Effect of presence of kin on dispersal and social behaviours in *Drosophila melanogaster*

Thesis submitted in partial fulfilment of the requirements for the BS-MS Dual Degree Programme

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CERTIFICATE

This is to certify that this dissertation entitled "Effect of presence of kin on dispersal and social behaviours in *Drosophila melanogaster*" towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Aarcha Thadi at Indian Institute of Science Education and Research under the supervision of Professor Sutirth Dey, Department of Biology, during the academic year 2019-2020.

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Date: 5/4/20

DECLARATION

I hereby declare that the matter embodied in the report "Effect of presence of kin on dispersal and social behaviours in *Drosophila melanogaster*" are the results of the work carried out by me at the Department of Biology, Indian Institute of Science Education and Research, Pune, under the supervision of Prof. Sutirth Dey and the same has not been submitted elsewhere for any other degree.

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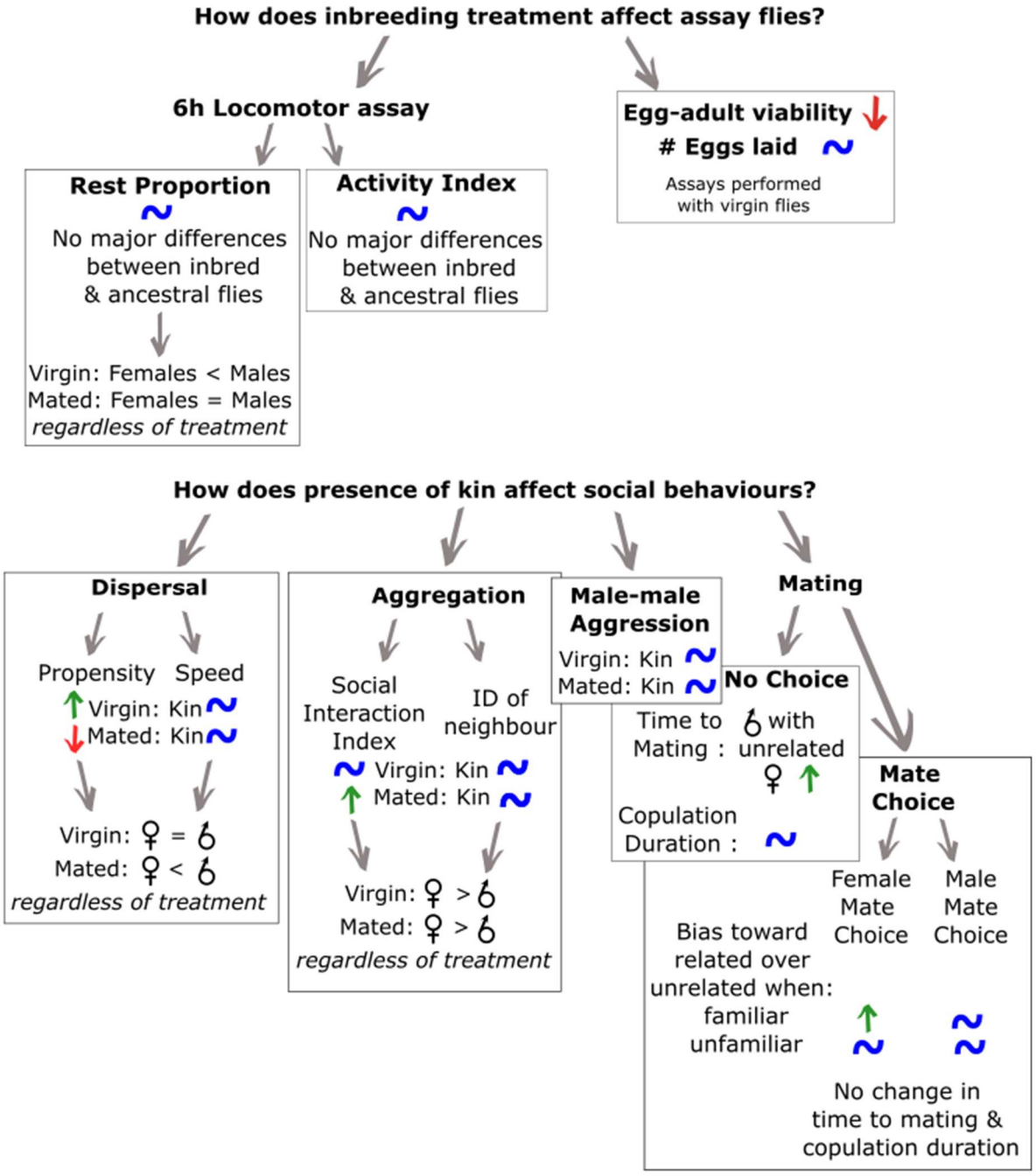
ABSTRACT

Behaviour can be altered by the presence of kin, and this is seen in multiple organisms. This phenomenon is comparatively less studied in organisms that do not show group-living behaviour, like *Drosophila melanogaster*. Our study examines how the presence of kin affects behaviours in *Drosophila melanogaster*, with a focus on how sex and mating status modulate these responses. We study dispersal, aggregation, aggression, and mating behaviours in both a mate choice and no-choice setup. We see that mating behaviours can be kin-biased depending on the context in which mates interact. We also see that having been previously mated leads to closer aggregation between related same-sex groups, and virgin flies do not show this behaviour. We provide the first experimental proof that mating status and relatedness can interact to modulate dispersal propensity, with related groups of flies dispersing less than unrelated groups when mated but more when virgins. Mating also leads to reduced dispersal speed and increased rest levels of females, which has further implications in spatial sorting of populations. Our study shows that traditionally 'non-social' organisms like the fruit fly can be used as models to understand social behaviour between kin. We pave the way for future studies to investigate how the social environment of an organism should affect its behaviour, and advocate for further studies to elucidate the motivation behind the behaviours we capture in our experiments.

GRAPHICAL ABSTRACT OF RESULTS



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1. INTRODUCTION

Kin selection theory argues that interactions between organisms can be influenced by attempts by individuals to increase their own inclusive fitness (Hamilton, 1964). Related individuals have genes in common, which can be passed onto the next generation indirectly through their kin. In order to benefit from the propagation of these common genes, organisms may choose to aid kin over non-kin, and help related conspecifics while expending their own time and energy. Inclusive fitness theory suggests that these situations wherein individuals seemingly lose reproductive opportunities, or put themselves in danger for the benefit of their kin are in fact instances of selfish behaviour (Alcock, 2001, p. 423-424).

An example of such a conflict is when related individuals share resources such as food, or nesting sites with each other. This can ensure the longevity and fitness of one's kin, and can encourage further sharing of resources and information between these organisms in the future (R. Trivers, 1971; 2006). However, when resources are limited, kin often end up competing with each other for survival which can negatively impact all individuals involved (Queller, 1994; Wade, 1985). Secondly, organisms may sometimes prefer mating with kin, rather than unrelated individuals (Puurtinen, 2011). Producing offspring highly similar to oneself ensures the continuation of the genetic line, though this can come with consequences. The progeny resulting from mating between closely related individuals are often seen to display a reduction in fitness, which is termed as inbreeding depression. Moreover, inbred organisms often are sensitive to changes in their environment, as inbreeding has been shown to lead to reductions in adaptive plastic phenotypes (Whitlock & Fowler, 1999). From these examples, it is clear that there exists a conflict in performing behaviours that are positively kin-biased, as they are often 'high risk, high reward'. Studying behaviour in the presence of kin becomes paramount in the understanding of how kin-biasing of behaviour occurs in populations of a variety of organisms.

Historically, kin-biased behaviours were studied in organisms that are group living. Group-living organisms depend on social relationships for their survival, sometimes in the form of distribution of work between relatives, and often the establishment of hierarchy within a group, serving to enforce cooperation between members of the group. Insects such as bees, wasps and ants exhibit these group-living tendencies.

These organisms have been extensively investigated to understand predicts of kin theory, however the prevalence these behaviours in other insects that are not traditionally ‘social’ or group-living is understudied. *Drosophila melanogaster* are examples of organisms that are considered to be ‘non-social’. Social structure within populations of this species is assumed to be too simple for dependencies on other conspecifics to exist. Further, they have a polygamous mating system, with the absence of parental care and colony-living. Despite this, recent studies demonstrate that fruit flies can learn from conspecifics (Battesti et al., 2012; Sarin & Dukas, 2009), and they build complex networks of interactions between themselves (Schneider et al., 2012) and may choose to interact with only a subset of the flies in their social environment (Kulkarni, 2019), demonstrating that there may be some basis upon which flies discriminate between conspecifics. Further, some experiments have shown that the social environment of a group of males can impact the mate-harm experienced by a female after multiple matings (Carazo et al., 2015). These results suggest that fruit flies have the ability to evaluate their social environment, modify their behaviour towards others within it, and form social relationships with these other individuals.

Drosophila melanogaster have demonstrated their utility in the study of behaviour, particularly those related to mating. In nature, fruit flies are known to be polygamous, promiscuous individuals with a complex mating behaviour repertoire. A large body of work is constantly being developed upon, dedicated to characterizing behaviours in this repertoire, as well as dissecting their neurological basis, and factors leading to plasticity in these behaviours (Griffith & Ejima, 2009; Villella & Hall, 2008). The decision to attempt courtship and mating in males is made on the basis of pheromone signals (Averhoff & Richardson, 1976), and informed by previous courtship experience with other females (Dukas, 2005). Upon the commencement of courtship behaviour, females can gauge whether or not to accept the male’s advances based on pheromone and acoustic signals generated by the male during courtship (Markow, 1987), and the female’s physiological readiness to mate (Laturney & Billeter, 2016). Thus, complex behavioural displays and decisions need to be made by both parties for mating to occur. It is in a male’s interest to mate with multiple females, but also to ensure that those females he has mated with produce his offspring and not the offspring of other males. To ensure this, the seminal fluid

contains factors such as sex peptide which induces a drastic change in female behaviour (Kingan et al., 1993). Females' priorities may shift towards increased egg laying, they actively reject advances of potential mates males, and sperm usage and storage changes (Chapman, 2001). Such a post-mating behavioural switch is commonly observed in insects, and the sex peptide receptor is highly conserved (Yapici et al., 2008). Due to this post-mating behavioural change, the resources required by individuals may change, and this may affect how individuals interact with their surroundings and other conspecifics.

Thus, the mating status of individuals can play a role in modulating further interactions between organisms. Mate choice, as well as post-mating decisions such as sperm allocation can play an important role in shaping population genetic structure. The level of kinship in a population is dependent on mating behaviours displayed by organisms. A study of relatedness in a large, naturally-occurring population of *Drosophila melanogaster* revealed that these flies showed higher than expected levels of inbreeding (Robinson et al., 2012a). This suggests a high frequency of interacting and mating with kin in these populations. The conflict of performing kin-biased behaviour resurfaces in mating – is it advantageous to mate with one's kin to ensure the propagation of common genes, though this could lead to the creation of local populations wherein organisms are closely related, resulting high levels of kin competition detrimental for all individuals? Therefore, the study of how mating and relatedness interact is important to understand how these factors can affect resource management and spatial distribution of a population.

One way that kin-biased behaviours may be realised is through dispersal. Dispersal can be defined as "any movement of individuals or propagules with potential consequences for gene flow across space" (Ronce, 2007). Dispersal can be an effective strategy for organisms to use, to avoid competition for resources with one's kin, and to avoid inbreeding (Clobert et al., 2012). There have been a number of theoretical and experimental studies investigating the effect of presence of kin on dispersal. Emigration of first-instar larvae of the mango shield scale, *Milviscutulus mangiferae* was observed at a higher rate in siblings (Kasuya, 2000). Kin competition was shown to promote dispersal in male pollinating fig wasps (Moore et al., 2006). Bitume *et al.* (2013) showed that genetic relatedness increased the dispersal

distance of the two-spotted spider mites they assayed. However, prairie dogs were seen to disperse from their original habitats only once all their kin have disappeared (Hoogland, 2013). Other studies found that some animals that exhibit cooperative breeding tend to disperse towards their kin (Baglione et al., 2003). Many factors can play a role in dispersal, and there is no one-size-fits-all for predicting dispersal behaviours in different species.

Mating can also play a role in determining the spatial structure of a population. Sex-biasing of dispersal can change depending on when organisms mate (prior, during or after dispersing) and when they are able to oviposit (Shaw & Kokko, 2016). Mating status can alter dispersal distances, with the whitefly parasitoid *Eretmocerus eremicus* showing a reduction in dispersal distance when individuals were mated (Bellamy & Byrne, 2001). Previous research in dispersal behaviours of *Drosophila melanogaster* revealed that mating can change dispersal propensity observed in both sexes (Simon et al., 2011). Drosop*hila melanogaster* populations in our lab have proven to be an effective model system to study dispersal in the past (Mishra et al., 2018; Tung et al., 2018). Here, we investigate how the presence of kin affects the dispersal propensity and speed of both virgin and mated flies.

To assay large numbers of kin, we had to produce groups of highly related flies. However, producing related flies involves inbreeding, which comes with its own suite of negative effects. A decrease in fecundity, at the stage of egg laying, or egg-adult survivorship can both be indicators of inbreeding depression. In *Drosophila melanogaster,* research shows that alongside fitness costs, inbreeding can have a multitude of other effects. Deleterious alleles have a higher chance of expression due to inbreeding, implying a higher likelihood of expression of morphological and physiological defects in organisms. Such defects can negatively affect an individual's ability to move and navigate its own surroundings, and therefore interaction with other conspecifics can be hampered. Effects of inbreeding of this kind have been observed in *Drosophila melanogaster*, leading to impairments in various aspects of mating behavior (Ala-Honkola et al., 2013; Dolphin & Carter, 2016; Miller et al., 1993), and learning abilities (Nepoux et al., 2010). A study by Manenti et al. (2015) demonstrates that the negative effects of inbreeding can manifest as changes in locomotor activity patterns. Locomotory activity has been shown to be cue-

dependent, with abiotic factors such as light, and temperature influencing locomotion (Tomioka et al., 1998). Their experiments showed that both an increase or decrease in total activity, as well changes in activity-rest patterns could occur due to inbreeding. Studying locomotion can therefore point toward changes in one's ability to interpret environmental cues as well as other physiological and morphological defects, and thus serve as a proxy to understand whether inbreeding can alter behaviour.

Thus, to test the harshness of the inbreeding treatment we imposed on our flies, we performed assays to check the effect of the inbreeding rearing treatment on the flies used in our experiments. Through these assays we aimed to quantify the effect, if any, of inbreeding depression on (a) fitness, and (b) physiological impairment that could affect behaviours of the flies.

