Behavioural traits correlated with evolution of increased dispersal in laboratory populations of *Drosophila melanogaster*

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CERTIFICATE

This is to certify that this dissertation entitled "*Behavioural traits correlated with evolution of increased dispersal in laboratory populations of 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 V.R. Shree Sruti at IISER Pune under the supervision of Dr. Sutirth Dey, Associate Professor, Biology Division, IISER Pune during the academic year 2016-2017.

Dr. Sutirth Dey
Biology Division, IISER Pune

Date: 20-March-2017

DECLARATION

I hereby declare that the matter embodied in the report entitled "*Behavioural traits correlated with evolution of increased dispersal in laboratory populations of Drosophila melanogaster*" are the results of the work carried out by me at the Department of Biology, IISER Pune, under the supervision of Dr. Sutirth Dey and the same has not been submitted elsewhere for any other degree.

V.R. Shree Sruti Date: 18/03/2017

Abstract

Dispersal is an important phenomenon that helps organisms escape stressful conditions. Evolution of dispersal can help organisms in keeping up with an ever-changing environment, especially with decreasing habitat qualities, increasing habitat fragmentation and human encroachment. In our lab, we have been selecting for dispersal in populations of *D. melanogaster* and have observed that the lines selected for dispersal have evolved a greater tendency to leave their habitat as well as move to a greater distance. The present study uses these populations to examine the effects of dispersal evolution on three behavioural traits which can contribute significantly to an individual's Darwinian fitness, namely, aggression, exploration and mating behaviour. The populations that had evolved greater dispersal were found to be significantly more aggressive and exhibited greater exploratory behaviour than the corresponding controls. However, there were no differences in mating behaviours of the dispersal-selected and the control lines. Further, despite the existence of very different kinds of selection pressures on the two sexes during selection for dispersal, sex-specific differences in mating behaviours did not evolve in the selected lines. Understanding the behavioural traits that can change due to selection for dispersal leads to a better appreciation of dispersal evolution. This in turn becomes useful in investigating various ecological phenomena like the spread of invasive species, community composition, gene flow in fragmented populations, etc.

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1. Introduction

Dispersal is defined as a permanent movement of individuals or propagules away from a source or an origin (Lowe and McPeek, 2014) "which may result in gene flow across space" (Ronce, 2007). Dispersal may be a beneficial strategy for an individual when exposed to stressful environments (Wenny, 2001) like environments of low-resource quality (Mathieu et al, 2010) and can allow organisms to seek habitats that can offer better mate availability and quality (Szostek et al, 2014). Dispersal can also help prevent the harmful effects of inbreeding (inbreeding depression), kin competition, and overcrowding or resource limitation (Clobert et al, 2012). Further, at the population-level, immigrants can lead to re-colonisation of habitats, thus reducing extinction risk (Bowler and Benton, 2005), and rescue a population from extinction (Brown and Kodrick-Brown, 1977). These effects of dispersal on spatial dynamics can in turn affect the costs and benefits of dispersal and thus influence the evolution of dispersal (Bowler and Benton, 2005). The evolution of dispersal ability can be profitable for individuals in the presence of ephemeral habitats and habitat fragmentation, as it allows them to track favourable environmental conditions (Ronce, 2007). Studying dispersal evolution has become relevant especially in the face of increased habitat fragmentation due to climate change and human activities (Visser, 2008).

In our lab, artificial selection for dispersal is underway on populations of *Drosophila melanogaster* (for details, refer Methods). In brief, the selection involves a source, and a path leading to a destination. Flies are introduced into the source and the flies that reach the destination within a fixed period of time are allowed to contribute progeny to the next generation. This selection has been imposed on these populations at every generation for 60 generations. Furthermore, our selection is directional: the path length has been gradually increased from 2 metres at the beginning to 17 metres after 60 generations of selection. A direct response to selection has been observed with the dispersal-selected populations having a significantly greater dispersal propensity (defined as proportion of flies exiting the source) as well as a significantly greater dispersal ability (defined as distance travelled) (Tung et al, 2016). This makes our selection a very good system to study the evolution of dispersal as well as correlated responses in other traits, both life history and behavioural.

