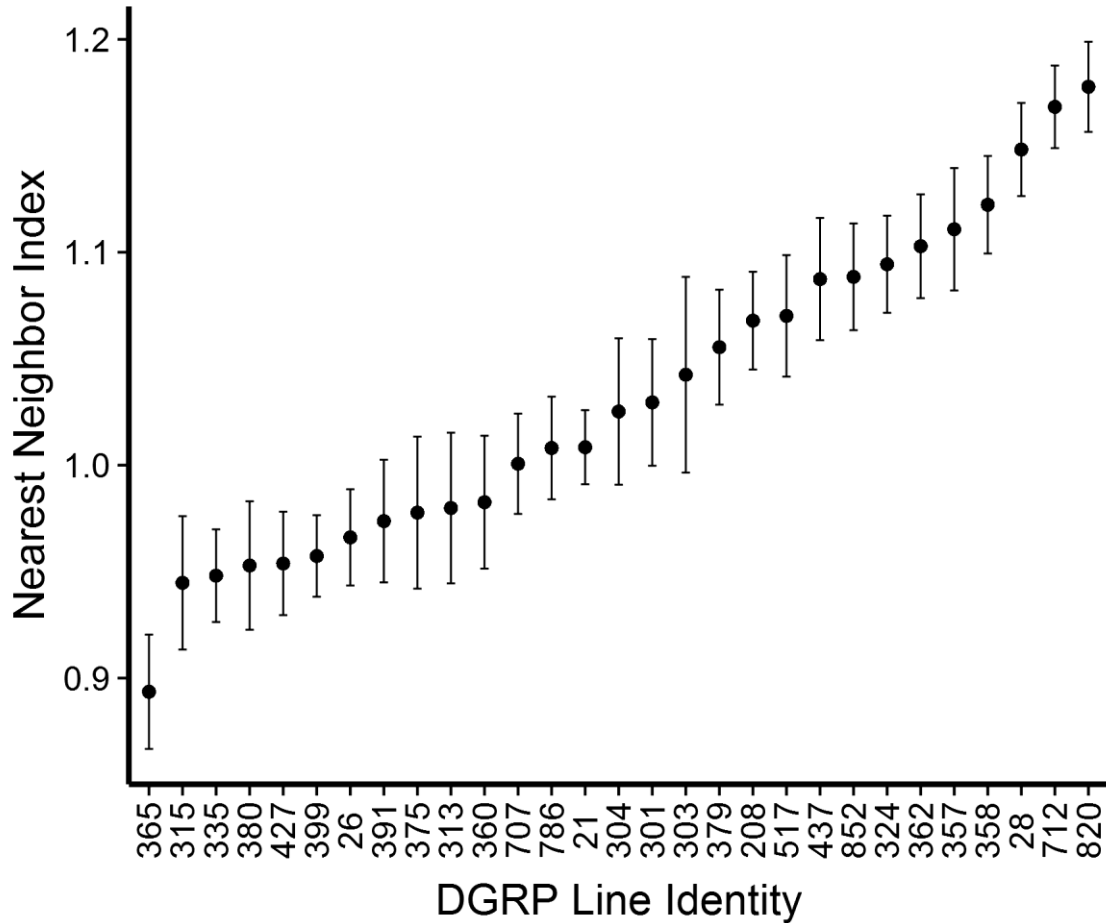
Figure 1: Mean ± SE nearest neighbor indices for groups of larvae from the 29 DGRP lines assayed. For the 12 lines assayed in both the first and second screenings, the data was pooled together.
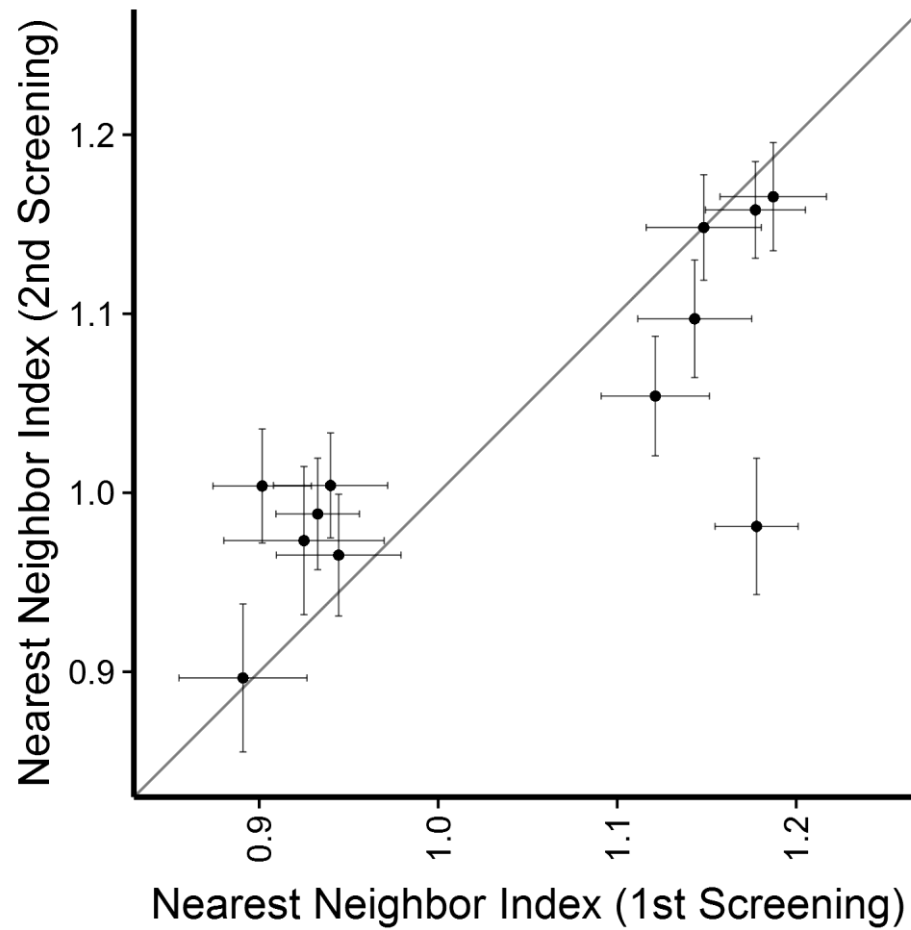
Figure 2: Correlation between nearest neighbor indices obtained during our first and second screening for the 12 DGRP lines tested in both. Error bars represent ± SE.