Grouping behaviours in organisms is shaped by the presence of resources and their social environment. Being in close proximity to conspecifics can lead to higher rates of social interaction, which can influence the spread of disease (Patterson & Ruckstuhl, 2013), but also positively impact exchange of cues and information (Fernández-Juricic et al., 2004). In *Drosophila melanogaster*, aggregation is often observed at food, i.e. in the presence of resources and oviposition sites (Saltz & Foley, 2011). Odours and pheromonal cues left by other conspecifics at these sites can be enough to promote aggregation, even in the absence of food (Navarro & del Solar, 1975). cVA, a fly pheromone, was shown to influence aggregation behaviour between same-sex group of flies (Simon et al., 2012). Interestingly, cVa is also known to be released into the reproductive tract of females by their male partners after copulation, to convey to future suitors that these females are recently mated (Laturney & Billeter, 2016). This dual role of cVA could point towards an interaction between mating status and aggregation.

To understand whether relatedness influences aggregation behaviours in groups, we use a social space assay, conceived by Simon et al. (2012). The assay involved loading flies into an arena, and calculating the distances between them once their positions stabilise. We calculated Social Interaction Index (SII), where a higher SII implies higher levels of interactions between flies. We aimed to test two things. Firstly, which groups have higher SII and therefore increased social interaction when

these groups are related or unrelated. Secondly, we repeated the experiment, with two unrelated sets of flies, where flies within a set are related. The number of each individual's neighbours that are related or unrelated to them would help us determine whether flies choose to aggregate preferentially with kin.

Much previous work on kin-biased interactions in *Drosophila melanogaster* has focused on interactions between pairs or small groups of flies, ranging from 2-4 flies under observation. Studying various aspects of reproductive behaviour of flies with their kin has been at the focus of these efforts, but with varying results. Some groups were unable to find any indication of an ability to discriminate between kin when studying both pre and post-copulatory mating behaviours (Ala-Honkola et al., 2011; Tan et al., 2012). In experiments performed by Mack et al. (2002), males seem to be potentially allocating less sperm to females that they are closely related to, while Ala-Honkola et al. (2014) observed a slight reduction in copulation duration of previously mated flies with related mates. The work of Robinson et al., however, demonstrates a preference for females to accept courtship faster from closely related males in laboratory populations (2012b), matching their previous finding of assortative mating between related individuals in nature (2012a). Chippindale et al. (2015) suggested that as different lab populations arose from different places, the ecology and genetic backgrounds of these flies could explain why inconsistent results were seen by groups attempting the replicate the same experiments. We study mate choice between related and unrelated flies, for both males and females in our experiments. We further observe the time taken to begin mating, and copulation duration, under a both a no-choice and mate-choice setup, to further understand whether mating behaviours differ when mating with kin or non-kin.

Previous research in *Drosophila* has shown that larval familiarity can play a role in whether or not kin-biased behaviours occur (Le Page et al., 2017). Larval familiarity refers to having shared a rearing environment as larvae, and thus be an indication of However, other work that has incorporated both familiarity as well as relatedness as factors in checking for the presence of kin-biased behaviours, concluded that familiarity did not play a role in such interactions (Chippindale et al., 2015; Hollis et al., 2015a). In our mating experiments, we take both relatedness and familiarity in

account as factors. By doing this, we hope to be able to check whether familiarity plays a role in mediating kin-biasing in social behaviours.

Finally, we study aggression in between pairs of males, that are either related or unrelated to each other. In nature, organisms may often compete aggressively for resources, however studies show that aggressive behaviours can reduce when competing with kin (Walls & Roudebush, 1991; Watson et al., 1994). Previous experiments in *Drosophila melanogaster* report observing lowered aggression observed in groups of kin males (Carazo, 2014; 2015), but over multiple days, and these experiments do not account for one-on-one interactions within the group affecting aggressive displays (Penn et al., 2010). We observe aggressive behaviours during initial encounter of individuals under study, in the presence of food and a female, which represent resources over which males may compete.

2. MATERIALS AND METHODS

2.1. Experimental Populations

The flies used in this study were derived from an outbred laboratory population of ~2500 individuals, DB_4 (**D**ey **B**aseline 4). This population shall be referred to as the ancestral population in this report. These flies were maintained at 25°C in constant light on a 21-day discreet generation cycle, and fed on banana-jaggery medium. For details of the maintenance regime, refer to Sah et al. (2013).

2.2. Generation of Flies for Assays

Inbreeding Rearing Treatment

Eggs were collected from the DB_4 population at a density of 60 eggs per vial of ~6ml food. Once flies began to eclose, generally on the 8th day post-egg collection onwards (for not longer than 2 days), they were sorted by sex under light CO_2 anaesthesia every 6 hours. This was to done to ensure that the flies obtained were virgins. Virgin adults were placed in same-sex vials at a density of 20 flies per vial. These flies constituted the F_0 generation. On the 10th day post eclosion, the flies were provided with *ad libitum* live yeast paste to boost their fecundity. On the 11th day, single male and female virgins were allowed to mate. After mating, the female was aspirated into a vial with 6ml food for 20 hours to deposit eggs. **The offspring of a given female will be referred to as a 'lineage', and such each lineage was maintained separately from the others.** The male was kept singly in a 3ml food vial which was changed every 2 days.

F_1 generation flies were collected as virgins and provided with yeast as described previously. On their 11th day post egg collection, 6 virgin females were backcrossed with the male from F_0 (i.e. their father) successively. When copulation lasted for longer than 10 minutes, we could be reasonably sure that sperm transfer would have occurred (G. L. Fowler, 1973). We observed all pairings manually to ensure that reliable mating had occurred, and to reduce delays between each mating. After each mating, the females were removed from the vial by aspiration, and allowed to deposit eggs as described earlier. Males remained singly in these vials for an hour (after 1st and 2nd mating), 1.5 hours (3rd and 4th mating) and 2 hours (after 5th mating),

before encountering their next mate. The F_2 generation flies were collected as virgins as in the previous generations, and were sorted for assays on the 11th day (Fig. 1).

Fly Collection for Virgin & Mated Assays

In preparation for assays that involved virgin individuals, the flies were sorted on the 11th day for their respective assays. For assays requiring mated flies, male and female sibling flies (i.e. related-familiar flies) were pooled, so they could mate on their 10th day. They were then separated by sex under light CO_2 anaesthesia on the 11th day and sorted for assays. Assay-wise specifications for this sorting is described in their respective sections.

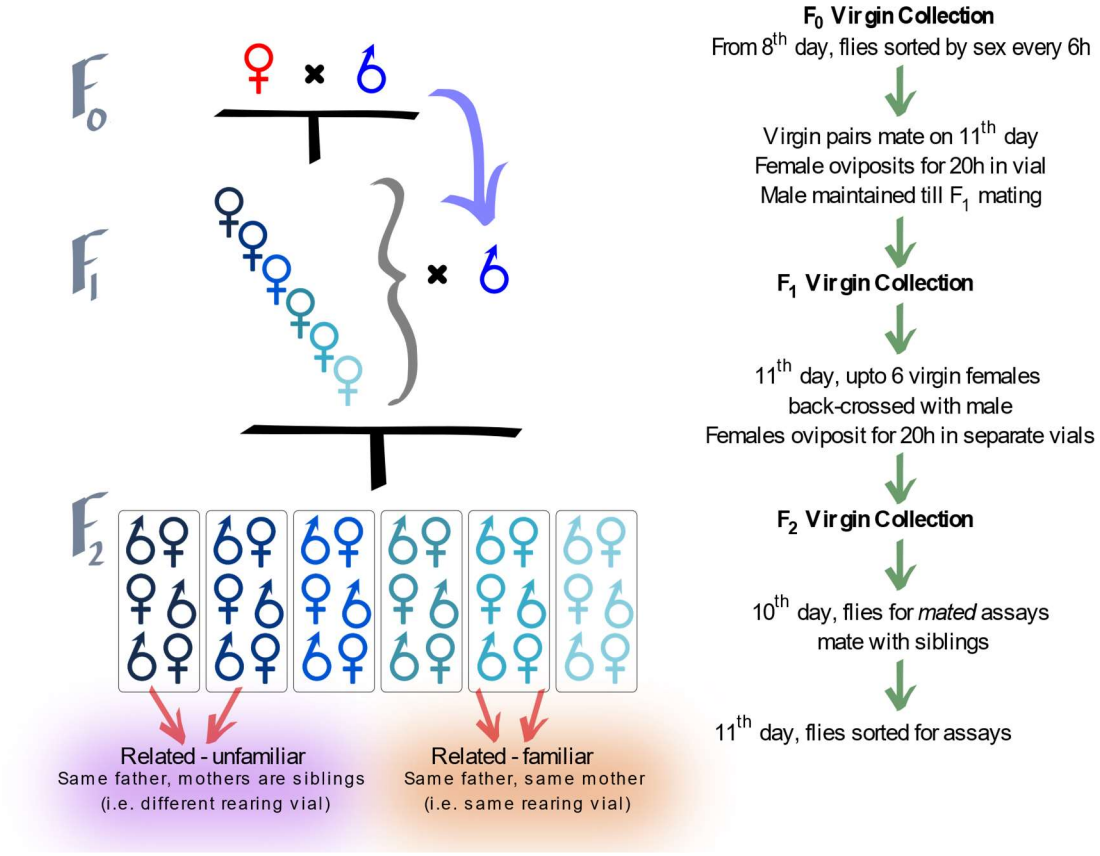


Fig. 1: Process followed to generate flies for assays. The diagram demonstrates how a single lineage was produced. For each assay, multiple lineages were created from random F_0 mating pairs.

2.3. **Assays**

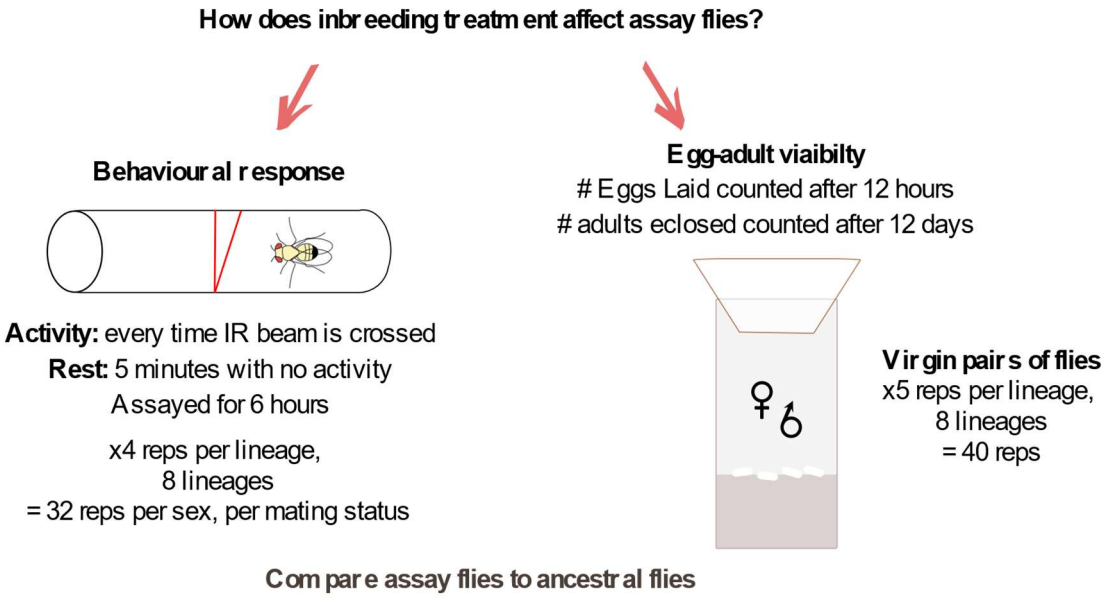


Fig. 2: How we studied effect of inbreeding on flies used for assays. Process followed to generate flies for assays explained in 2.3.1 and 2.3.2.

2.3.1. Egg-adult viability

Collection of Flies

Flies were bred as detailed in Section 2.2. At the same time, eggs were collected separately from the DB_4 ancestral population at a density of ~60 eggs per vial in ~6ml of banana-jaggery medium. Inbred flies used for this assay were taken for vials having similar egg densities of ~60 eggs per vial. F_2 inbred flies and DB_4 flies were thus age-matched for the assay. For this assay, virgin flies were used. Similar to the F_2 inbred flies, DB_4 flies were collected as virgins between the 8^{th} and 10^{th} day post egg-collection.

Setup & Protocol

On their 11^{th} day post egg-collection, pairs consisting of a virgin male and female were placed in a vial containing ~6ml of banana-jaggery food, and allowed to mate and oviposit for 12 hours. For the inbred set, the male and female pair were siblings, and 5 replicates were set up for each of the 8 lineages used in the assays. For the

ancestral set, two random flies were paired for all 40 replicates. The vials were kept in the incubator at 25°C under constant light. After 12 hours, the flies were discarded and the number of oviposited eggs on the food was counted. This food had been coloured grey with charcoal (1gm per 1L of food) for easy visibility of eggs during counting. The vials were placed back in the incubator and after 12 days, the number of adults in each vial was counted. Most flies complete egg-to-adult development within 10 days, and so 12 days was chosen to ensure that all viable adults had sufficient time to develop.

Data Collection and Statistical Analysis

Replicates in which no eggs had been laid (4 in inbred, 2 in ancestral), or in which egg counts were lower than adult counts (2 in inbred, 7 in ancestral) were discarded. Final sample size was 34 replicates in the inbred treatment, and 31 in the ancestral set. Inbreeding depression was studied as egg -adult viability, and calculated as given below.

$$Egg - adult\ viability = \frac{Number\ of\ \ adults\ eclosed}{Number\ of\ \ eggs\ laid}$$

In the inbred rearing treatment, the mean of the replicates corresponding to flies from the same lineage were calculated, which was treated as a proxy for the average inbreeding depression shown by a single genotype. Similarly, for the ancestral flies, the mean of every 5 replicates was calculated, ignoring the removed data as listed above.

As egg-adult viability was in the form of a proportion between 0 and 1, it was then arcsine-square root transformed for further analysis (Zar, 1999). The data was analysed using a General Linear Model (GLM) with egg-adult viability as a dependent variable and rearing treatment as a fixed factor (2 levels: Inbred/Ancestor).

We used a GLM to evaluate whether the overall number of eggs laid was affected by the rearing treatment. Similar to egg-adult viability, lineage-wise means were calculated here as well, and a GLM was constructed as in egg-adult viability analysis.