Behaviour is defined as "the internally coordinated responses (actions or inactions) of whole living organisms (individuals or groups) to internal and/or external stimuli, excluding responses more easily understood as developmental changes" (Levitis et al, 2009). Thus, "behaviour" is a broad term that includes a wide variety of actions (or inactions) that an organism performs during the course of a day, or its entire lifetime. Since organisms interact with their environment and other organisms around them through many behaviours, if these behaviours had varying successes, it could reflect on the fitness of the organism. The behaviours that an organism exhibits can thus have profound effects on its fitness (Baum, 2013). For example, individuals with varying risk-taking behaviour (bold vs. fearful) can have different fitness based on the habitat as well as the predator's hunting mode (Preisser et al, 2007). Dispersal, as discussed in previous paragraphs, is a complex trait which includes many behaviours that are performed at various stages of the dispersal process. Thus, many behavioural traits may directly be a part of this complex dispersal trait or strongly correlated to it. Hence, the evolution of dispersal can be expected to result in the evolution of the linked behavioural traits as well. This brings us to the focus of my study which is to understand how different behavioural traits have changed (or stayed the same) with the selection on dispersal that is conducted in our lab. Different behaviours are elicited in different contexts based on, among other things, the environment and the internal state of the individual. These behaviours can affect different components that contribute to the Darwinian fitness of an individual, like survival or reproductive success.

Intra-specific aggression, a common social behaviour in many animals, is a well investigated agonistic act that can serve as the means for procuring and/or retaining resources (or territory) and mates (Hinde, 1970). On the flip side, aggressive behaviour can be energetically expensive and a failure can result in loss of territory/resources, injury or even death (Thomas et al, 2015). It naturally follows that aggression and dispersal could be related since movement can serve an effective way to avoid conflict. In such conflicts, more aggressive individuals may drive individuals that are not as aggressive away from the territory, such as when population densities in territories increase (Gaines and McClenaghan Jr, 1980). In the cichlid *Neolamprologus multifasciatus*, submissive females are driven out of their territory by the more aggressive females (Schradin and Lamprecht, 2002). In roe

deer *Capreolus capreolus* in Sweden, it was observed that the disperser males were at the receiving end of most aggressive acts (Wahlström, 1994). On the other hand, dispersing individuals, on reaching new territory, might need to be aggressive to establish themselves there against the resident populations (Duckworth, 2008). In Western bluebirds, it has been seen that there is a distinct variation in dispersal strategy and that this is tightly correlated with aggression as well (Duckworth and Badyaev, 2007). Males that are more dispersive are also seen to show greater aggression towards conspecifics and a closely related congener, the mountain bluebirds (Duckworth and Badyaev, 2007). Thus, the relation between aggression and dispersal is context-dependent and there is no common mechanism that can be used to predict their relationship. The extant literature though rich in elaborate studies of ecological contexts and their influence on aggression and dispersal, is lacking on evolutionary studies to understand how either trait will change with the evolution of the other.

The mating success of an individual, can have a large influence on the reproductive success and hence the fitness of an individual. The mating behaviour displayed by an individual is thus presumably under strong selection pressure. Dispersal and mating are often associated because dispersal offers an effective mechanism for individuals to seek mates in new territories as well as escape heavy competition for mates (Clobert et al, 2010). Sex-biased dispersal in birds is dependent on the type of mating system followed (Greenwood, 1980). While most birds exhibit female-biased dispersal where the female moved away from her natal site (Greenwood, 1980), in very few species, male-biased dispersal was observed. The difference between the two types was in their mating systems: while the former were mostly monogamous or facultatively polygamous, examples of the latter were entirely from a single family of birds (Anatidae) that formed pairs during spring migration or in the wintering grounds (Greenwood, 1980) and returned to the natal territory of the female to establish themselves. Along with data from mammals, Greenwood (1980) concluded that this sex-bias in dispersal arose from differences in the mating systems, i.e., whether the resources like territory were prerequisites for the male to obtain mates or not. Familiarity of the natal site thus increases the chances of success for the males and hence females disperse, whereas this is not required in other mating systems and may hence encourage dispersal of males as well as females (Clarke et al, 1997). In