Experiment 1.2: Larval Activity

Methods

At 1 pm on the day following egg laying, we transferred groups of 20 recently hatched larvae from each of the 29 DGRP lines to 35 mm petri dishes (containing 8 ml of standard food) to maintain equal densities. We maintained experimental larvae at high (75%) humidity, 25°C, and on a 12 h photoperiod (with lights on at 1 am) until observations. We conducted all observations between 8:30 am (approximately 44 hours post-hatch) and 12:30 pm, during which we transferred single larvae into 35 mm petri dishes containing standard food (colored black to improve visibility), placing up to 10 dishes at a time into one of two boxes (53 cm x 30 cm x 30 cm; length x width x height) for observations. The boxes were uniformly illuminated by two 3 W LED bulbs suspended above on opposite sides of the box lid (35.5 cm apart). Within 5 minutes of being placed in the boxes, we filmed the larvae for 10 minutes using a high resolution webcam through a hole in the center of the box lid. This 10 minute duration was sufficient to capture movement, but short enough that burrowing behaviour did not become apparent.

We integrated video frames over 0.5 second time windows to reduce pixel variation prior to analysis, which consisted of calculating the centroid coordinates of larvae at each time point, and partitioning the cumulative distance travelled by each larva into 2 minute bins. To reduce noise, movement occurrences were only scored if the larvae had moved more than 0.5 mm from its previously recorded location. All automated analyses were verified in real time by blind observers. We omitted data from 24 larvae which burrowed

into the substrate during the 10 minutes of observation. In total, data was collected from 497 larvae over a span of 2 days.

We analyzed the data using a general linear mixed effect model, including *Wolbachia* infection status and day as fixed effects, genotype, day, dish, time of day, and box as random effects, and elapsed time as a repeated measure. The contributions of genotype and time of day were evaluated using parametric bootstrapping.

Results

A significant proportion of the variation in larval path length was explained by genotype (P<0.001; Fig 3). Path length was found to exhibit a very weak, but significant decline over the 10 minutes individuals were tested (Wald χ^2_1=7.80, P<0.01). Additionally, activity appeared to increase steadily across the four hours of data recording, peaking before the start of the night cycle (P<0.001). There was no effect of *Wolbachia* infection status on larval activity (Wald χ^2_1=0.01, P=0.76), though there was a significant effect of day (Wald χ^2_1=4.93, P<0.05).

When comparing measures of larval activity and social behaviour between the 29 lines tested, we found no significant correlation between the two ($F_{1,27}$=2.18, P=0.15; Fig 4).
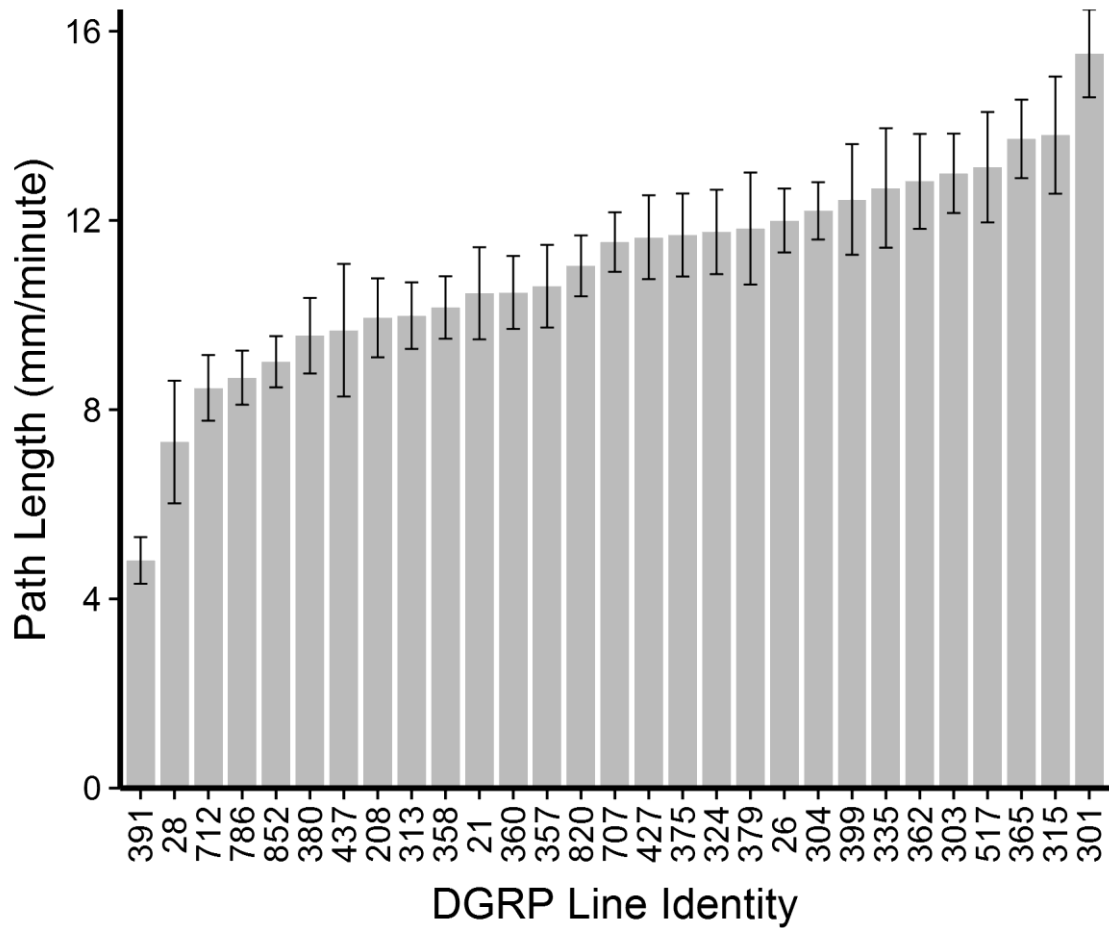
Figure 3: Mean ± SE movement rate of larvae obtained over 10 minutes of observation amongst the 29 DGRP lines tested, ordered in terms of increasing values.
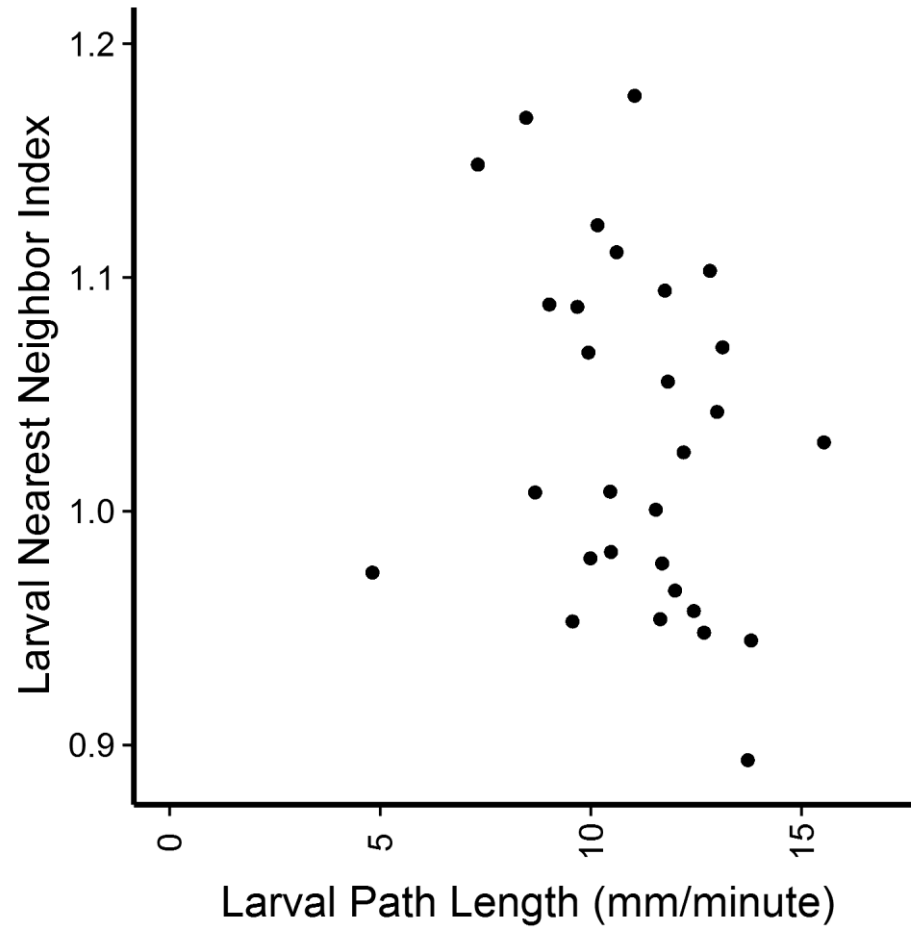
Figure 4: Comparison between observed measures of larval activity and social behaviour for 29 DGRP lines tested.