2.3.2. Locomotor Activity and Rest

Collection of Flies

Flies were bred as detailed in Section 2.2. At the same time, eggs were collected separately from the DB_4 ancestral population at a density of ~60 eggs per vial in ~6ml of banana-jaggery medium. F_2 inbred flies and DB_4 flies were thus age-matched for the assay. Inbred flies used came from vials having similar egg densities of ~60 eggs per vial. Similar to the F_2 inbred flies, DB_4 flies were collected as virgins between the 8^{th} and 10^{th} day post egg-collection, and then allowed to mate on the 10^{th} day for the mated assay, or kept separate for the virgin assay.

Setup and Protocol

Both sexes were assayed, to check for any sex-specific effects of the inbreeding treatment. Inbred flies were compared to flies of the same age from the ancestral population DB_4. The DAM (*Drosophila* Activity Monitor) data collection systems (Trikinetic Inc., USA) were used to measure these parameters. The system measures the activity of a fly in a glass tube as the number of times it crosses the two parallel infrared beams that bisect the DAM channels perpendicular to the tube. Activity data was obtained as the number of crosses every minute.

Flies were loaded by aspiration singly into 5mm glass tubes, which were then plugged with cotton on both sides and devoid of food. Aspiration, rather than CO_2 anaesthesia was done as the latter can affect the flies' activity levels if the readings were taken without sufficient time for recovery post anaesthesia (van Dijken et al., 1977). The glass tubes were loaded into the monitor and then were placed undisturbed in an incubator at 25°C with constant light for the duration of activity recording. The flies were monitored for 6 hours. For each rearing treatment, 32 were monitored per sex.

Data Collection and Statistical Analysis

The first 15 minutes of the data recorded was not included in the data analysis to allow the flies to acclimatise to the environment. Two parameters were scored for each fly, activity index and proportion of rest. Activity Index (AI) was measured as

the total number of activity counts divided by the duration that the fly spent awake i.e. not resting (Gilestro, 2012; Kayser et al., 2014). When there was no activity for at least 5 minutes, this was scored as rest (Hendricks et al., 2000). The fraction of assay duration spent resting was scored as the proportion of rest. This value was arcsine-square root transformed for further analysis (Zar, 1999). These two parameters are independent, as a fly that spends a longer time in rest need not have either a higher or lower activity index.

With either AI or Rest Proportion as the dependent variable, data was analysed using a GLM with rearing treatment (2 levels: Inbred/Ancestor) and sex (2 levels: (Male/Female) as fixed factors.

2.3.3. **Dispersal Propensity and Speed**

We conduct our dispersal experiments in the absence of food. Previous observations in our lab show that flies disperse at a much lower rate when food is present in the environment. Further food is utilised differently by mated and virgin flies, as it can serve the additional purpose of an oviposition site for mated females. We chose to keep the environments homogenous so that the behaviours of mated and virgin flies could be contrasted.

In flies, mate-following can play a significant role in modulating dispersal patterns as individuals are sometimes seen to disperse through tracking potential mates (Mishra et al., 2018). However, inbreeding avoidance, a phenomenon which is not well-understood in fruit flies, can potentially manifest through movement away from potential mates (Lehmann & Perrin, 2003). Therefore, our experiments were done on same-sex groups of flies, so as to avoid potential confounds of the interaction of mate-following behaviours and inbreeding avoidance.

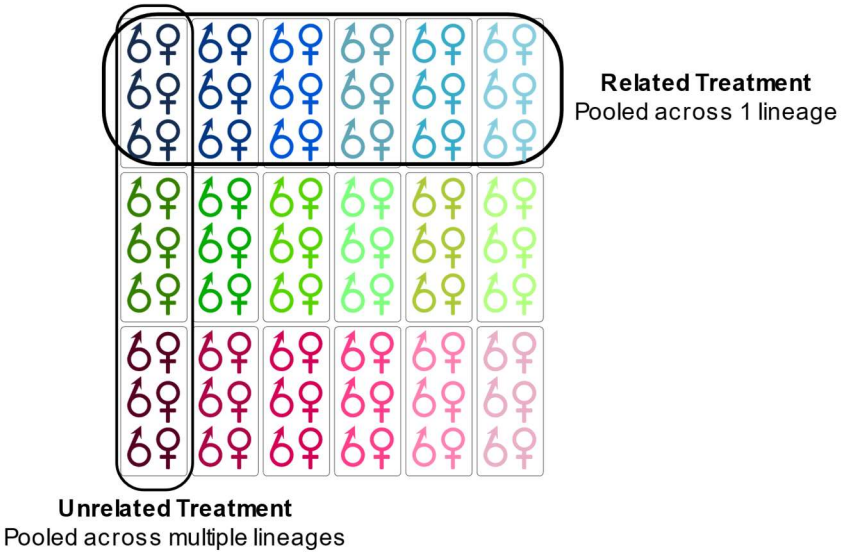


Fig. 3: Representation of collection of flies as described in dispersal (2.3.3) and aggregation (2.3.4) assays. To obtain related treatments, male OR female flies from a single lineage were pooled and sorted into 12 vials. For unrelated treatments, this was done across 12 lineages. The vials contained either 10 or 3 flies, to make the total number of flies per replicate 120 or 36, for the respective assays.

Collection of flies

Flies were reared as in Section 2.2. This assay was performed separately for virgin and mated flies. On the 11th day, flies were pooled into related and unrelated treatments for assays, as per Fig. 3. They were sorted randomly into holding vials having ~3ml of food, at a density of 10 flies per vial. A related treatment replicate consisted of 12 such vials, to give 120 flies in total. For the unrelated groups, 12 vials of 10 flies each were made from 12 different lineages. This was done for both sexes, separately. The average relatedness of flies in a related group was approximately 10 times greater than flies in the unrelated groups (see Section 4 for calculation). All sorting was done under light CO_2 anaesthesia. 8 replicates for each treatment (Sex × Relatedness) were thus made. Due to logistical constraints, half of the replicates for each treatment were assayed in the morning and the other set were assayed in the afternoon of the same day.

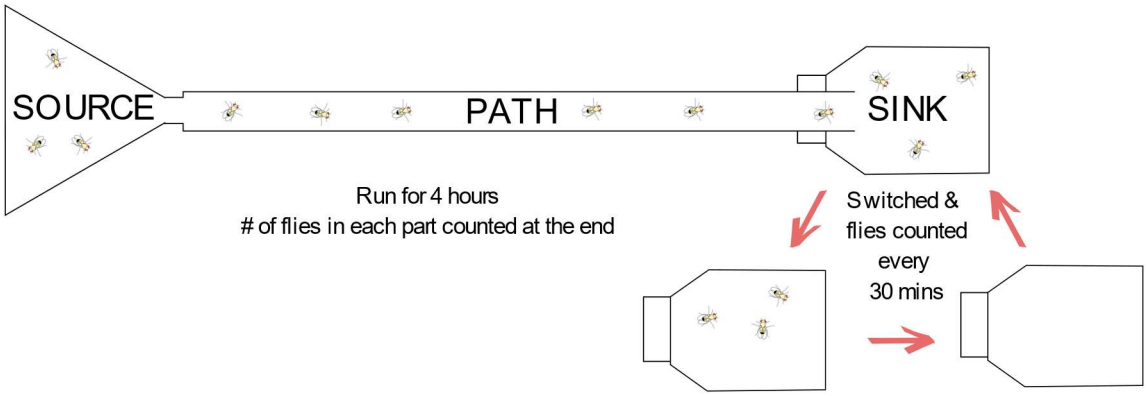


Fig. 4: Set-up for dispersal assay

Setup and Protocol

The set-up consisted of a source, a path and a destination, all devoid of food and water. The path was a 2m long transparent pipe. A 100ml glass flask was used as

the source and a 250ml plastic fly bottle as a destination, or sink (Fig. 4). The mouth of the was fitted with a sponge plug with a hole in it, through which the end of the path pipe passed. The pipe protruded into the destination through a plastic nozzle, done to prevent backflow of the flies (Tung et al. 2018).

Pooling the previously sorted vials having ~10 flies each, 120 flies of the same sex, of either the related or unrelated treatment, were transferred to the source and allowed to disperse. After every thirty minutes the sink container was removed and replaced, and the number of flies in it was counted. This process was repeated for 4 hours, and then the setup was dismantled flies in the source and the path were also counted.

Data Collection and Statistical Analysis

The following dispersal traits were measured (Mishra et al., 2018):

- **Dispersal Propensity** – the proportion of flies that left the source and initiated dispersal. This value was arcsine-square root transformed for further analysis (Zar, 1999).

$$Dispersal\ Propensity = \frac{\Sigma_i(n_i) + n_p}{N}$$

- **Dispersal Speed** – the average speed at which the dispersers completed source-sink movement.

$$Dispersal\ Speed = \frac{\Sigma_i \left(\frac{d}{T_i} \times n_i\right)}{\Sigma_i n_i}$$

Where, d = path length (2m, here), N is the total number of flies in the setup, n_i is the total number of flies that reach the destination in the i^{th} time interval, n_p is the number of flies in the path at the end of the assay and T_i is the total time in hours since beginning of the assay at the end of the i^{th} interval.

Both dispersal speed and dispersal propensity data were analysed using GLMs, with Sex (2 levels: Male/Female) and Relatedness (2 levels: Related/Unrelated) as fixed factors, with a random factor added of timing (2 levels: Morning/Afternoon) to account for half the replicates being done at different points in time. The mated and virgin experiments were analysed separately as they were done on separate occasions.

2.3.4. Social Aggregation

2.3.4.1. Related and Unrelated groups

Fig. 5: Set-up for social aggregation assay

Collection of flies

Flies were bred as in Section 2.2. As explained in 2.3.3, flies were sorted into related and unrelated treatments (Fig. 3). In this case, at a density of 3 flies per vial. Each related treatment replicate consisted of 12 such vials, to give 36 flies in total. For the unrelated treatment, 12 vials of 3 flies each were made from 12 different lineages. This was done for both sexes, separately. 8 replicates for each treatment (Sex × Relatedness) were made in this fashion.

Setup and Protocol

The test chamber used has been previous described and standardised by Simon et al (2012). It consists of three square plexiglass plates (19 × 19 × 0.3 cm), placed on top of each other. The middle plate has a triangular cut-out (base = height = 15.2 cm), and this is the area within which the flies are confined to a two-dimensional space. On the 13th day post egg collection, 36 flies of a single sex, where all flies were either from the related or unrelated treatment, were loaded by aspiration into the chamber. This was done through a small hole at the bottom of the middle section, which was then plugged with cotton. The set-up was mechanically disturbed to ensure all flies were at the bottom of the test chamber, and was then placed

vertically. The setup was backlit with an LED panel to ensure even lighting and contrast of the flies against the background. The setup was video recorded (Sony HDR, GoPro Hero 7) and flies were given 30 minutes to acclimatise and reach stable positions. At a time, one replicate from each treatment were set up simultaneously.

Data Collection and Statistical Analysis

Digital images were isolated from the videos at 30 minutes post setup. Using ImageJ, the Nearest Neighbour Distance (NND) for each fly was measured followed protocols described in McNeil et al. (2015). From this, we calculated the below quantity for flies in each replicate.

$$Social\ Interaction\ Index\ (SII)\ = \frac{Number\ of\ flies\ with\ NND\ < 0.5cm}{Total\ number\ of\ flies}$$

A GLM was used to analyse this data, wherein Sex (2 levels: Male/Female) and Treatment (2 levels: Related/Unrelated) were fixed factors in the analysis.

2.3.4.2. Studying Kin-Specific Grouping

Collection of flies

Flies were bred as in Section 2.2. Flies were sorted into related and unrelated groups as explained in 2.3.4.1 (Fig. 3). 12 hours prior to the experiment, half the vials of each replicate were coloured either pink or green, with fluorescent powder (DayGlo, Cleveland, OH, USA).

Setup and Protocol

The assay was carried out in a similar fashion as the previous social aggregation experiment. For each treatment, a single replicate for this experiment consisted of the test chamber 18 pink coloured flies from one lineage, with 18 green flies from another lineage. These flies were loaded by aspiration, with flies of differing colours loaded separately to ensure that the coloured powders did not mix. The setup was recorded (Sony HDR-PJ410, Sony HDR-CX405), and flies were allowed to acclimatise to the test chamber for 30 minutes till they attained stable positions. The lighting for this experiment was done differently than the previous social aggregation experiment, with the LED panel placed opposite to the test chamber, to ensure that the colours were easily differentiable in the recording.

Data Collection and Statistical Analysis

Photos were captured from the recordings 30 minutes after setup. Using ImageJ, all pairwise-distances between flies were calculated. For each fly, the number of neighbouring flies (within 0.5cm) that were either related (same lineage), or unrelated (different lineage) was noted. Within a replicate, the mean across all individuals of the number of related and unrelated neighbouring flies was calculated. These means were analysed using a GLM with Sex (2 levels: Male/Female), lineage ID of neighbouring fly (2 levels: Related, Unrelated) as fixed factors, replicate number as a random factor, and mean number of neighbouring flies as the dependent variable. The analysis was done separately for mated and virgin flies.

2.3.5. No-Choice Mating Experiment

Male and females can both exercise control over the different aspects of the mating process. The length of copulation is said to be under mainly male control (MacBean & Parsons, 1967), whereas females have to accept mating attempts from males in order for mating to begin. However, males can modulate their courtship efforts (Griffith & Ejima, 2009), and which can result in a reduced time till first mating. In this assay, we study the behaviours of both males and females in the presence of an unrelated or related individual of the opposite sex.

Collection of flies

For each test fly lineage, two vials with similar egg densities in the vial were chosen, to ensure that rearing density did not confound with mating behaviour observed for flies within the same lineage. Egg density is often known to affect body size, and body size can factor into attractiveness of a mate (Lefranc & Bundgaard, 2000). Following virgin collection over the 8^{th} – 10^{th} days, the F_2 generation flies were kept in vials with food at a density of ~20-30 flies per vial. For this assay, flies from 10 different 'lineages' were chosen as test flies, and one 'lineage' was chosen as the unrelated control flies.

In type A experiments, 4 replicates had a male fly from the first vial of that particular lineage, and 4 from the other vial. Similarly, type C replicates were constructed for females. An unrelated fly of the opposite sex placed in both type A and C vials. In type B, all combinations of male × female from the two vials were constructed, with 2 replicates of each kind. This gave a total of 8 vials, of which 4 pairings were familiar male-female pairings, and 4 were unfamiliar vial pairings (refer Fig. 6).

Setup and Protocol

The assay was carried out on the 11^{th} day post egg collection for the flies. In this assay, one virgin male-female pair of flies were introduced by aspiration in a vial with agar to prevent desiccation. Once both flies had been introduced into the vial, the cotton plug was pushed in, to restrict space and thus ensure that the pair came in contact with each other. The vial was placed horizontally and the behaviour of the

pair was recorded using a video camera (Sony HDR-PJ410, Sony HDR-CX405). 12 pairs were recorded by a single camera, for a duration of ~1 hour (Fig. 7).