insects with non-resource based mating systems, the dispersal of males can depend on the female behaviour. When females are expected to come to predictable mating sites, males are seen to crowd there, resulting in a lek system and if not, males have to undertake prolonged searching to obtain mates (Wickman and Rutowski, 1999). Further, selection experiments in the flour beetle, *Tribolium castaneum*, have shown that males from lines selected for increased dispersal (walking) have a greater mating success than those from lines selected for reduced dispersal while females from both lines show no difference (Matsumura and Miyatake, 2015). Selection pressures acting on the two sexes could select for different characters in them because often different strategies and behaviours are beneficial for males and females. The inevitability of mating in sexually reproducing organisms will interact heavily with the selection pressures that are acting on the two sexes to decide the fate of the traits of an individual. Though larger ideas of mating systems and mating success have been studied in the context of dispersal, the exact behaviours that are performed by organisms as a part of mating and how they change due to selection pressures that are acting on them has remained relatively less investigated. The difficulty of studying the mating behaviour with many animals in the wild hinder detailed studies in this regard. Further, unlike *D. melanogaster*, the entire set of behaviours that are performed before and during copulation have not been characterised for too many animals. Aggression and mating behaviours largely affect the reproductive success portion of an individual's fitness. Survival, the other major component of fitness, includes traits that ensure that an individual stays alive to be able to reproduce. In the case of dispersing individuals, traits like exploratory behaviour ensure the survival of an organism through its ability to sense and assimilate information about its surroundings.

Exploratory behaviour and dispersal have often been implicated together. Any organism that disperses from one habitat patch to another would largely benefit from exploring its surroundings and hence exploratory behaviour could serve to increase a dispersing individual's fitness. Many studies across different organisms have showed that exploratory behaviour and dispersal are strongly correlated to each other. For example, in the great tit *Parus major*, juvenile males that explored novel environments more and faster were also the ones that dispersed more in their adulthood (Dingemanse, 2003). Further, the same study showed from natural

populations that immigrants to a territory were far more exploratory than the residents (Dingemanse, 2003), thus linking exploration and dispersal abilities very tightly. In the mosquitofish *Gambusia affinis*, exploration was strongly correlated with activity, such that individuals that were more active showed greater exploratory behaviour (Cote et al, 2010). Here, though, exploration and dispersal itself were not correlated to each other which was explained by authors as due to the features of the assay itself. On the other hand, in the Trinidad killifish, dispersal distance in natural streams is correlated to an individual's exploratory behaviour (Fraser et al, 2001). In mammals like the Belding's ground squirrel as well, exploration and dispersal along with a set of other traits have been shown to be triggered by an 'ontogenic switch' during adult development (Holekamp, 1986). Similarly, in root voles *Microtus oeconomus*, the dispersers are seen to be more active as well as exploratory (Hoset et al, 2010). Thus, greater exploratory behaviour and higher dispersal are seen to be linked with each other across many animal taxa (although see Myers and Krebs, 1971).

In my study, I have attempted to understand the relationships between the above mentioned traits of aggression, mating behaviours and exploratory behaviour with dispersal by studying how these traits change when dispersal traits evolve. Firstly, I compared the aggression of the dispersal-selected lines and the corresponding control lines. Secondly, I compared two different components of mating behaviour observed in *D. melanogaster* between the dispersal-selected lines and their controls. Thirdly, to check for sex-specific evolution of these mating behaviour components, I analysed and compared mating behaviours for the dispersal-selected lines and the control with a common mate. Further, to also see if mate choice made by the two lines are any different, I compared the choices made by them when given a choice between mates of their own kind (i.e., dispersal-selected or control) and the other kind (i.e., control or dispersal-selected, respectively). Finally, I compared the exploratory behaviour of the dispersal-selected and the control lines to understand how the selection for dispersal has changed exploratory behaviour. The main advantage that my study has over most studies in this topic is that since the selection for dispersal is being performed under completely controlled conditions, it gives me the power to disentangle the effects of the selection for dispersal without interference from other confounding factors that are inevitable in field studies.

2. Materials and Methods

2.1. Experimental populations

Four large, outbred laboratory populations of *Drosophila melanogaster*, called DB_{1-4} (**D**ey **B**aseline) are maintained in the lab on banana-jaggery food, on discrete 21-day generation cycles (for detailed maintenance regime, refer to Sah et al, 2013).