Adult Behaviour

Experiment 2.1: Adult Social Behaviour

Methods

We collected experimental adults within 6 hours of eclosing, sexed them under light CO_2 anesthetic, and placed them into mixed sex vials containing 14 males and 14 females. Approximately 70 hours post eclosion, at 9 am, we briefly anesthetized experimental adults and placed groups of either 12 males or 12 females, inside 35 mm experimental petri dishes. These dishes contained 8 ml of standard food, with corn meal omitted to reduce the heterogeneity of the food surface texture. The volume of food in each dish was sufficient to minimize headspace, effectively constraining flies to 2 dimensions. The flies were left to acclimatize for 5 hours, after which we placed up to 10 dishes into each of 6 boxes for observations (see experiment 1.2). Flies were allowed to acclimatize for 30 minutes after being placed inside the box, before being filmed for 30 minutes. All observations were taken during one of two test sessions, beginning at either 3:00 or 3:30 pm, with no more than a single group of males and females from a single line being tested on the same day. In total, we collected data from 823 dishes of flies, observed across 26 days.

Video analysis consisted of sampling single frames at 30 second intervals, determining the Cartesian coordinates of each fly's centroid, and calculating nearest neighbor distances. Since the number of visible individuals was always 12, we used the median nearest neighbor distance as a measure of spatial distribution rather than computing a nearest neighbor index, as correcting for number of individuals was unnecessary. This measure was found to be less variable over short time scales than the nearest neighbor index

previously used, since average measurements were strongly skewed mobile outlying individuals. All automated video analysis was manually verified, and in the case of errors, corrected by observers blind with regards to DGRP genotype. Because nearest neighbor distances were measured from the centroid rather than periphery, we applied a correction to account for body size differences between the sexes. To do so, we measured the length (anterior antennae to posterior abdomen) and width (width of thorax) of 25 males and 25 females, and used these measurements to calculate the diameter of an equivalent area circle (1.63 and 1.85 mm for males and females, respectively), which was subtracted from all nearest neighbor distances.

Nearest neighbor distances were square root transformed to eliminate positive skew. We analyzed the data using a general linear mixed effects model with sex, time, *Wolbachia* infection status, and test session as fixed effects. We included day, box, and dish as simple scalar random effects. A random effect of sex, varied by genotype, was included in our full model. We then constructed two nested models in which this term was either reduced to a simple scalar random effect of genotype, or omitted entirely. These nested models were sequentially compared using parametric bootstrapping to test both the significance of the genotype by sex interaction, and the main effect of genotype.

Results

Median nearest neighbor distances showed significant variation with respect to genotype (P<0.001), as well as a significant genotype by sex interaction (P<0.001; Figure 1). There was no significant main effect of sex (Wald χ_1^2=2.91, P=0.09), nor were there differences between test sessions (Wald χ_1^2=0.49, P=0.48), nor changes over time (Wald χ_1^2=0.59,

P=0.44). There was a marginal effect of *Wolbachia* infection status (Wald χ^2_1=3.64, P=0.06), with infected lines showing shorter nearest neighbor distances than uninfected lines.
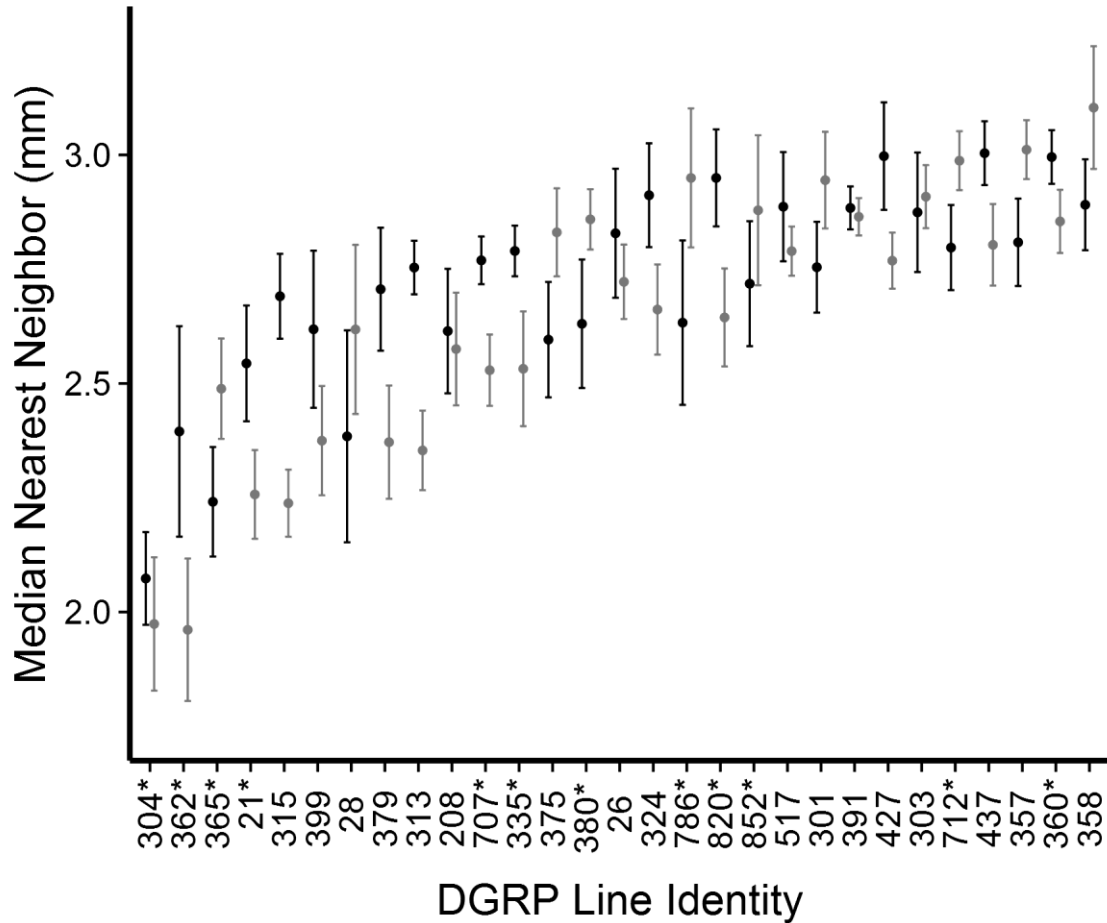
Figure 5: Mean ± SE median nearest neighbor distance for male (black) and female (grey) flies from 29 DGRP lines assayed, arranged by increasing line averages. *Wolbachia* infected lines are denoted with an asterisk (*).