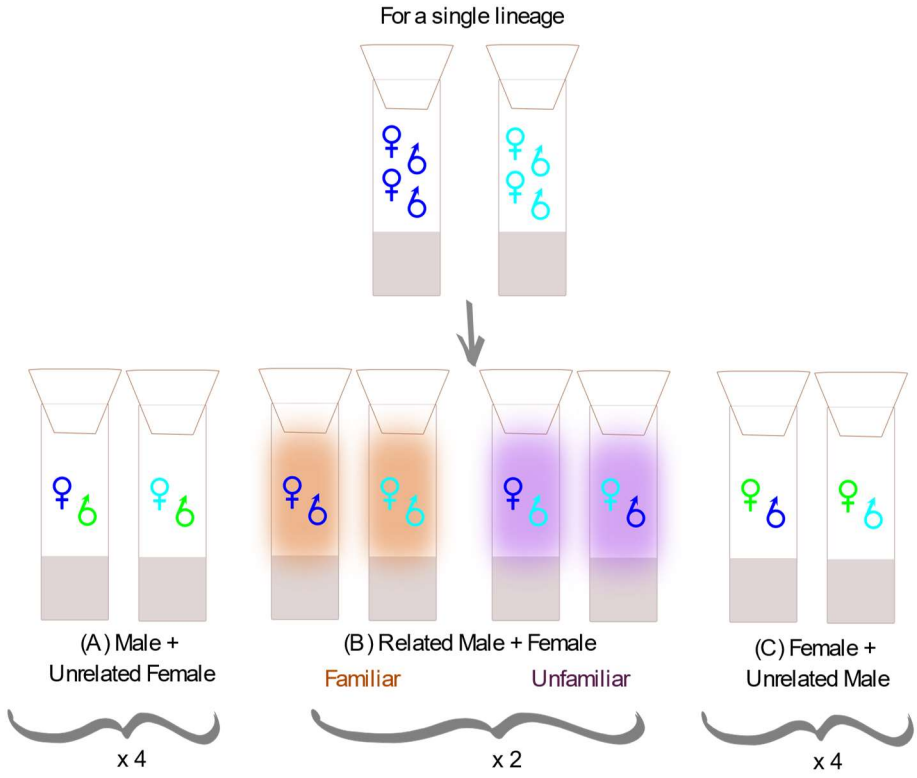


Fig. 6: Mating pairs set-up for no-choice mating assay. Blue symbols are from the same lineage (dark and light blue from different vials), and green represents the unrelated control flies. Diagram shows how pairs were made for 1 lineage, and 10 lineages were used for the experiment.

Data Collection and Statistical Analysis

These videos were manually scored and the time of setup of each vial, as well as beginning and end times of mating was noted. From these times we determine 'Time Taken to Mate' (time taken to initiate the first successful copulation, **TM**) and 'Copulation Duration' (time from beginning to end of mating, **CD**). Any replicates where time taken to mate exceeded 1 hour were not included in the analysis. A bout of copulation was deemed successful if it lasted for 3 minutes or more (G. L. Fowler, 1973). We compared the dependent variables of TM, and CD through GLMs, with fixed factor of Mate (2 levels: Related/Unrelated) and lineage of test fly as a random factor.

For treatment (C), TM and CD of replicates in which the flies were related and unfamiliar were compared to those in which flies were related and familiar. This was also done using GLMs as specified above.

2.3.6. **Mate Choice Experiment**

This assay was performed to check if flies were biased towards mating with either related or unrelated mates when given a choice, and to further check whether mating behaviours varied between treatment groups in a mate choice scenario.

We assayed both males and females separately, to check if either sex displayed a mate choice bias. For the male mate choice experiment, each replicate consisted of a male in a vial, with a female from its own lineage and an unrelated female. This related male could either be familiar or unfamiliar, as explained in the introduction. This same setup was followed but with the sexes swapped for the female mate choice experiment.

Collection of flies

Vials for each test fly lineage, were selected as in Section 2.3.5. Following virgin collection over the 8^{th} – 10^{th} days, the F_2 generation flies were sorted under light CO_2 anaesthesia, and the flies in each vial were randomly assigned to 3 groups and kept in separate vials. Flies in two of these groups would be coloured either pink or green for use as the related mate in the mate choice set up. Reciprocally coloured replicates would then be setup to ensure that there was no colour bias in mate choosing. The final vial would provide test flies for mate choice for that particular sex. 10 lineages were used for the assay, and this meant a total of 80 replicates each for male and female mate choice experiments. Of this, 40 had related-familiar mates as the related mate option, and 40 had related-unfamiliar mates (refer Fig. 8).

Setup and Protocol

12 hours prior to the assay, flies to be used as the competing mates were coloured using either pink or green fluorescent powder (DayGlo, Cleveland, OH, USA). At the time of the assay on the 11^{th} day post egg-collection, the two competing mates (related/unrelated to test fly) were introduced first by aspiration in a vial. This vial

contained agar to prevent desiccation. Right before recording, the test fly was aspirated into the vial. Once all three flies are introduced into the vial, the cotton plug was pushed in to restrict space and thus ensure that the flies came in contact with each other. As in the no mate choice experiment, 12 such vials were placed horizontally (Fig. 7) and the behaviour of the pair was recorded using a video camera for an hour (Sony HDR-PJ410, Sony HDR-CX405).

Data Collection and Statistical Analysis

These videos were manually scored. We noted whether the test male/female mated with an unrelated or related fly. Using one-tailed chi-square tests, we compared the numbers of related vs unrelated mates chosen. Depending on whether the related mate provided in each setup was familiar or unfamiliar to the choosing fly, these numbers were collated and compared separately. Additionally, to check if colour of the mate influenced the choice made, the colour of the chosen mate for each mate was noted, and the numbers of pink and green mates chosen were also compared using a chi-square test.

Alongside identifying the type of mate chosen in each replicate, the time of setup of each vial, as well as beginning and end times of first mating were noted. From these times we determined 'Time Taken to Mate' (time taken to initiate the first successful copulation, **TM**) and 'Copulation Duration' (time from beginning to end of mating, **CD**). Any replicates where time taken to mate exceeded 1 hour were not included in the analysis. A bout of copulation was deemed successful if it lasted for 3 minutes or more (G. L. Fowler, 1973). With either TM or CD as the dependent variable, a GLM was used to analyse this data, with fixed factors of relatedness of mate chosen (2 levels: Related/Unrelated) and familiarity of related mate provided (2 levels: Familiar/Unfamiliar), and lineage of the choosing fly as a random factor.

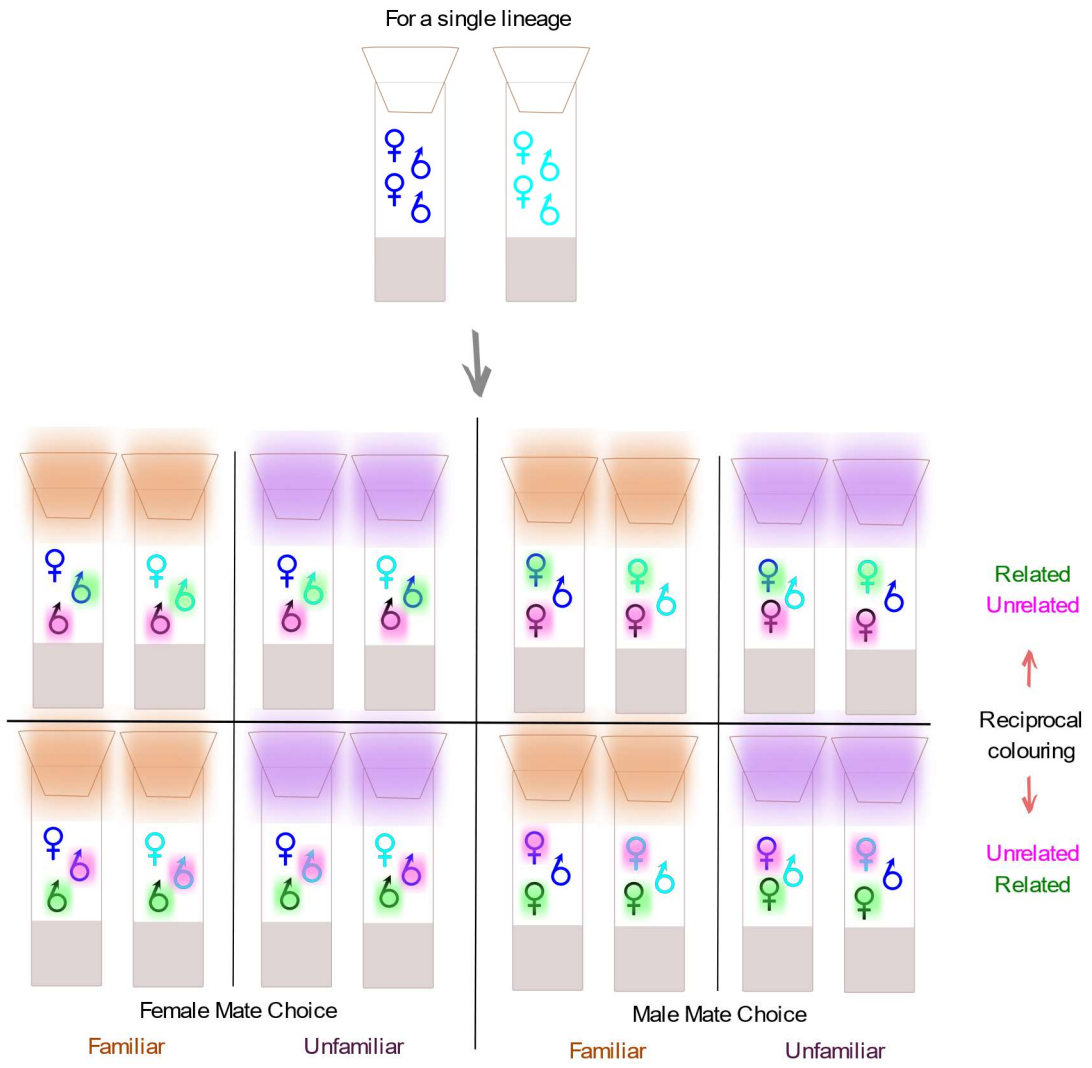


Fig. 7: Mating treatments set-up for mate choice assay. Blue symbols are from the same lineage (dark and light blue from different vials), and black represents the unrelated control flies. The green and pink indicate the fluorescent powder colouring that was done to be able to identify unrelated and related same sex flies in each vial. Diagram shows how pairs were made for 1 lineage, and 10 lineages were used for the experiment.

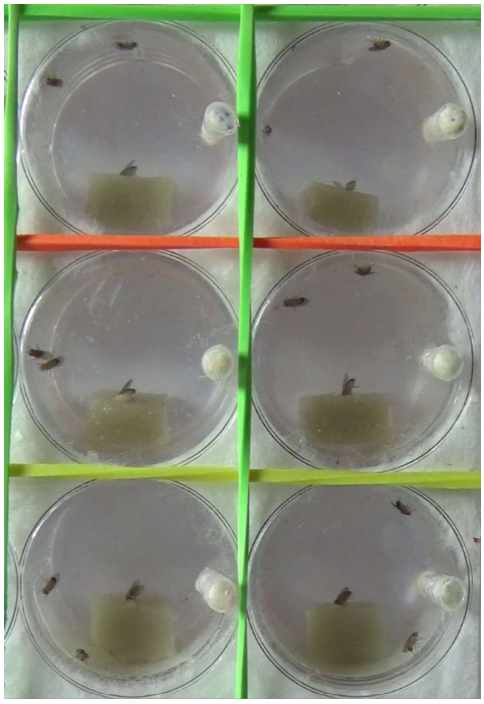


Fig. 8: Set-up to record male-male aggression

2.3.7. Male-male Aggression

Collection of flies

Flies were reared as in Section 2.2. We assayed both mated and virgin flies simultaneously. On the 11th day, male F2 flies were separated by sex under light CO2 anaesthesia, and males were kept in isolation for the next 48 hours (1 fly in one 5mm glass tube, provided with banana-jaggery food on one side). It has been observed that fighting is reliably observed after isolation (Chen *et al.*, 2002).

Setup and Protocol

The apparatus for the assay was a twelve-well tissue culture plate (Corning®, NY, USA), of which six wells were used, each well serving as one replicate. A rectangular piece of banana-jaggery food was placed at the bottom of each well. A decapitated female (taken from a different lineage from all the lineages from which the males were used in the assay) was stuck using yeast paste to the centre of each food piece. Both the food and the female serve as points of defence and thus conflict for the males, promoting aggressive encounters (Chen et al., 2002). Individual wells

were isolated by using cotton to cover all sides of the well, to ensure that there is no exchange of visual cues between the replicates.

On the 13th day, two male flies were introduced into each arena, either from the same lineage or from two different lineages. The set-up was placed vertically to ensure clear visualization of their movements (Fig. 2), with an LED backlight to provide ample light conditions for video recording. Their interactions were recorded for 30 minutes for video camera. 48 such replicates were assayed for both the treatments of kin or non-kin. wherein half were flies that had previously been mated, and half were virgins.

Data Collection and Statistical Analysis

Each replicate was given a five-minute period of acclimatization after which scoring was done for 20 minutes. The beginning and end time of any interaction wherein fighting, chasing or boxing occurred (as defined on the next page), was noted.

- Fighting: One fly raises one of its forelegs and taps/pushes/holds down the other fly. An encounter may have multiple instances of fighting.
- Boxing: Both the flies have both their forelegs raised and are attacking each other with them.
- Chasing: One fly runs after another fly.
- The end time of each bout was determined when one of the flies retreated or turned away from the other. The proportion of total time spent in all aggressive encounters was summed and calculated, and this value was arcsine-square transformed for further analysis (Zar, 1999). With the proportion of aggression as the dependent variable, analysis using a GLM was done, where treatment (2 levels: Related/Unrelated) and mating status (2 levels: Mated/Virgin) were fixed factors.

2.4. **Statistical Analysis**

Mated and virgin treatments were analysed separately for all assays except the aggression experiment, as they were performed separately. For mate choice tests, we used chi-square goodness of fit tests to evaluate whether bias was seen in

choice of mate and colour of mate chosen. All other analyses used General Linear Models done using Statistica 8. For mixed models involving a random factor, all interactions between fixed factors were evaluated, and the random factor was added but not crossed with the other fixed factors as we were not interested in the higher order interactions of the random effect. Models wherein the random factor was significant were further analysed with crossing the random factor with fixed factors, however the interpretation of the results remained essentially unchanged and therefore these analyses are not reported.