From each of four replicate baseline populations (DB_{1-4}), a set each of selected populations, called VB (**v**aga**b**ond) and a corresponding set of control populations called VBC (**VB C**ontrol) have been derived. Each set of the selected and the control populations, VB_i and VBC_i (referred to as a block, derived from DB_i) were always assayed together and treated as a blocking factor during statistical analysis.

2.2. Selection regime

This section describes the selection regime followed for the VB and the VBC populations and their maintenance. These populations are maintained in large plexi-glass cages (of around 7.5 litre capacity) at densities of ~2500 adult flies per cage and provided with *ad libitum* banana-jaggery food. The life-cycle of these flies starts out as eggs that are collected from the previous generation of flies. Around 60-80 eggs are collected into a single plastic food vial containing around 6 ml of the banana-jaggery food. 80 and 40 such vials are made for each of the VB and the VBC populations respectively. On the 12^{th} day after egg collection, the selection for dispersal is performed (detailed protocol below). After the completion of dispersal selection, adults are transferred into plexi-glass cages and provided with banana-jaggery food for around 24 hours. They are then provided with live yeast paste for a period of 30 hours after which they are provided with fresh banana-jaggery food for egg-laying. Yeast paste boosts the fecundity of female flies and hence allows for a large number of eggs to be collected. These eggs are then distributed over 80 and 40 food vials (for VB and VBC respectively) and adults that emerge from these vials constitute the next generation.

The dispersal selection protocol for the VBs involves a simple source-path-destination setup (refer Figure 1). The source is large plastic cylindrical bottle of ~2L

volume with a funnel attached to one end of the bottle. The source leads into a pipe of around 1cm diameter through an adapter. The far end of the pipe leads into a cylindrical bottle with dimensions similar to the source bottle which made the destination (or sink). Through 60 generations, the path that the flies have to traverse to reach the sink has been increased gradually from 2 metres to 17 metres.

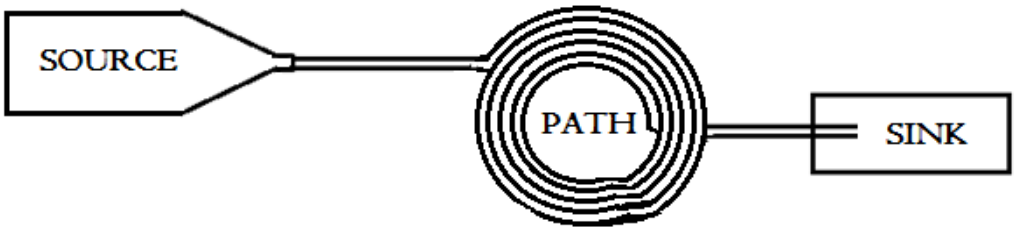

Figure 1. The dispersal selection setup. Flies are introduced into the source and allowed to disperse through the path into the sink. When 50% of flies introduced reach the sink, the selection is stopped.

For the selection, ~2400 flies were introduced into the source bottle and the dispersal run was set up by connecting the source to the pipe. The source provides no food or moisture for the flies. Flies that traverse the entire length of the path and reach the sink are rewarded with water that is provided in the sink in the form of a moist cotton strip. The dispersal setup is dismantled when either 50% of all flies introduced into the source reach the sink or at the end of 6 hours, whichever is earlier. A time limit of 6 hours was set since experiments revealed that there were no deaths due to desiccation within 6 hours. At the same time, the control flies are introduced into a bottle similar to the source for dispersal selection, but the exit from the bottle is closed with a cotton plug. At the time of dismantling, all the VBC flies are transferred into the cage and hence get a chance to reproduce. In the VBs on the other hand, as only 50% of flies introduced into a setup are selected to constitute the breeding population, two such replicates are made for all VB populations to ensure equal breeding population sizes for both the VBs and the VBCs. Thus, the adults that reach the destination from both replicates are mixed to form the breeding adult population for that generation. Further, since the VB flies that reach the sink are provided with water while the VBC flies are not, to prevent any selection for

desiccation tolerance, the VBCs are provided with water through a moist cotton plug when 25% of all VB flies introduced into the source reach the sink. Throughout the entire duration of the dispersal selection, the temperature (25°C) and light are maintained at even and constant levels.