Experiment 2.2: Adult Activity

Methods

Concurrent with our observations of adult social behaviour, we assayed the 29 DGRP lines

for levels of activity, quantified as the walking path length of isolated flies. We transferred

flies individually into experimental petri dishes. Four hours later, we placed up to 10 dishes

in each of 6 boxes and allowed flies to acclimatize for 30 minutes before video recording

them for 30 minutes. All observations were taken starting at either 1:30 or 2:00 pm, with a

maximum of a single male and female from each line tested on the same day. Video analysis

was identical to the previous experiment, with the exception that data was partitioned into

5 minute bins to reduce the occurrence of zeros in the data set caused by minimally active

flies.

Cumulative distance measures were cube root transformed to eliminate substantial

positive skew. We analyzed the data using a general linear mixed effects model with sex,

time, *Wolbachia* infection status, and test session as fixed effects. As simple, scalar random

effects, we included day, box, and dish. Similar to the analysis of adult social behaviour, a

random effect of sex varied by genotype was included in our full model, which was

compared to two nested models in which this term was omitted or reduced to test for the

effect of genotype and its interaction with sex, respectively.

Results

We found a significant main effect of sex (Wald χ_1^2=13.43, P<0.001; Fig. 6) and genotype

(P<0.001), as well as a significant interaction between the two (P<0.01). Overall, males

displayed more movement than females, however the extent of this effect varied across the

29 lines. Despite 30 minutes of acclimatization before beginning data recording, there was a significant reduction in activity over time (Wald χ^2_1=187.70, P<0.001), as well as between the first and second testing session (Wald χ^2_1=6.24, P<0.05). Lines infected by *Wolbachia* were found to be significantly less active than uninfected lines (Wald χ^2_1=4.68, P<0.05).

When comparing measures of activity and social behaviour, we found that median nearest neighbor distances were not significantly correlated with measures of activity for either male ($F_{1,27}$=2.94, P=0.10; Fig. 7) or female flies ($F_{1,27}$=1.82, P=0.19).
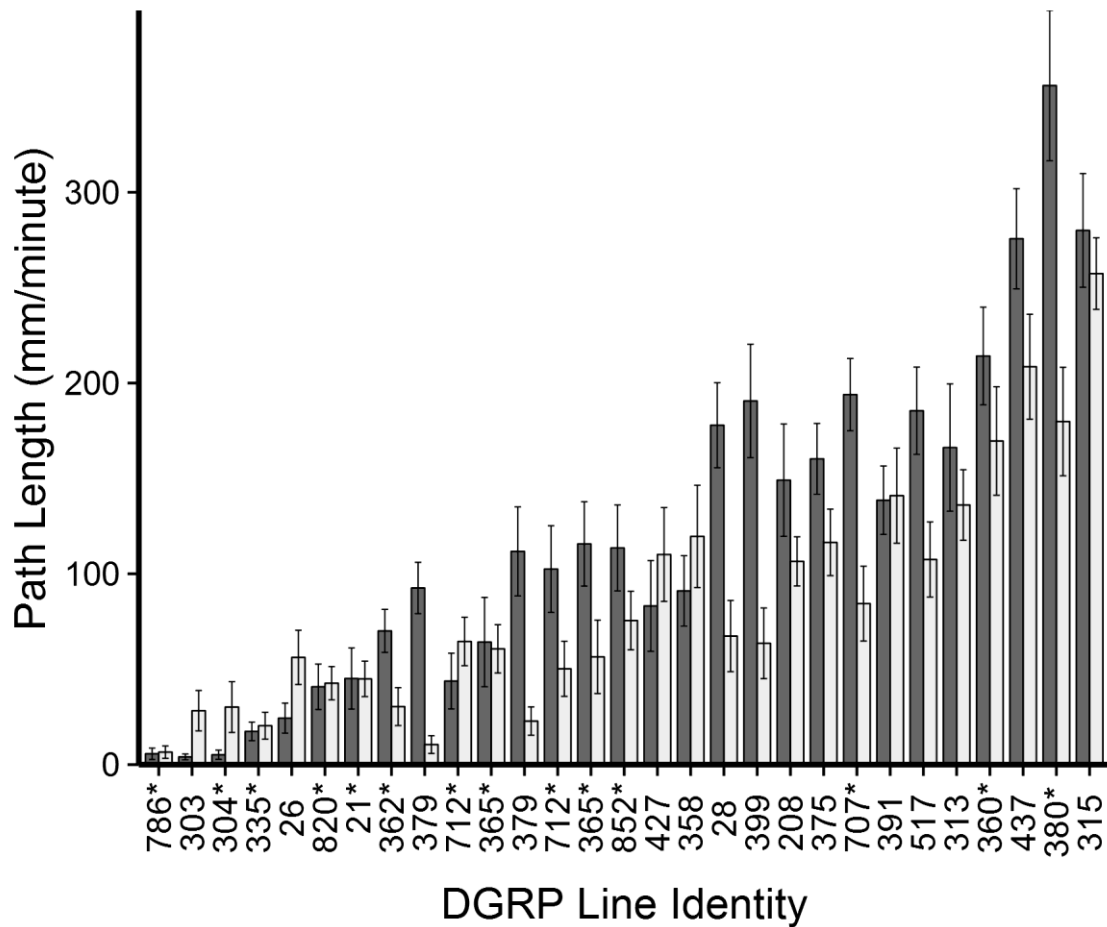
Figure 6: Mean ± SE distance walked per minute for males (dark bars) and females (light bars) from 29 DGRP lines assayed, arranged by increasing line mean. *Wolbachia* infected lines are denoted with an asterisk (*).
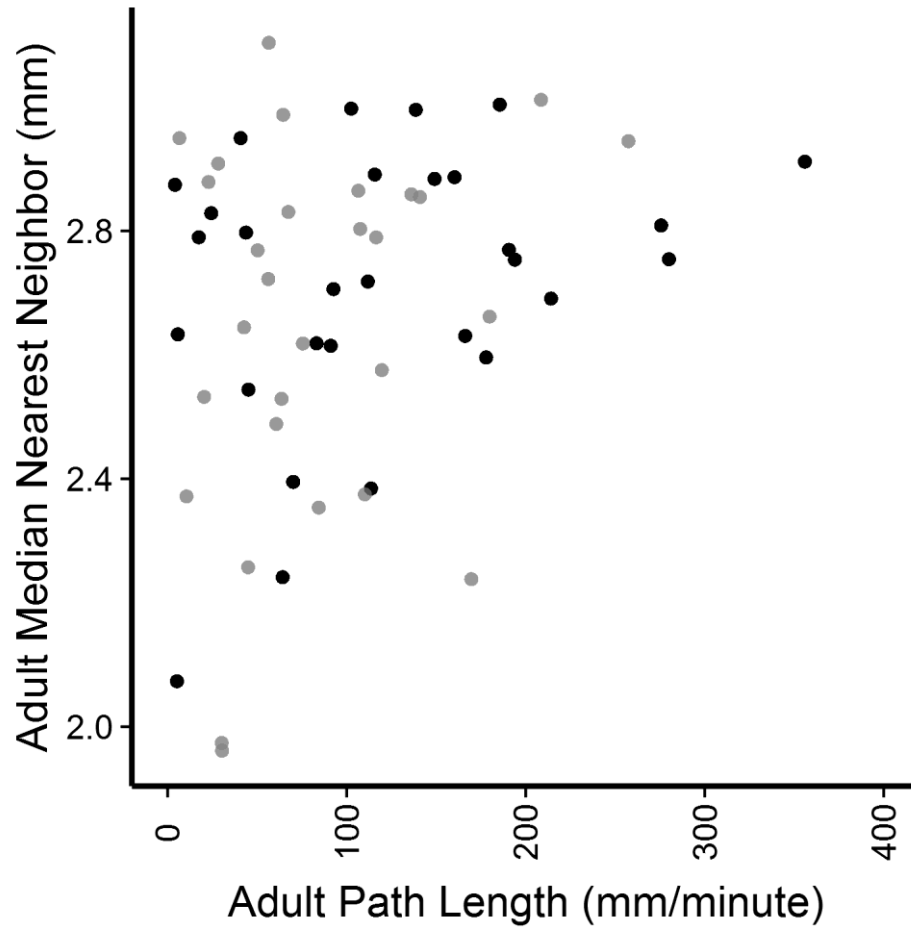
Figure 7: Relationship between adult activity and social behaviour for both males (black) and females (grey).