2.5. **Calculation of Relatedness**

Assumptions

- All organisms are equally unrelated at F_0.
- Calculations assume simplistic 2 chromosome per individual model with no recombination.
- All combinations of genetic material are allowed and equally probable.

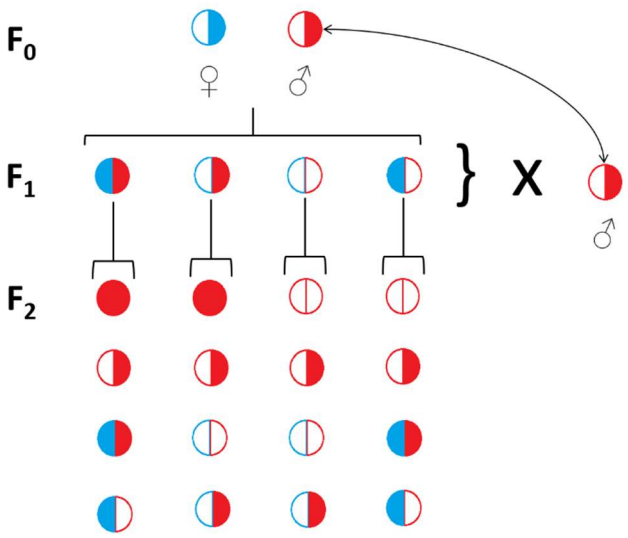


Fig. 9A: Representation of inbreeding treatment described in Section 2.2. Semi-circles represent chromosomes, where each colour represents a different chromosome. Here, 2 colours have been used for simplicity, however there is no relation between filled and empty semi-circles as both are meant to represent different genetic backgrounds.

As per this model, F_1 siblings can be related to each other as given by the three cases below (Fig. 9B).

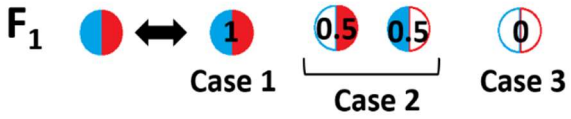
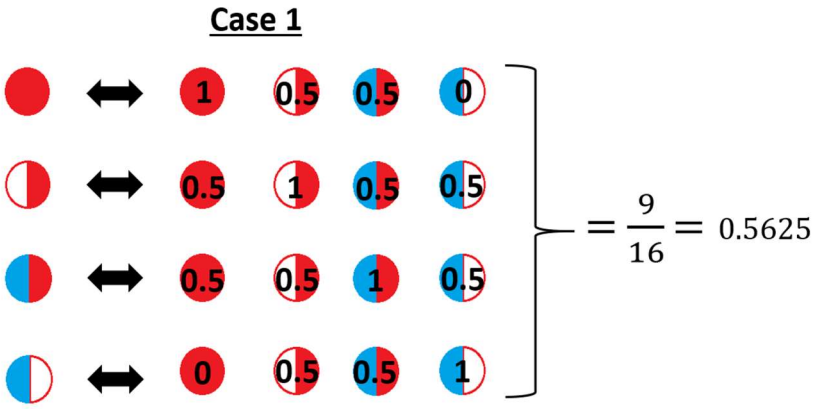
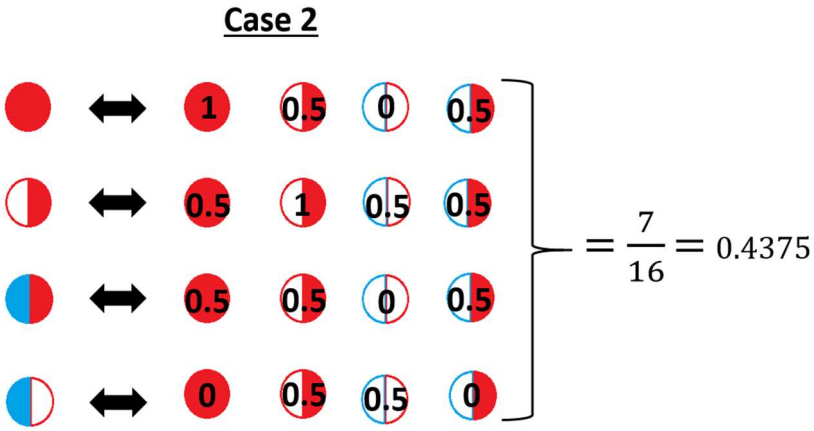


Fig. 9B: Relatedness between F_1 siblings. Here, we randomly choose to compare solid blue – solid red to each possible siblings. Two F_1 siblings can have a relatedness coefficient of 1, 0.5 or 0.

Below, we calculate average relatedness between the offspring of two F_1 mothers.





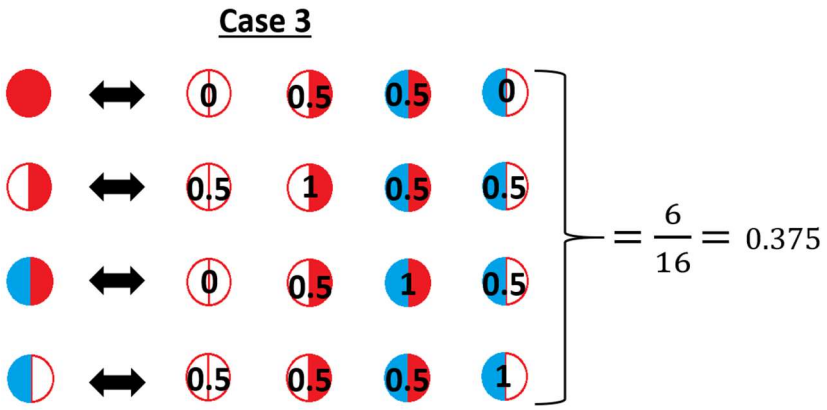


Fig. 9C: We have calculated the relatedness for the offspring of F_1 solid blue – solid red with the offspring of siblings related to it by a relatedness coefficient of 1 (solid blue – solid red), 0.5 (empty blue – solid red) or 0 (empty red – empty blue). The final number demonstrates the average relatedness for two randomly chosen F_2 offsprings of these F_1 mothers.

Average relatedness of flies:

a) from two different vials = $(\frac{9}{16} + \frac{7}{16} + \frac{7}{16} + \frac{6}{16}) \div 4 = \frac{29}{64} = 0.453$

b) in dispersal or social aggregation assay (section 3.5)

- Related group = $(\frac{1}{6} \times \frac{9}{16}) + (\frac{5}{6} \times \frac{29}{64}) = 0.471$
- Unrelated group = $(\frac{1}{12} \times \frac{29}{64}) = 0.038$

3. **RESULTS**

In all boxplots, the points represent the data for all replicates of the particular group with small random jitter on the x-axis (provided to aide in the visualisation of the data), the edges of the box denote the 25th and 75th percentiles, the black solid line represents the median. The mean is represented by a black filled triangle. The whiskers extend to the extreme data point, which is no more than 1.5 times the inter-quartile range from the top or bottom of the box.

3.1. **Inbreeding Depression**

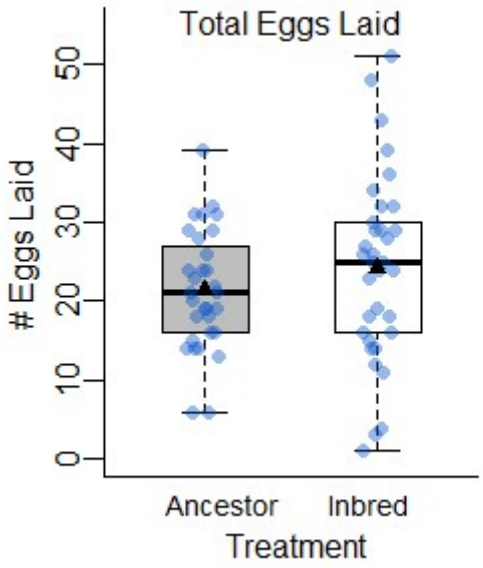


Fig. 10: Number of eggs laid by flies from ancestral population and flies from the inbred rearing treatment.

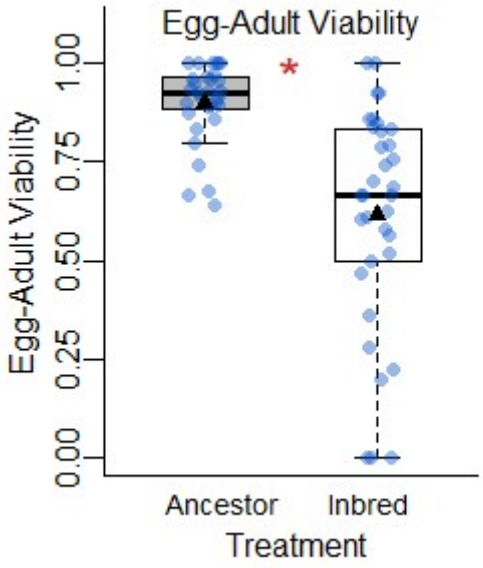


Fig. 11: Egg-adult viability in eggs laid by flies from ancestral population and flies from the inbred rearing treatment.
** indicates p < 0.05 for treatment*

There was no significant difference in the number of eggs laid by flies from the inbreeding rearing treatment and the flies from the ancestral population (Fig. 10, $F_{1,14} = 1.303$, $p = 0.273$). Therefore, we did not have to adjust the egg-adult viability ratios for egg numbers.

Flies from the inbreeding treatment had significantly lower egg-adult viability than the ancestral population (Fig 11, $F_{1,14} = 14.4675$, $p = 0.0018$).

3.2. **Locomotor Activity and Rest**

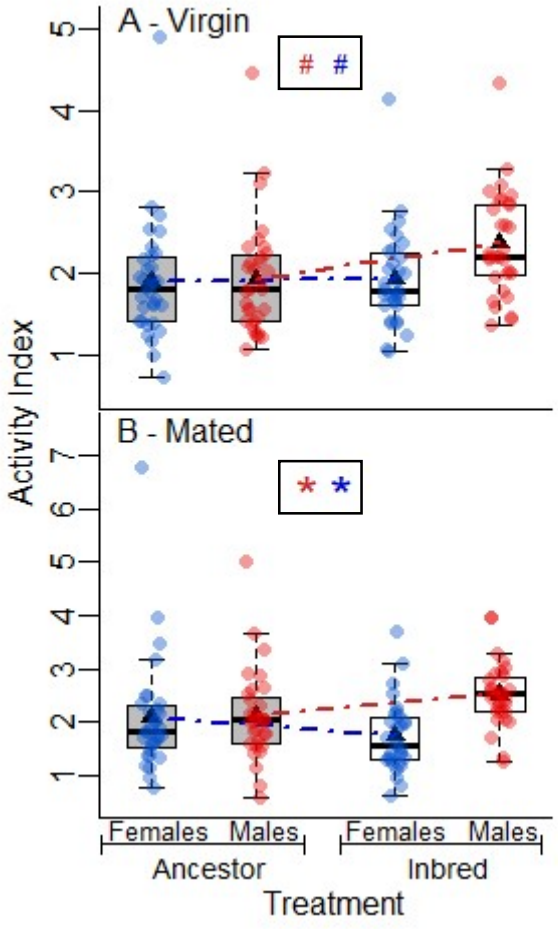


Fig. 12: Activity Index over 6 hours for both sexes of A. virgin flies and B. mated flies, of inbred rearing treatment and the ancestral control.

** indicates p < 0.05 for interaction*

** indicates p < 0.05 for sex*

 # indicates p < 0.1 for treatment

 # indicates p < 0.1 for sex

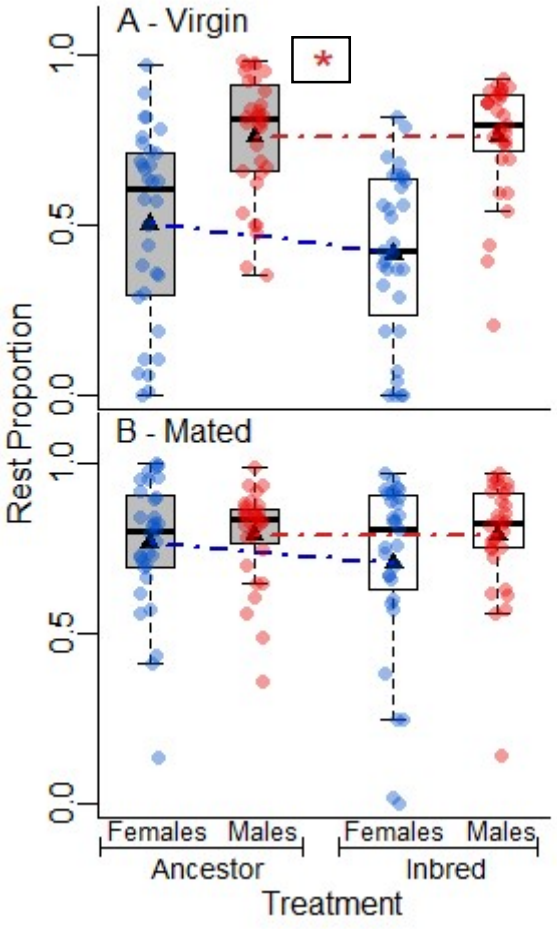


Fig. 13: Proportion of time spent resting over 6 hours for both sexes of A. virgin flies and B. mated flies of inbred rearing treatment and the ancestral control.

** indicates p < 0.05 for sex*

In virgin flies (Fig. 12A), there was no interaction of the rearing treatment with sex on activity index (refer to Table 1 for results of statistical analysis). Sex had a marginally insignificant effect, with a small effect size ($\eta_p^2 = 0.030$) of males being more active than females. We also see a marginally insignificant, small effect size trend of inbreeding leading to a higher AI ($\eta_p^2 = 0.029$).

For mated flies (Fig. 12B), while there was a significant, but small effect size interaction of rearing treatment and sex ($\eta_p^2 = 0.048$), on performing Tukey's HSD post-hoc test, we saw that there were no pair-wise differences between inbred and ancestral groups for either sex (for males $p = 0.241$, females $p = 0.348$). Activity index was not affected by the rearing treatment. Males had a higher AI than females, however this had a small effect size ($\eta_p^2 = 0.061$).

The proportion of time spent resting was not affected by treatment in virgin flies (Fig. 13A), though male flies seemed to spent more time resting compared to females overall. There was no interaction of treatment and sex on rest for virgin flies. Mated flies also did not have differing rest proportions depending on rearing treatment (Fig. 13B). Here, males and females did not differ in the proportion of time they spent resting. There was no interaction of treatment and sex for mated flies either.