2.3. Preparation of flies for assays

Before assaying for any trait, the selection for dispersal is relaxed for one generation and the experiments are performed on the progeny of these flies that did not undergo selection. This is done to reduce non-genetic parental effects that may arise due to the selection (Rose, 1984). Further, for all assays, eggs were collected at a density of ~50 eggs in 6 ml of food to avoid larval crowding and effects of larval crowding on the adults that emerge.

2.4. Aggression

This experiment was performed after 52 generations of selection for VB and VBCs on virgin flies.

2.4.1 Collection of flies for aggression

To generate flies for the experiment, eggs were collected from VB and VBC flies on which the selection was relaxed. As mentioned in Section 3, around 50 eggs were collected into each of 20 food vials. On the 8^{th} day after egg collection, all the adults that had eclosed till then were discarded. This was done to ensure that all the flies used for the experiment were unmated flies. Every 6 hours after the discard, flies that had eclosed in that time period were collected, separated by sex under light CO_2 anaesthetisation and isolated in tubes which provided them with food. The aggression assay was performed on the 12^{th} day after egg collection. Thus, the individual flies were housed in the isolation tubes for around 3 days. 24 hours before the experiment, i.e., on day 11, the males were coloured using fluorescent powder (DayGlo, Cleveland, OH, USA) by dipping the cotton plug of the isolation tubes in the powder.

2.4.2 The aggression setup

The aggression assay was performed in 6 wells of a twelve-well culture plate (Corning®, NY, USA). Each well served as the enclosure for one replicate of the aggression assay. In the centre of the well, a small plastic cup was glued on, which contained regular banana-jaggery food. A freshly decapitated female was stuck to the middle of the food cup using yeast paste. The food and the female together serve as two resources for the males to fight for, one a nutritional resource and the other a reproductive resource. One pair consisting of a VB and a VBC male were then introduced into the setup and their interaction was recorded for 45 minutes using a video camera (Sony HDR-PJ410, Sony DCR-SR20E). 30 such replicates were assayed for each of the four populations of VBs and the corresponding VBCs. Individual wells were visually isolated from each other using cotton to ensure no visual cues were being exchanged between replicates. Uniform lighting and constant temperature (25°C) were maintained.

2.4.3 Scoring for aggression

The scoring for aggression was done after an initial five minute acclimation period. For each of the replicates, the number of successful chase-aways from the food cup for both the VB male and the VBC male was counted and tabulated. A successful chase-away is one in which one male completely chases the other male from the entire top surface of the food cup. A male that manages to complete three consecutive successful chase-aways usually is the dominant male and tends to successfully chase away the other male in all future encounters (Yurkovic et al, 2006). Thus, the male who performed these three consecutive successful chase-aways was considered the winner in each replicate, established norms (Yurkovic et al, 2006). The winners for each of the replicates for all 4 populations were tabulated.

2.4.4 Statistical analysis

The total number of wins for the VBs and the VBCs for each of the 4 blocks was tabulated and a Mann-Whitney U test was performed on the total number of wins. This was done since Mann-Whitney test being a non-parametric test, does not demand normal distribution of the data.

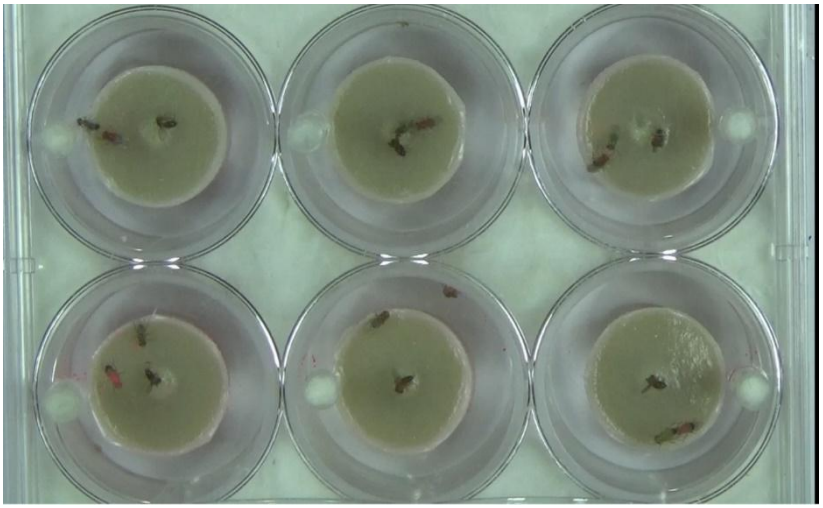

Figure 2. The aggression assay setup. Each well in this setup is one replicate. A decapitated female is stuck on to the centre of the food cup with yeast paste. One VB male (here, pink) and one VBC male (here, green) completes a replicate.