Correlation between Life Stages

Results

We performed a series of linear regressions to test whether there was a positive correlation in social behaviour, or activity, between the larval and adult flies of each genotype. We found no significant correlation between measures of aggregation in larval and adult flies ($F_{1,27}$=1.39, P=0.25; Fig. 8), nor was there any significant correlation in measures of activity between the two life stages ($F_{1,27}$=0.46, P=0.50; Fig. 9). None of these correlations between larval and adult traits approached significance when considering adult males and females separately (all P>0.27).
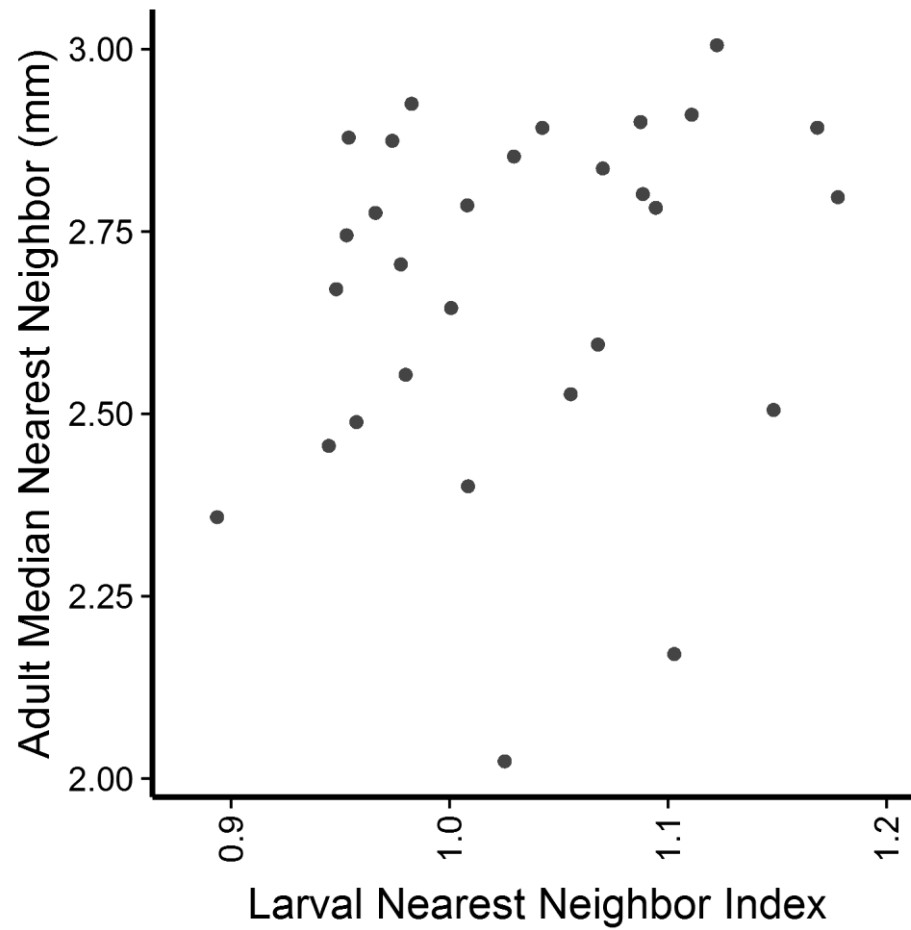
Figure 8: Correlation between measures of social behaviour during both the larval and adult stages, for 29 DGRP lines.
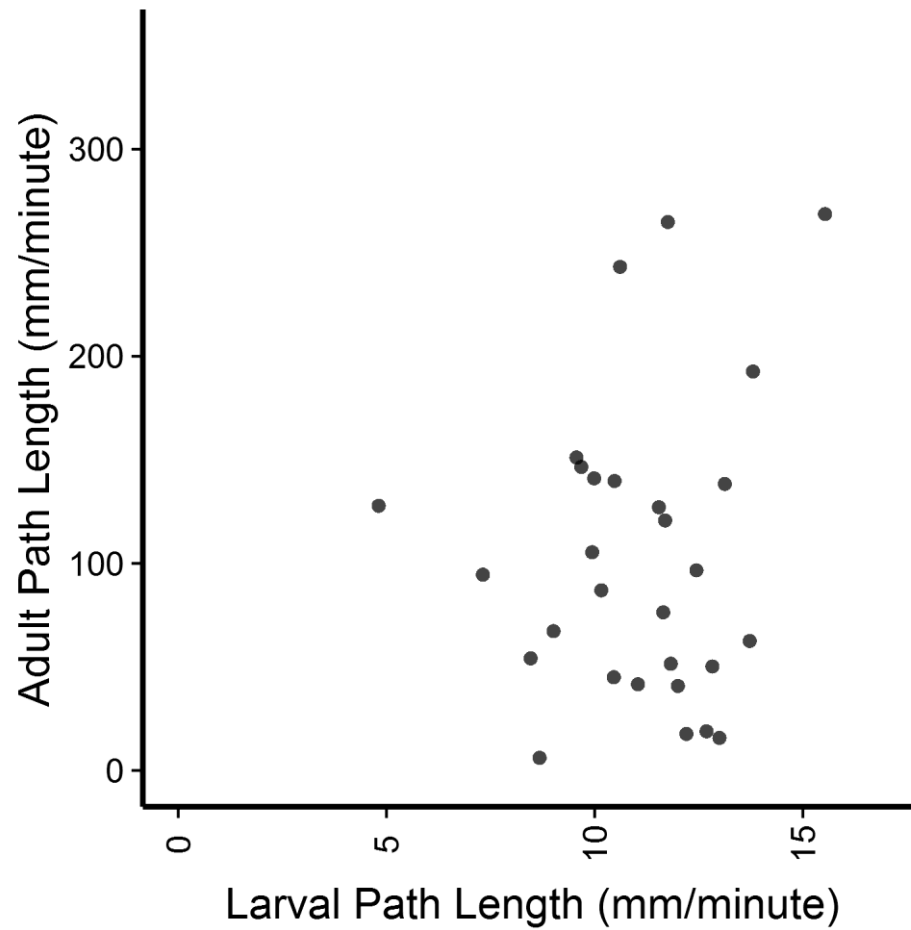
Figure 9: Correlation between measures of activity during both the larval and adult stages, for 29 DGRP lines.