The locomotor activity and rest results show that inbred and ancestral flies do not differ greatly (as effect sizes were small) in their activity indices and rest proportions, indicating that the flies used in the subsequent experiments have not incurred morphological or physiological damage that could affect their behaviour. We thus believe all subsequent results presented in this thesis can be interpreted reliably and used as a guideline in approaching kin-biased social interactions in *Drosophila melanogaster*.

3.3. **Dispersal Propensity and Speed**

For virgin flies (Fig. 14A), relatedness affected dispersal propensity, with related groups dispersing more than unrelated groups (refer to Table 3 for results of statistical analysis). There was no significant effect of sex, and no interaction between sex and relatedness.

For mated flies (Fig. 14B), dispersal propensity was again affected by relatedness, in this case however, unrelated flies dispersed more than related flies. Sex had a significant effect, with males dispersing more than females. There was no effect of interaction between sex and relatedness on propensity. Timing of the setup did not change dispersal propensity for either virgin flies ($F_{1,25} = 0.990$, $p = 0.329$) or mated flies ($F_{1,23} = 0.788$, $p = 0.384$).

In virgin flies (Fig. 15A), dispersal speed was not altered by either sex or relatedness, and there was no interaction between sex and relatedness. In mated flies too (Fig. 15B), there was no effect of the interaction between sex and relatedness on dispersal speed. Sex had a significant effect, with males dispersing faster than females. Relatedness did not affect dispersal speed. Timing did not affect dispersal speed in either virgin ($F_{1,25}$ = 1.484, p = 0.234) or mated flies ($F_{1,23}$ = 2.709, p = 0.113).

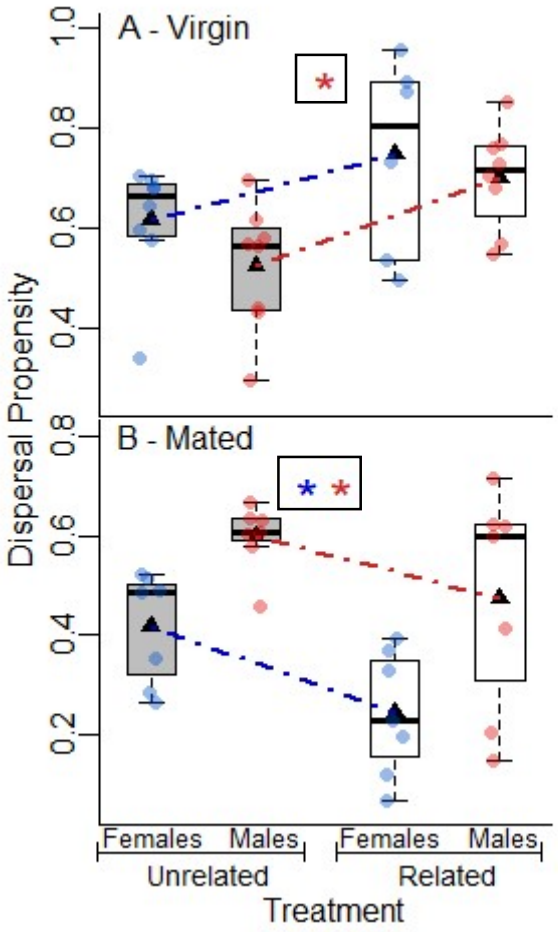


Fig. 14: Propensity to disperse away from the source for both sexes in A. virgins and B. mated flies for related groups and their unrelated control groups.

* *indicates p < 0.05 for relatedness*

* *indicates p < 0.05 for sex*

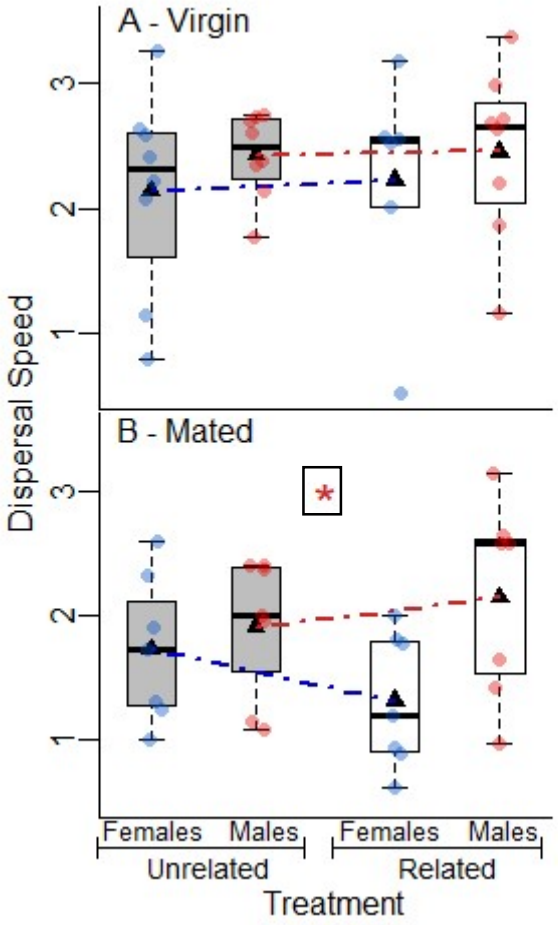


Fig. 15: Dispersal speed for both sexes in A. virgins and B. mated flies for related groups and their unrelated control groups.

* *indicates p < 0.05 for sex*

Thus, we see that the dispersal propensity of groups of *Drosophila melanogaster* changes depending upon the relatedness between themselves and others in the group. The direction of this change in related groups, relative to unrelated groups, is further modulated by whether or not the flies were previously mated with their siblings. Dispersal speed, however, does not change for related or unrelated groups for either virgin or mated flies. However, mated females disperse much less, and at a slower rate than mated males.

3.4. **Social Aggregation**

In virgin flies (Fig. 16A), there was no effect of relatedness on SII (refer to Table 3 for results of statistical analysis). There was a main effect of sex, with males having a lower SII than females. There was no interaction of sex or relatedness.

In mated flies (Fig. 16B), on the other hand, there was a marginally insignificant main effect of relatedness on SII, with a medium effect size ($\eta_p^2 = 0.111$). Related flies had a higher SII than unrelated flies, indicating that they showed a higher tendency to have social interactions. Sex had a marginally insignificant effect, with a medium effect size ($\eta_p^2 = 0.107$), where males showed a reduced SII compared to females. There was no interaction of sex or relatedness.

In virgin flies (Fig. 17A), the ID of neighbouring flies did not have a significant effect on the average number of neighbours. There was a significant effect of sex, with females having more neighbouring flies within 0.5cm than males. There was no interaction between the ID of the neighbouring flies and sex. The random factor of replicate had a significant effect ($F_{7,21} = 4.291$, $p = 0.004$) on the average number of neighbours, as one particular replicate had a higher average number of neighbours than all the others.

In mated flies (Fig. 17B), there was no significant effect of the interaction between ID of neighbouring fly and sex on the average number of neighbours. Neither sex or ID significantly altered the average number of neighbours. Replicates in this case did not seem to differ from each other, with the random factor of replicate not showing a significant effect ($F_{7,21} = 1.23$, $p = 0.331$).

To summarise, incidences of social interactions was higher in related groups, and for females, but only when flies were mated. The ID of neighbouring flies however was not biased towards either related or unrelated flies, for any of the treatments assayed.

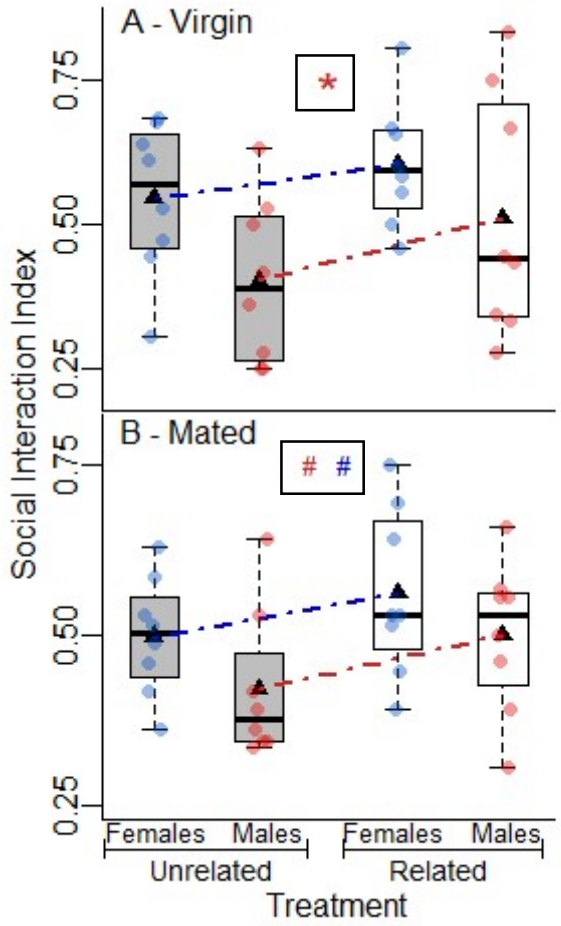


Fig. 16: Social Interaction Index for both males and females, for A. virgin, and B. mated fly groups that are related to each other and their unrelated controls.

** indicates p < 0.05 for sex*

 # indicates p < 0.1 for sex

 # indicates p < 0.1 for relatedness

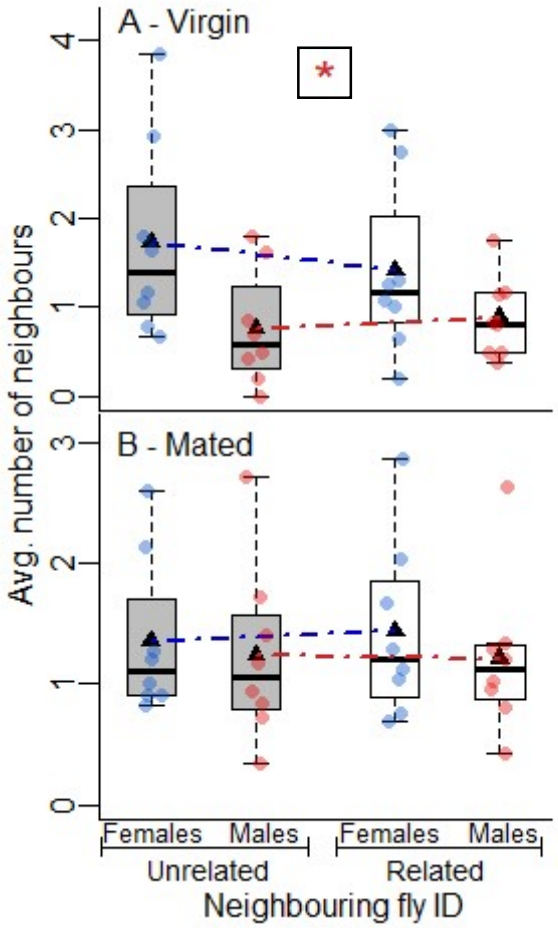


Fig. 17: Avg. number of neighbouring flies in both males and females, which are either related or unrelated, for A. virgin, and B. mated fly groups.

** indicates p < 0.05 for sex*

3.5. **No-Choice Mating Experiment**

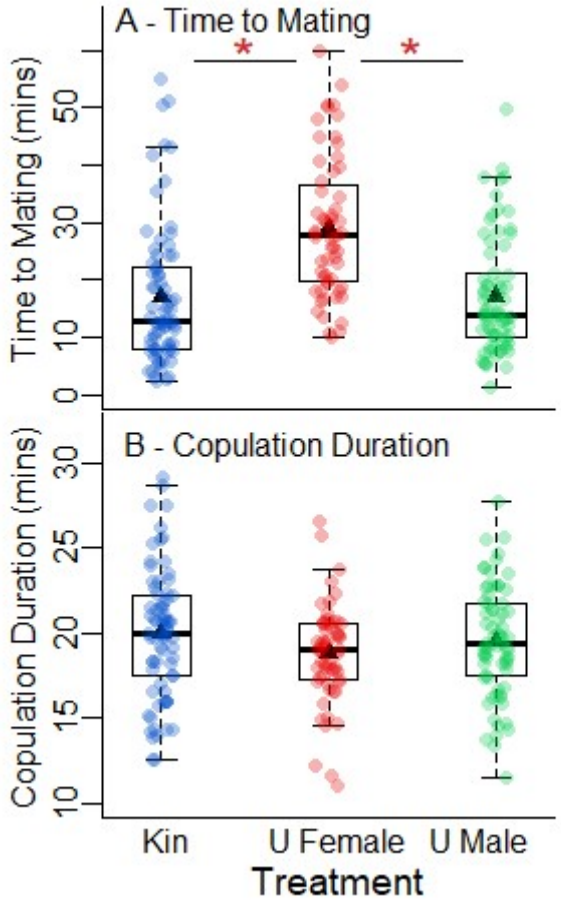


Fig. 18A: Time to mating for pairings between kin pairs, test males and unrelated females (U Female) and test females and unrelated males (U Male).

Fig. 18B: Copulation duration for pairings between kin pairs, test males and unrelated females (U Females) and test females and unrelated males (U Male).

* *indicates $p < 0.05$ for Tukey's HSD*

Time taken to start mating varied with treatment (Fig. 18A, refer to Table 2 for results of statistical analysis). Post-hoc test of Tukey's HSD showed that this was due to the UF treatment being significantly different from the other two treatments ($p = 2.17E-05$ in both cases), though MF and UM did not differ from each other. The random factor of lineage did not affect the time taken to start mating.

Treatment did not have a significant effect on the copulation duration (Fig. 18B). However, the random factor of lineage used in each replicate did affect the copulation duration.

Familiarity did not appear to play a role in either time taken to start mating (Fig. 19A), or copulation duration (Fig. 19B). The random factor of lineage had a significant effect on the length of copulation, but not on the time taken to start mating.

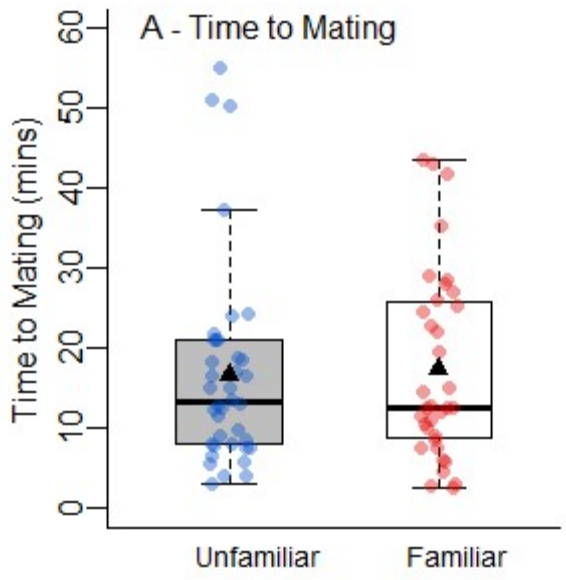


Fig.19A: Time to mating for test females mating with either related-familiar males or related-unfamiliar males.