2.5. Mating assay

The mating assay was done to check for differences between mating behaviours in the VB and the VBCs. In this assay, one virgin male-female pair of VB or VBC was introduced into an enclosure and their behaviour was recorded using a video camera (Sony HDR-PJ410, Sony DCR-SR20E). This assay was performed after 52 generations of selection.

2.5.1 Collection of flies

For the assay, eggs were collected from VB and VBC flies on which the selection was relaxed. Around 50 eggs were collected into vials with ~6ml of banana-jaggery food. Similar to the procedure followed for aggression, on the 8^{th} day from egg collection, all adults that had eclosed till then were discarded. Every 6 hours after the discard, all flies that had eclosed in that period were collected, separated by sex under light CO_2 anaesthetisation and isolated in tubes that provided food. The assay was performed on the 12^{th} day from egg collection i.e., the day on which selection for dispersal is imposed on the VB populations.

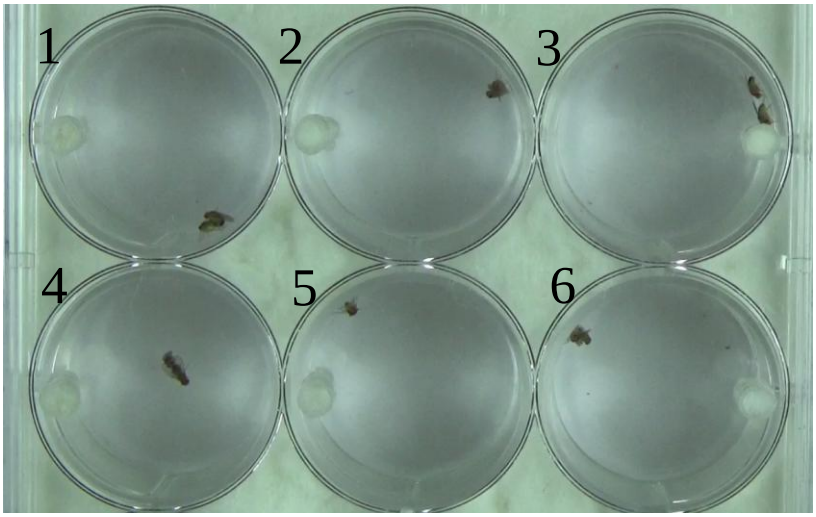

Figure 3. The mating assay setup. Each well is a replicate of the assay. A male and a female are introduced into the well and allowed to mate. Here, we can see the flies mating in wells 1, 2, 4 and 6

2.5.2 The mating assay setup

The mating assay was performed in 6 wells of a 12-well culture plate (Corning®, NY, USA). The wells were visually isolated from each other using cotton to ensure that the flies in one replicate were not influenced by what flies in other replicates were doing. Each of the 6 wells served as one replicate for either VB or VBC. On the day of the experiment, one female and one male were introduced into each of the wells and once 6 pairs had been introduced into the wells, the plate was recorded using a video camera for a duration of ~45 minutes. 24 such replicates were assayed for each block of VB or VBC.

2.5.3 Parameters measured

For each replicate, the time from the start to when the first copulation was initiated (Mating Latency) and the duration of the copulatory event (Copulation Duration) were noted down. For any event to be considered a copulatory event, it had to be longer than 3 minutes (O'Dell, 2003) since it is known that it takes many minutes for the males to successfully transfer sperm to the female.