Discussion

Consistent with our predictions, we found that direct genetic effects explained a significant degree of the variation in social behaviour for both larvae and adult flies of both sexes. In the larvae, we found that differences between lines were reliable between screenings, and across generations. While limited, we have similar evidence for repeatable line differences as adults (see 2nd chapter). While the lack of differences between the sexes may be partially confounded due to measurement error associated with our body size correction, or variation in the degree of sexual body size dimorphism between lines, it is apparent that the interaction between sex and genotype contributes much more than sex alone.

Similarly, there was significant variation in activity observed between lines for both larvae and adults of both sexes. In the adults, we observed a significant effect of sex, with males generally being more active than females. Since we reared experimental adults in mixed sexed groups, oviposition behaviour by females may partially explain their reduced levels of activity, though the degree of the sex difference varied significantly by genotype. We found that *Wolbachia* infection was associated with a significant reduction in adult locomotor activity, and a marginal decrease in nearest neighbor distances. While non-significant, the latter result is consistent with the notion that reduced aggression may lead to increased group densities upon a food patch. In neither adults nor larvae did we find evidence of any correlation between measures of activity and social behaviour, suggesting that neither encounter rates nor movement behaviour have significant influence on the preferred distribution of individuals.

Contrary to our predictions, we found no evidence for a positive correlation in either social behaviour, or activity between the larval and adult stages. These results agree with the notion of an adaptive decoupling of genes between the larval and adult life stages. While it was previously reported that the *for* gene may have correlated effects on larval foraging behaviour and adult dispersal, we failed to find significant genetic correlation between life stages in terms of activity. This may be due to the fact that the dispersal from a discrete food patch previously observed (Edelsparre et al., 2014) is distinct from locomotor behaviour upon a continuous substrate tested here. It is also possible that there is a significant degree of gene-environment interplay involved, as genes mediating larval foraging behaviour have been shown to produce profound changes in adult physiology and behaviour depending on the nutritional conditions available (Burns et al., 2012; Urquhart-Cronish & Sokolowski, 2014). Thus, weak positive genetic correlations may still exist under a set of highly specific environmental conditions not critically evaluated here.

Chapter 2: Indirect Genetic Effects and Social Behaviour

Introduction

While many traits in individuals can be readily measured, there are a large number of traits which cannot be explicitly defined in the absence of an interacting partner. These generally include those relating to social interactions, including dominance, competition, and courtship, where the behaviour of one individual is partially, determined by the individual it interacts with. Traditionally, the factors that influence the expression of an individual's phenotype have been partitioned into two main components, the effects of genetic factors, and the effects of environment. However, this is perhaps too simplistic, as genes can give rise to traits that modify the environment of the individual, which in turn influences the fitness of the individual. These "extended phenotypes" (Dawkins, 1999) put forth the notion that aspects of the environment can also evolve, as they are determined by the genes of the individuals occupying them. A simplified approach has reduced extended phenotypes to the study of "interacting phenotypes" between dyadic pairs, or within small groups (Moore, Brodie, & Wolf, 1997).

Interacting phenotype approaches have revealed the importance of indirect genetic effects (IGE), which refer to how the genes of one individual influence the phenotype of an interacting individual through changes to their social environment (Moore et al., 1997). IGEs can occur within a single shared trait, for example, when aggression exhibited by one individual induces aggressive responses in the interaction partner (Cairns & Scholz, 1973), or between two distinct traits, such as when experience with male field cricket song influences subsequent female mate choosiness (Bailey & Zuk, 2012). The magnitude of the

effect one individual's phenotype has on its interacting individual can be referred to as the interaction effect coefficient (Ψ), which may be positive or negative, depending on if the IGE increases or decreases trait expression, respectively. This effect may also be reciprocal, in which case a positive feedback loop forms where the change in an individual's phenotype due to its interacting partner elicits further changes in the interacting partner. If both Ψs act in the same direction, the rate of phenotypic evolution within a population is expected to increase exponentially. Alternatively, if both Ψs act in opposite directions, the rate of phenotypic evolution will be slowed, or completely halted if both Ψs are of equal magnitude (Moore et al., 1997). The term 'phenotypic evolution' is distinct from the traditional definition of evolution because, through IGEs, phenotypes expressed within population can change even when the genes directly contributing to that phenotype remain static (Wolf, Brodie III, Cheverud, Moore, & Wade, 1998). The existence of IGEs can thus have broad evolutionary consequences and may explain, to some extent, why phenotypes related to inter-individual interactions such as sexual ornamentation have appeared to undergo more rapid evolution than other traits (Fisher, 1915; Wolf, Brodie III, & Moore, 1999).