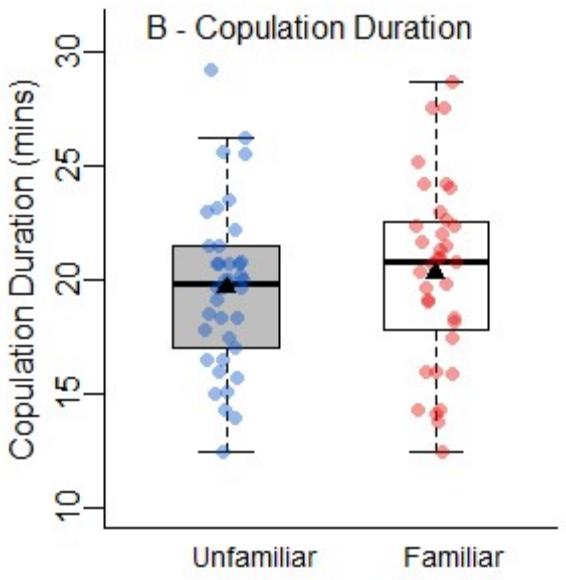


Fig. 19B: Copulation duration for test males mating with either related-familiar females or related-unfamiliar females.

3.6. **Mate Choice Experiment**

Single tailed chi-square tests were performed to check for a bias in colour preference for the mate chosen in male and female mate choice experiments (refer to Table 5 for results of statistical analysis). There was no colour preference displayed by females (Fig. 20A), both when the related fly provided was familiar and unfamiliar (N = 36, 18 pink flies were chosen and 18 green flies were chosen). For the male mate choice experiment, there was no colour bias present when the related mate provided was familiar, but a significantly larger proportion of green flies were chosen when the related mate was unfamiliar (Fig 20B). Hence, for male mate choice, we cannot be sure that mate choice is not influenced by colour of the mate. For females, however, we can interpret further results as uninfluenced by colour bias in mate choice.

For the female mate choice experiment (Fig. 21A), we see that there is a significant bias toward choosing related flies when they are familiar, but this bias was not present when the related fly was unfamiliar. There was no such bias observed in males either when the related mate provided was familiar or unfamiliar (Fig. 21B).

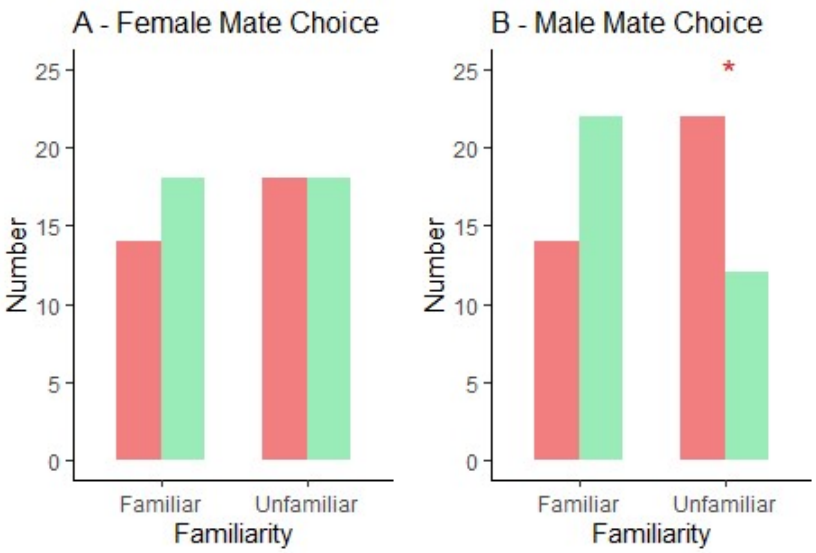


Fig. 20: Colour of mate chosen by A. females, and B. males when the related mate provided is either familiar or unfamiliar.
** indicates chi-square*
$p < 0.05$ for colour of mate

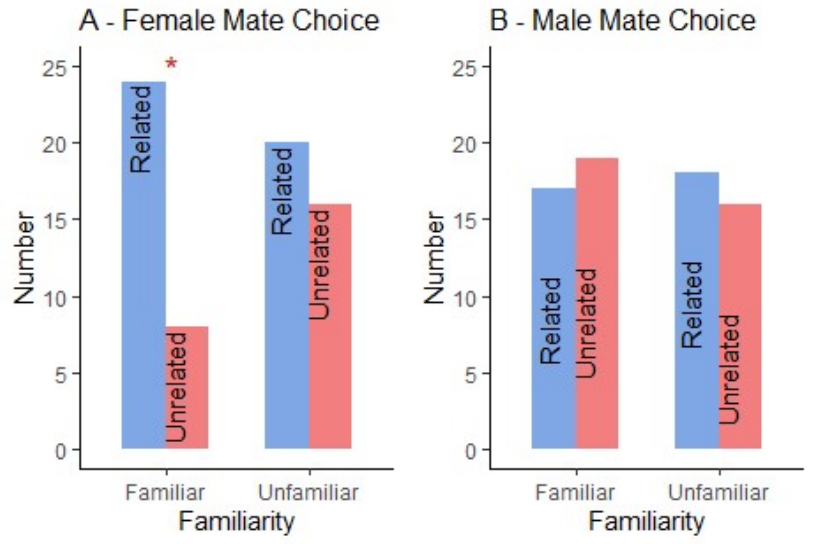


Fig. 21: Mate chosen by A. females, and B. males when the related mate provided is either familiar or unfamiliar.
** indicates chi-square*
$p < 0.05$ for relatedness of mate

In the male mate choice experiment (Fig. 22B, 23B), there was no interaction effect of the mate chosen and the familiarity of the related mate provided on either mating behaviour studied (refer to Table 4 for results of statistical analysis). The main effect of familiarity did not significantly affect either the duration of copulation or time taken till mating. Though mate chosen did not affect copulation duration, there was a marginally insignificant, but low effect size trend of time taken till mating being longer when the mate chosen was related ($\eta_p^2 = 0.064$). There was no effect of the random factor of lineage on time taken till mating, however copulation duration was affected significantly, though this had a small effect size ($\eta_p^2 = 0.271$).

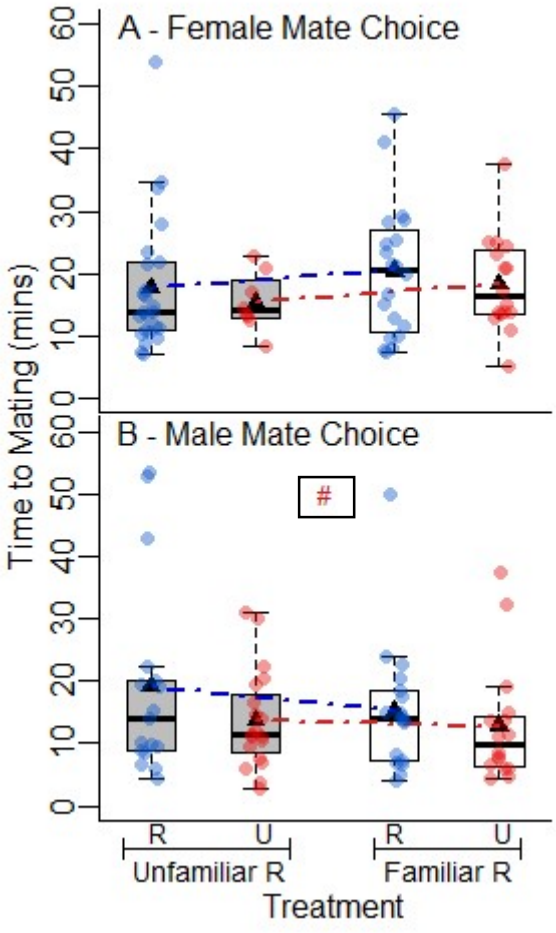


Fig 22: Time to mating in A. Female Mate Choice and B. Male Mate Choice experiments. The treatments compared are those in which the related mate provided was either familiar or unfamiliar, and within this, the mate chosen was either related or unrelated.

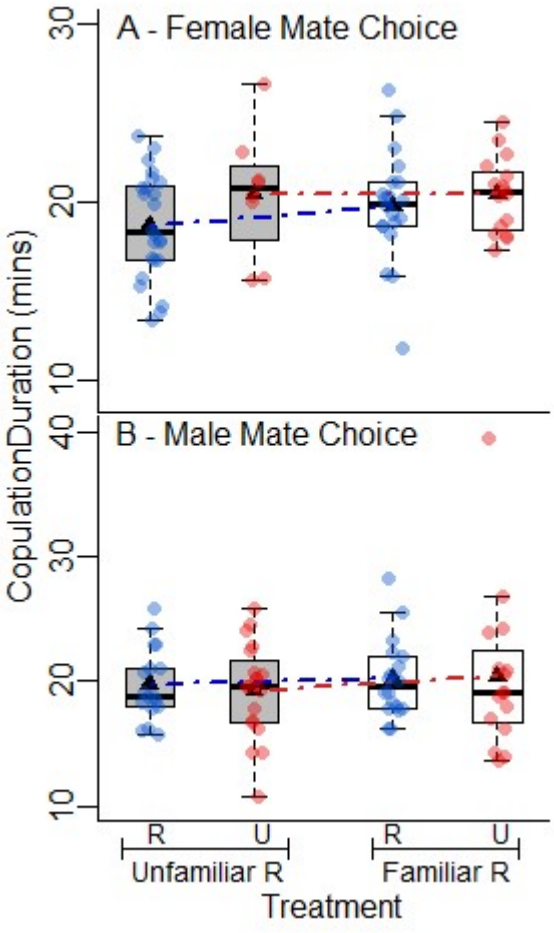


Fig 23: Copulation duration in A. Female Mate Choice and B. Male Mate Choice experiments. The treatments compared are those in which the related mate provided was either familiar or unfamiliar, and within this, the mate chosen was either related or unrelated.

In the female mate choice experiment (Fig. 22A, 23A), there was no interaction of the mate chosen or the familiarity of the related mate provided on either time to mating or copulation duration. The familiarity did not significantly affect either mating behaviour. Similarly, the mate chosen did not affect these behaviours. There was no effect of the random factor of lineage on copulation duration, however time taken till mating was affected significantly ($\eta_p^2 = 0.364$).

Overall, the time taken till mating and copulation duration data do not provide strong evidence for changes in mating behaviour with kin in either male or female flies,

though there is a weak trend of increased time taken to start mating when males have to choose between related and unrelated females. However, we do observe a bias in the female mate choice, wherein females had a higher likelihood of mating with related-familiar males than unrelated males.

3.7. **Male-male Aggression**

The proportion of time wherein the flies were engaged in aggressive interactions (Fig. 24) was not affected either treatment ($F_{1,84}$ = 0.8713, p = 0.353) or mating status ($F_{1,84}$ = 0.779, p = 0.380). There was also no significance of the interaction of treatment and mating status ($F_{1,84}$ = 0.104, p = 0.747).

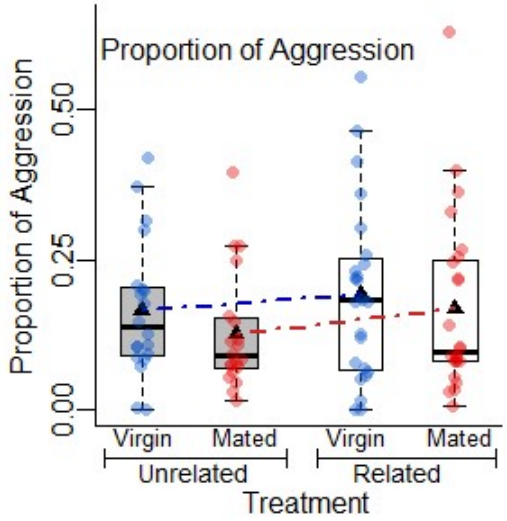


Fig 24: Proportion of time spent in aggression activities between pairs of related or unrelated males which are either both virgins or mated

Assay	p-value			df (effect, error)			F		
	Sex	*Treatment*	*Sex x Treatment*	*Sex*	*Treatment*	*Sex x Treatment*	*Sex*	*Treatment*	*Sex x Treatment*
Activity Index (Virgin)	5.25E-02	5.59E-02	1.04E-01	(1,123)	(1,123)	(1,123)	3.833	3.725	2.793
Activity Index (Mated)	5.98E-03	8.71E-01	1.40E-02	(1,121)	(1,121)	(1,121)	7.830	0.027	6.215
Rest Proportion (Virgin)	2.89E-11	1.86E-01	3.33E-01	(1,123)	(1,123)	(1,123)	53.507	1.765	0.947
Rest Proportion (Mated)	1.83E-01	3.34E-01	3.14E-01	(1,121)	(1,121)	(1,121)	1.795	0.939	1.021

Table 1 (above): p-values, test statistics for locomotory activity and rest assays conducted on inbred and ancestral flies. Table 2 (below): p-values, test statistics for no-choice mating assay. For p-value: red $p < 0.05$ (significant); blue $p < 0.1$ (marginally insignificant).

Assay	p-value		df (effect, error)		F	
	Treatment	*Lineage (Random)*	*Treatment*	*Lineage (Random)*	*Treatment*	*Lineage (Random)*
NMC: Time to Mating	2.36E-09	1.31E-01	(2,191)	(9,191)	22.083	1.555
NMC: Copulation Duration	2.34E-01	3.78E-04	(2,191)	(9,191)	1.464	3.589
NMC: Time to Mating (Familiarity)	7.44E-01	1.34E-01	(1,62)	(9,62)	0.108	1.602
NMC: Copulation Duration (Familiarity)	4.16E-01	4.13E-07	(1,62)	(9,62)	0.672	7.244

Assay	p-value			df (effect, error)			F		
	Sex	*Relatedness*	*Sex x Relatedness*	*Sex*	*Relatedness*	*Sex x Relatedness*	*Sex*	*Relatedness*	*Sex x Relatedness*
Social Aggregation: R vs U (Virgin)	4.23E-02	1.28E-01	6.31E-01	(1,28)	(1,28)	(1,28)	4.526	2.459	0.236
Social Aggregation: R vs U (Mated)	7.70E-02	7.18E-02	8.53E-01	(1,28)	(1,28)	(1,28)	3.370	3.500	0.035
Social Aggregation: Kin-Specificity (Virgin)	2.68E-03	6.40E-01	3.18E-01	(1,21)	(1,21)	(1,21)	11.576	0.226	1.048
Social Aggregation: Kin-Specificity (Mated)	4.82E-01	9.14E-01	8.41E-01	(1,21)	(1,21)	(1,21)	0.512	0.012	0.041
Dispersal Propensity (Virgin)	1.44E-01	3.57E-03	8.63E-01	(1,25)	(1,25)	(1,25)	2.272	10.348	0.031
Dispersal Propensity (Mated)	1.41E-03	1.20E-02	5.86E-01	(1,23)	(1,23)	(1,23)	13.166	7.434	0.305
Dispersal Speed (Virgin)	3.27E-01	8.25E-01	9.02E-01	(1,25)	(1,25)	(1,25)	1.001	0.050	0.016
Dispersal Speed (Mated)	3.95E-02	6.98E-01	1.74E-01	(1,23)	(1,23)	(1,23)	4.765	0.155	1.969

Table 3 (above): p-values, test statistics for social aggregation and dispersal assays. Random factor statistics are not included. For p-value: red $p < 0.05$ (significant); blue $p < 0.1$ (marginally insignificant).