2.5.4 Statistical analysis

The data were pooled across both VBs and VBCs and a univariate ANOVA was performed with either Mating Latency (ML) or Copulation Duration (CD) as

dependent variables and Selection (fixed factor with levels: "VB" and "VBC") crossed with Block (random factor with levels: "1", "2", "3", "4") as independent variables. Further, the variances of ML and CD values for the VBs and the VBCs were compared with a test of homogeneity of variances using the Levene's statistic (refer Discussion for motive).

2.6. Mating assay with common mate

This assay was performed to check if there were any differences in sex-specific differences in mating behaviours between the VB and VBCs. To do that, virgin fly pairs of either a VB or a VBC male and a DB female or vice versa (VB or VBC female with DB male) were introduced into the arena and their mating behaviours recorded. This assay was performed after 59 generations of selection for VB-$VBC_{1,2}$ and after 60 generations of selection for VB-$VBC_{3,4}$.

2.6.1 Collection of flies

The collection of eggs for this assay was done in a similar manner as that for the previous assay (refer Section 2.5.1). In addition, eggs were collected from the corresponding DB_i population as well, at similar densities. On the 8^{th} day from egg collection, flies that had eclosed till that time were all discarded and from then on flies were collected every six hours to ensure that they were virgin flies. These flies were then immediately separated by sex under light anaesthetisation using CO_2 and introduced into isolation tubes that contained food where they stayed till the day of the experiment. The mating assay was performed on the 12^{th} day from egg collection.

2.6.2 The mating assay setup

The setup used for this experiment was the same as that used for the previous assay (refer Section 2.5.2). In this assay, four different types of mating pairs were made; VB male-DB female, VBC male-DB female, VB female-DB male and VBC female-DB male. 36 replicates were assayed for each of the four types of mating pairs. After the pairs were introduced into the arena, they were video recorded for around 45 minutes.

2.6.3 Parameters measured

Similar to the previous assay, two types of data were recorded by watching the videos. The first parameter was the time taken to initiate the first successful copulation (Mating Latency, ML) and the duration of the successful copulation event (Copulation Duration, CD). A successful copulation was one that lasted for 3 minutes or more (O'Dell, 2003).

2.6.4 Statistical analysis

For the analysis, the data across the VB and the VBC males, when paired with DB females, for both ML and CD were pooled. A univariate ANOVA was performed with either ML or CD as dependent variables and Selection (fixed factor with levels: "VB" and "VBC") crossed with Block (random factor with levels: "1", "2", "3", "4") as independent variables.

Similarly, the data for ML and CD from VB and VBC females when paired with DB males were pooled. A univariate ANOVA was performed with either ML or CD as the dependent variable and Selection (fixed factor with levels: "VB" and "VBC") crossed with Block (random factor with levels: "1", "2", "3", "4") as independent variables.

2.7. Male Choice Mating Assay

This assay was performed to check if VB and VBC males had any preference of females of their own type when given a choice of mate. For this assay, each replicate consisted of either a VB or a VBC male in an arena with one VB female and one VBC female. This assay was performed after 63 generations of selection for VB-$VBC_{1,2}$ and after 64 generations of selection for VB-$VBC_{3,4}$.

2.7.1 Collection of flies

The collection of eggs was similar to that in the previous two assays (refer Sec 5.1). On the 8^{th} day after egg collection, all adult flies that had eclosed till then were discarded and from then on flies were collected every 6 hours to ensure they were virgin flies. The flies that were thus collected were immediately separated by sex under light anaesthetisation using CO_2. Once separated, they were introduced into

isolation tubes which contained food. On the 12th day after egg collection, the females were marked using fluorescent powder (DayGlo, Cleveland, OH, USA) by covering the cotton plug of the isolation tubes with the powder and letting the colour get transferred on to the fly inside. VB females were marked with one colour (pink) and VBC females with another (green) for one half of the replicates and vice versa in the other half of the replicates. One hour after colouring the flies, the male choice mating assay was performed.

2.7.2 The mating assay setup

The setup used for this assay was similar to that used in the previous two mating assays. The only difference was in the number of flies introduced and their identity. While the previous two assays had only one male and one female in each replicate, in this assay, each replicate consisted of one male (either VB or VBC) and two females (one VB and one VBC). 48 replicates were made for each VB_i or VBC_i population. Once the flies were introduced into the setup, their behaviours were recorded on video.