Despite the relevance of IGEs to ecological systems, they represent a small, yet exponentially growing topic of study (Wootton, 2002). While a classical empirical example of IGEs includes maternal genetic effects (Kirkpatrick & Lande, 1989), IGEs have also been demonstrated in deer mice aggression (Wilson, Gelin, Perron, & Réale, 2009), *Arabidopsis* growth (Mutic & Wolf, 2007), and social behaviour in microorganisms (Crespi, 2001). Studies in fruit flies have demonstrated that IGEs influence aggression between males (Saltz, 2013), male courtship behaviour (Krupp et al., 2008) and cuticular

hydrocarbon expression (Petfield, Chenoweth, Rundle, & Blows, 2005). For the male pheromone cVA, as well as a subset of cuticular hydrocarbons, social environment has been shown to explain a greater proportion of phenotypic variation than direct genetic effects (Kent, Azanchi, Smith, Formosa, & Levine, 2008). In addition to its role as an aggregation pheromone, cVA is also transferred to females during mating, where it reduces courtship behaviour of interacting males (Jallon, Antony, & Benamar, 1981), through the actions of Or67d expressing neurons (Kurtovic, Widmer, & Dickson, 2007). Artificial stimulation of this pathway has been shown to increase male-male aggression, suggesting cVA signaling may regulate male density by promoting male dispersal when aggressive interactions upon food sites become too frequent (Wang & Anderson, 2010). While we did not examine intersexual interactions, female flies also express a sex specific cuticular hydrocarbon, 7,11-heptacosadiene (7,11-HD; Jallon & David, 1987), which is detected by gustatory neurons on the male forelegs expressing *ppk23* (Toda, Zhao, & Dickson, 2012). The presence of 7,11-HD in the male environment has been shown to decrease lifespan, increase activity levels, and stimulate neuropeptide F expression (Gendron et al., 2014), the latter of which has been linked to increased levels of aggression in male flies (Dierick & Greenspan, 2007). Expression of 7,11-HD has been shown to vary among flies, in part due to a polymorphism that decreases production of 7,11-HD in favor of its isomer 5,9-heptacosadiene (Dallerac et al., 2000), which consequently changes male courtship behaviour (Capy et al., 2000). It is apparent that the relationship between social environment, and the expression of cues mediating interactions in fruit flies is reciprocal in nature. Given that a genetic basis has been demonstrated for both social environment choice

and pheromone expression, there is an implied role of IGEs, though the extent to which IGEs influence social behaviour in fruit flies remains largely unknown.

Rationale

There are very few experimental demonstrations of indirect genetic effects, yet they have been shown to play a major role in a subset fruit fly behaviours, including mating and aggression. Under the assumption that male attraction into groups is balanced with dispersal through aggressive actions, we took a trait based approach (Moore et al., 1997) and assessed how preferred group density amongst a group of Canton-S (CS) males varied as we altered the genetic component of their social environment. We predicted that Ψ would be positive, meaning that when a group of highly social individuals were introduced to the focal environment, that focal flies would maintain a denser group, and vice versa when a group of minimally social flies comprised their social environment.

Methods

We maintained 3 populations of flies, one line of our CS, as well as well as 2 DGRP lines (427 and 304). Males from all three lines were used as our interacting (treatment) individuals, though only CS flies were used as focals. Our focal CS were collected from different egg laying vials than our treatment CS. The two DGRP lines used were chosen based on a previous experiment (Experiment 2.1) suggesting that, out of 29 lines assayed, they represented extreme cases of social behaviour on a similar measure to that used here. Males of line 304 exhibited the smallest inter-individual distances (highly social), while males of line 427 exhibited the greatest inter-individual distances (low social) when left to distribute themselves upon a food substrate. All populations were maintained at low density

in vials containing 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 off at 10 pm.

Egg laying for experimental males was conducted across several consecutive days, in which mated females were left to lay eggs in fresh vials containing a sprinkle of live yeast. Following pupation, vials were cleared daily at 8 am, before collecting experimental adults at 4 pm in the afternoon and sexing them under light anesthetic. Both treatment and focal males were kept in mixed sex vials containing 14 males and 14 females in order to provide social experience. On the third morning following sexing, at 8 am, treatment and focal adults were dusted with either pink or blue fluorescent powder, which was counterbalanced across days. An hour after coloration, adults were very briefly anesthetized under light CO_2 and 6 focal males as well as 12 experimental males were transferred together into 85 mm petri dishes. 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, two hours before the start of observations, we transferred up to 6 dishes of flies to each of 4 testing boxes (see experiment 1.2) containing a 5 cm raised bottom platform. The boxes were not internally illuminated, though the boxes were semi-opaque, and as such were permeable to ambient room lighting while still blocking external visual cues. Following acclimatization, groups of flies were recorded for 60 minutes through a small (3x8 cm) hole in the center of the lid using a high resolution webcam.

During video analysis, Cartesian coordinates of each fly were sampled at 60 second intervals, and observers blind to treatment genotype verified the position of all 18 males and distinguished the 6 focals from the interacting males based on color. For each dish, we applied a body size correction (see experiment 2.1) and calculated the average nearest neighbor distances independently for the 6 focals and 12 DGRP. The data was analyzed using a linear mixed model with genotype and focal color as fixed effects, day, box, and dish as random effects, and time as a repeated measure.

Results

We found that the average nearest neighbor distances of focal males differed significantly based on the genotype of the interacting males they were paired with ($F_{2,114}=3.16$, $P<0.05$), where focals paired with our highly-social DGRP exhibiting smaller nearest neighbor indices than those paired with either our low-social DGRP or CS (Figure 10), though there was a significant effect of coloration ($F_{1,114}=12.3$, $P<0.001$). A post-hoc analysis comparing calculated nearest neighbor indices for the focal and interacting CS males suggested that, when correcting for the number of sampled points, the 6 focal males aggregated less than the 12 interacting CS (t-test; $T_{59.2}=4.71$, $P<0.001$).
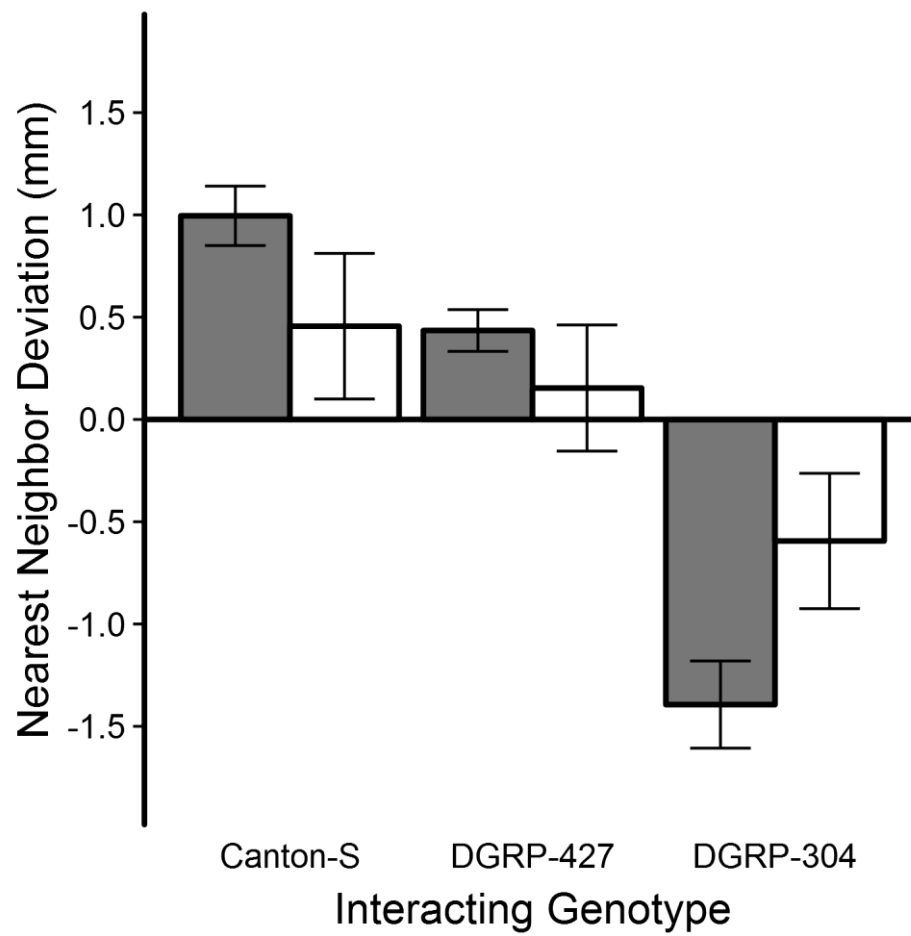
Figure 10: Deviation of mean ± SE nearest neighbor distances for both the 6 focal males (white) and 12 interacting males (grey) from their overall averages (15.43 and 8.87 mm, respectively).