Assay	p-value				df (effect, error)				F			
	Mate Chosen	*Famili -arity*	*Mate Chosen x Familiarity*	*Lineage (Random)*	*Mate Chosen*	*Famili -arity*	*Mate Chosen x Familiarity*	*Lineage (Random)*	*Mate Chosen*	*Famili -arity*	*Mate Chosen x Familiarity*	*Lineage (Random)*
MMC TM	5.3E-02	4.6 E-01	3.6E-01	1.9E-01	(1,57)	(1,57)	(1,57)	(9,57)	3.91	0.55	0.84	1.46
MMC CD	3.2E-01	2.4 E-01	3.7E-01	2.4E-02	(1,57)	(1,57)	(1,57)	(9,57)	0.55	1.39	0.81	2.36
FMC TM	9.6E-02	8.6 E-02	9.9E-01	1.8E-03	(1,55)	(1,55)	(1,55)	(9,55)	2.88	3.06	2.8E-04	3.50
FMC CD	2.1E-01	4.5 E-01	3.9E-01	5.3E-01	(1,55)	(1,55)	(1,55)	(9,55)	1.64	0.58	0.75	0.90

Table 4 (above): p-values, test statistics for mate choice assays. MMC – Male Mate Choice, FMC – Female Mate Choice, TM – Time to Mating, CD- Copulation Duration. Table 5 (below): p-values, test statistics for mate choice and colour bias chi-square tests. For p-value: red $p < 0.05$ (significant); blue $p < 0.1$ (marginally insignificant).

	Colour-bias			Mate choice bias		
Assay	**p-value**	**(degree of freedom, N)**	**X^2**	**p-value**	**(degree of freedom, N)**	**X^2**
FMC: Related-Unfamiliar	-	(-, 36)	-	0.252	(1,36)	0.444
FMC: Related-Familiar	0.24	(1,24)	0.5	0.002	(1,32)	8
MMC: Related-Unfamiliar	0.043	(1,34)	2.941	0.366	(1,34)	0.118
MMC: Related-Familiar	0.091	(1,36)	1.778	0.369	(1,36)	0.111

4. DISCUSSION

4.1. Inbreeding treatment has fitness consequences but not physiological consequences

Though the inbreeding rearing treatment did not result in a reduction in number of eggs laid (Fig. 10), there was a reduction in egg-adult viability in these flies (Fig.11). Inbreeding depression, manifesting at the stage of offspring viability is often observed in inbred populations of *Drosophila melanogaster* (Charlesworth & Charlesworth, 1987). We also see that the inbred flies have much more variation in egg-adult viability compared to the ancestral flies. Such increased variation in traits is often a hallmark of inbreeding in a species (López-Fanjul & Villaverde, 1989; Whitlock & Fowler, 1999).

Despite these fitness consequences, inbreeding did not appear to majorly impact locomotor activity or patterns of rest for either sex (Fig. 12 & 13), which supports a lack of injury or physical impairment in the inbred flies used in assays. Inbreeding effects are known to manifest in other life-history or behavioural traits as flies age. It is possible that by not checking for this, we are not capturing the complete extent of the effects that the inbreeding treatment may have had on the flies. However, as our experiments are performed with flies that had eclosed within a week of conducting the experiments, these age-related effects are unlikely to change our results.

4.2. Aggressive behaviour independent of relatedness between fighting males

We found that aggression did not vary with relatedness (Fig. 24), implying that males do not change their aggressive behaviours in response to kin competition. These results are in contrast to previous work (Carazo et al., 2014, 2015). This group investigated aggressive behaviours displayed between males over a number of days, with observations beginning after a day of setup. The experiments consisted of treatments of three males which were either related to each other or not, in the presence of an unrelated live female. In contrast, our experiments measured aggression during initial encounter between pairs of males, when the female present in the arena was decapitated. It known that male flies modulate their aggressive behaviours towards other dependent on their previous interactions (Penn et al., 2010). It is possible that the presence of a live female that could exercise preference

for a particular male, as well as the pairwise interactions between the flies in the studies by Carazo et al. (2014, 2015) played a role in the reduction of aggressive behaviours displayed between related males. Further experiments wherein pair-wise aggression is assayed both during initial encounter as well as over multiple days would help us further understand how social environment and time given to adjust to said environment play a role in behaviours between kin.

4.3. Time to mating reduces for males paired with related females

In the no mate-choice assay, we saw that when males were paired with unrelated control females, they took a longer time to start mating than when they mated with related females (Fig. 18A). This difference was not observed for females mating with related and unrelated mates. One way to explain this could be that since the unrelated females used in the experiment all had the same genetic background, this particular set of females in general were less receptive to mating attempts. To check this, the assay requires repeating with control females from multiple lineages. Another hypothesis is that males were directing less courtship efforts towards unrelated females as they were to related females, leading to a longer time required for these females to accept mating. In Section 4.4 below, we elaborate further on why the latter hypothesis may hold more weightage.

4.4. Relatedness and familiarity interact to bias female mate choice

Our mate choice experiments corroborate research which argue for kin-biased behaviours in mating (Robinson et al., 2012a, 2012b), wherein we observe that females, given a choice between an unrelated mate and a related mate that it was reared with (until adult stage), tend to choose the related individual (Fig. 21). It is important to note that this bias was not observed if the related mate provided did not share a larval rearing environment with the choosing female.

Unlike previous work done to establish the role of larval familiarity in modulating behaviours towards kin (Hollis et al., 2015b; Le Page et al., 2017), our related-familiar treatment consisted of flies with the same parentage and the related-unfamiliar treatment did not (different sibling mothers). This could have two potential drawbacks, where (a) this leads to a lower level of relatedness between the related-unfamiliar male and female, that is not related enough to be differentiable from an

unrelated individual. Alternatively, (b) an interaction between relatedness and familiarity, as suggested by Le Page et al. (2017), may be required for identifying kin and thus for kin-specific behaviours to occur. This could mean that in the female mate choice experiment, a higher rate of courtship by a related-familiar male may be what is leading to a bias in choice by the female. It should be noted however that there was no difference observed in the time taken to mating when flies were presented with related-familiar and related-unfamiliar mates (Fig. 19A), demonstrating that in a no-choice mating scenario, familiarity was either irrelevant or indistinguishable.

Previous work by Carazo et al. (2014) showed that groups of related males courted a females less frequently compared to groups of unrelated males. These results seem to support the notion that courtship behaviour of males could be modulated by relatedness and familiarity of the mate, depending on the situation in which the male is encountering this mate (i.e. when given a choice or not). Further work would be required to explore this hypothesis.

4.5. Aggregation increases in related groups when mated

Firstly, we see that females showed tendencies to aggregate closer than males (Fig. 16, 17A). This trend was not observed in previous studies wherein male and female aggregation behaviours were seen to be similar (Simon et al., 2012). Aggregation pheromones such as cVA are known to be transferred to females post-mating. Potentially higher amounts of such aggregation cues present on females may be influencing this trend.

Our experiments show that groups of related individuals showed increased grouping behaviours when mated (Fig. 16B). We did not observe this trend in virgin flies (Fig. 16A). Whether there exists an adaptive benefit of aggregation is unknown in *Drosophila melanogaster*, though some studies tout gregarious oviposition behaviours as an explanation of aggregation behaviours in females (Wertheim et al., 2002). This doesn't however explain why we see males display similar aggregation behaviours when mated.

However, we found no evidence for relatedness predicting the identity of neighbours that could interact with individuals in an aggregate (Fig 17).Though our work is the

first to attempt to quantify aggregation in related and unrelated *Drosophila melanogaster* groups, our method of analysis fails to take into account the plastic nature of these groups. Flies have a tendency to join large aggregates rather than smaller ones (Philippe et al., 2016; Saltz, 2011). Initial group formation could be dependent on relatedness, but once a group reaches a certain size it could attract flies independent of their relatedness to other members of the group. Using fly tracking software, we would be able to gain more insight into the role relatedness between flies plays in these other aggregation behaviours.

4.6. Mated females disperse slower than mated males

The relatedness within a group did not affect the speed at which dispersal occurred (Fig. 16), a result that is unsurprising considering that the effect of relatedness on an individual in a group decreases drastically as the individual disperses farther away from the group. We did see however, that a lower proportion of mated females dispersed, and did so much slower than their male counterparts did (Fig 14B). This result complemented our locomotor assays, which showed an overall trend of higher activity in males (Fig 12), a trend previous observed (Simon et al., 2011).

Further, we saw that the effect of mating on rest was highly pronounced, with mated females resting at similar levels as mated males, a pattern which was not observed in virgin flies (Fig. 12). Virgin females spent a lot less time resting than their male counterparts. This difference seen due to mating status in female dispersal speed and activity behaviour could be a result of mate harm, which occurs when females suffer injury and reduction in fitness post-mating due to transfer of Acps (accessory gland proteins) found in seminal fluid (Chapman et al., 1995), as well as male courtship behaviours (K. Fowler & Partridge, 1989). This harm experienced by the female flies could be why mated females appear to be much more restive.

4.7. Dispersal propensity modulated by interaction between mating status and relatedness in group

In virgin flies, we see that groups of related flies disperse much more than unrelated groups (Fig. 14A). A likely cause of this increased dispersal could be mate-searching behaviours, exaggerated by a need to avoid inbreeding. Though our mating experiments did not demonstrate any evidence for bias against mating with related

individuals in these fly populations, dispersal has been suggested as an alternate mode of inbreeding avoidance, especially when relatedness within the population is high (Clobert et al., 2012). Our experiments were performed on same-sex groups, so despite there not being any mates to avoid in the group, it is possible that the ability to identify other related individuals to oneself can promote this movement. More experiments would be required to evaluate this hypothesis.

Surprisingly, mated flies showed an opposite response in dispersing with related groups, and had a much lower propensity than their unrelated counterparts (Fig. 14B). Having already mated, the need to search for mates and avoid inbreeding would be much less pronounced, in contrast to the virgin fly treatment. In the social aggregation assay done between groups of mated related and unrelated flies, we see that related groups showed evidence of more interactions than unrelated groups (Fig. 16B). This supports the possibility of higher social interactions within the group leading to a reduction in dispersal. The fact that females dispersed less than males strengthens this explanation, as mated females were seen to have higher SII than males.

5. CONCLUSIONS AND FUTURE DIRECTIONS

Behaviours displayed by an organism can be modulated by the social environment in which they find themselves. In nature, organisms may often find themselves surrounded by their kin, and this means they may end up sharing resources with kin, or mating with them. If an organism is in the presence of its kin, kin selection theory predicts that it may often behave in a way that may decrease its own fitness and minimizes damage to its kin, in order to increase its total inclusive fitness. However, at high levels of relatedness, organisms have been seen to attempt to avoid kin, perhaps as a way to ameliorate adverse effects of inbreeding or competition.

Mating status also determines behavioural interactions in many organisms. Mated organisms show changes in their gene expression and neurotransmitter levels, which, coupled with varying natures of interaction with one's mate(s) can interact to govern behavioural changes. In all our experiments, flies in mated treatments had been mated with their siblings prior to experiments. Mating history with kin can impact subsequent mating behavior (Ala-Honkola et al., 2014; Tan et al., 2012) and consequences of mating (Carazo et al., 2015), but whether it plays a role in influencing other behaviours is currently unknown. Future work could be directed towards understand how mating history can affect other social behaviours, by investigating these behaviours when flies are mated with either related or unrelated individuals.

A salient feature of our study are the observations made about dispersal. We show dispersal propensity changes depending on the relatedness of the group that is dispersing. Further, we demonstrate that this change is dependent on the mating status of the individuals dispersing. Our work adds to the body of experimental and theoretical dispersal research that has studied these two factors separately. Yet to our knowledge, this is the first evidence of an interplay between mating status and relatedness of same-sex conspecifics influencing dispersal behavior in a population. Previous studies show that while dispersal in the presence of the opposite sex can promote dispersal through the need to track the movement of potential mates (Mishra et al., 2018), the presence of food causes dispersal propensity to reduce drastically (Simon et al., 2011). However, neither of these factors have been studied in related flies. Our experiments were performed in the absence of resources and

mates, and though we hypothesise that the need to avoid kin competition and inbreeding avoidance may explain our results, further experiments incorporating these two factors are needed to validate this.

Currently, there is no agreed upon mechanistic understanding of how fruit flies are able to discriminate between kin and non-kin, though research has suggested that microbiota, pheromones, and CHCs (**C**uticle **H**ydro**C**arbons) could be playing a role in this identification process. In *Drosophila*, these three topics are often in other contexts. Conducting social behaviour experiments after the removal/modification of microbiota in flies or use of mutants having defective CHC/pheromone receptors may help us to understand their contribution to a fly's ability to discriminate between other flies. This may translate towards a better understanding of social behaviours, and the mechanisms behind these interactions.

It is important to note that our experiments were performed on flies that have been maintained as laboratory populations for decades. We cannot predict how accurately the behaviours reported in this thesis will reflect those of wild drosophilids. However, this was not the intended purpose of this study. Our experiments aimed to investigate whether interactions between *Drosophila melanogaster* were modulated by the presence of kin. We observed that the presence of kin does affect some social behaviours, and that these behavioural changes can be sex-specific. We contribute to the growing literature database that shows that *Drosophila melanogaster*, a conventionally 'non-social' organism, can be easily manipulated in the lab to study sociality. These observations prompt a re-evaluation of how the study of kin-biased social behaviours is conducted, and advocate for the incorporation of 'non-social' organisms to understand the extent of sociality across taxa.

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