Discussion

Consistent with our prediction, the distribution of groups of focal individuals varied significantly with interacting male genotype, suggesting that group density is indeed influenced by indirect, as well as direct genetic effects. We found that our focal CS interacted less among themselves relative to the 12 CS with which they were paired. This may be indicative that, following changes to a group's social environment, within-group interactions decrease to garner information about their new social environment. Alternatively, this may simply be a shortcoming of the nearest neighbor index, which becomes increasingly variable at small sample sizes, and assumes that the likelihood of occupancy of all positions are equal, despite informal observations suggesting different regions in space, such as dish boundaries, may be preferred by exploring individuals.

We also found that nearest neighbor distances were greater among the CS males than they were among males from DGRP line 427. This is surprising, considering that line 427 was previously found to be the least social of 29 lines assayed. Our experimental CS were established from founding females collected from a cage-maintained population. In these cages, it may have been selectively advantageous to prefer less densely occupied substrates, as this would be associated with less competition for resources and mates (in males), and lower larval crowding (for ovipositing females), hence the reduced aggregation among CS males.

While we found that Ψ was positive for focals paired with DGRP from line 427 and 304, estimations of the magnitude of the IGE here are difficult due to our use of CS males as focals. While lab maintained populations of fruit flies can be expected to show

significantly higher degrees of inbreeding than wild populations (Latter & Mulley, 1995), most DGRP lines have a coefficient of relatedness above 0.98 (Huang et al., 2014). Kin selection theory suggests that interaction amongst individuals should vary with measures of relatedness, thus the magnitude of Ψ may be different among individual focal flies (Alemu, Berg, Janss, & Bijma, 2014).

General Discussion

Major Findings

Our major findings were threefold. First, social behaviour of both larvae, and adult flies will be expected to vary between wild populations owing to underlying direct genetic factors. Genetic variation also influences baseline differences in locomotor activity, but this does not explain the observed differences in social behaviour. Second, there is no correlation in the phenotypic traits measured between the larval and adult life stages. This suggests that the genes influencing social behaviour during either life stages are decoupled, that is, they are expressed differently on either side of the metamorphic boundary. Lastly, while direct genetic factors influence the degree of social interaction exhibited by an individual, there is also a significant indirect genetic influence. In order to estimate the phenotype of an individual, one must account for direct genetic effects and their environmental interaction, as well as the composition of their social environment, as the degree of social behaviour exhibited by an individual may conform to their social group.

Decoupling of Behaviour

The lack of correlation found in measures of social behaviour is consistent with an adaptive decoupling of behaviour between life history stages. Our original prediction was that a potential correlation in environmental factors may minimize any benefit of behavioural decoupling. However, given the ephemeral and variable nature of the resources utilized by fruit flies, there may be situations where the environmental conditions are negatively correlated between life stages, such as when food quality or abundance is poor. In such a case, larger aggregations of ovipositing adults may result in unsustainable densities of

larvae. In such a situation it may even be beneficial for larvae to leave their initial social environment in search of less densely occupied food sites. If the relationship between larval and adult environment is not consistent over evolutionary time frames, a decoupling in behaviour, such as that seen here would allow for increased frequencies of social behaviour to emerge during either life stage, without being constrained by the gene complexes underlying social behaviour in the other stage.

Adaptive Significance of IGEs

The presence of genes that influence choices in social environment have been suggested to have profound influences on both development, and the selection of social traits (Saltz, 2011). Here we have demonstrated that social environment choice by individuals is influenced by their current social environment, as well as genotype. Previous research has demonstrated that socially enriched flies exhibit far less aggressive behaviour than isolated flies (Wang, Dankert, Perona, & Anderson, 2008), and that socially reared flies maintain denser groups than socially inexperienced flies (Simon et al., 2012). The effect of social environment we observed on focal behaviour may explained by changes in the number of interactions among individuals during the acclimatization phase: when paired with more social interacting males, the focals habituate to a more social environment, and increase their propensity to aggregate accordingly.

We previously suggested that ecological pressures such as resource density may select for traits that increase propensities for aggregation. Here it is made evident that, through IGEs, the direction of causality may also be reversed. Thus social environment may facilitate increased aggregation behaviour, and the ecological pressures associated

with larval crowding may emerge as a result. While we demonstrated that there was a positive influence of interacting individuals upon our focals, our methodology lacked the ability to detect whether this IGE was reciprocal or not. Despite this, the reciprocal relationship between the social and non-social environment hypothesized here may lead to exaggerated selection pressures in much the same ways as would be expected of reciprocal IGEs, and subsequently, may increase rates of phenotypic evolution

Conclusion

This work contributes to a growing body of work attempting to elucidate both the proximate and ultimate underpinnings of social behaviour in a model organism. Having established that social behaviour during both the larval and adult stages is influenced by heritable genetic factors, future research may benefit from characterizing the selective pressures that elicit social evolution in natural populations, as well as the degree to which interacting genotypes influence rates of phenotypic change in fruit flies. The demonstrable role of social environment on interactions between individuals has far reaching implications, as these IGEs may be fundamentally involved in the evolution of social behaviour, as well as the maintenance of solitary living.

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