2.7.3 Parameters measured

From the video recordings, two types of data were noted down for each replicate, both based on the first female that the male mated with. One was the identity of the female, i.e., whether the female was a VB or a VBC female. The second was the colour of the powder with which the female that the male chose was coloured with. This was done to check for any bias that might have been introduced because of a preference or an aversion that the male might have.

2.7.4 Statistical analysis

The identity of the female (VB/VBC) every male mated successfully with first was noted. All the data were then classified into three categories - 'Same', 'Different' and 'No mating'. Same included both the instances when a VB male mated with a VB female and when a VBC male mated with a VBC female. The category 'Different' included instances when a VB male mated with a VBC female and when a VBC male mated with a VB female. The total number of 'Same' and 'Different' copulations were then compared using a Mann-Whitney U test. Additionally, for to check if males had a preference for one of the colours, the colour of the chosen female was pooled over

VB and VBC males and the numbers for pink and green were compared using a one-way ANOVA.

2.8. Exploration

This assay was performed to compare the exploratory tendencies of the dispersal-selected VBs and the control, VBCs, after 53 generations of selection.

2.8.1 Collection of flies for the assay

After 53 generations of selection on the VBs and the VBCs, selection was relaxed for one generation. Eggs were then collected from these flies for the assay. Around 50 eggs were introduced into each of ten vials containing ~6ml of banana-jaggery food for both the VBs and the VBCs. The assay was performed on the 12^{th} day after egg collection when flies were aspirated from the egg-collection vials and introduced individually into separate glass tubes based on their sex. From these glass tubes the flies were introduced into the arena and were recorded on a video camera (Sony HDR-PJ410, Sony DCR-SR20E).

2.8.2 The exploration setup

An open field apparatus (modified from Soibam et al, 2012) was used to assay exploration in the flies. The open field apparatus involved a Petri lid (Laxbro, Pune, India) of inner diameter 10 cm. A small hole was bored into the centre of the lid to introduce flies into the setup. The lid was placed on top of a sheet of paper, which apart from the trace of the lid boundary, also had a trace of the inner two thirds of the area (Liu et al, 2006) of the lid (refer Figure 4). The flies were introduced into the exploration arena from the glass tubes and their activities in the arena were video-recorded. 32 replicates were assayed for each sex of the VBs and the VBCs.

2.8.3 Parameters measured

After the flies were introduced into the arena, they were given one minute to acclimate to the new environment. They were then observed for the next ten minutes and the number of times they entered the inner circle, which was considered the

number of exploratory trips, and the total time they spent inside the inner circle were tabulated for each fly.

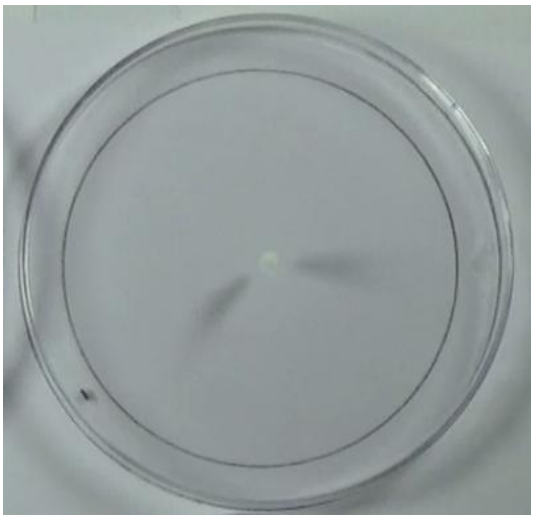

Figure 4. The exploration assay setup. The setup consists of a single fly introduced into a Petri lid. The paper below shows the inner two-thirds of the area of the lid. Here, we see the fly in the outer two-thirds.

2.8.4 Statistical analysis

The data were pooled across both sexes, the VBs and the VBCs and a univariate ANOVA was performed with either Number of Exploratory Trips or the Total Time Spent on the Inside as dependent variables, Selection (fixed factor with levels: "VB" and "VBC") and Sex (fixed factor with levels: "Male" and "Female") crossed with Block (random factor with levels: "1", "2", "3", "4") as independent variables.

The statistical analysis was done on STATISTICA 8 (StatSoft Inc, Tulsa, OK, USA) and graphs were plotted using SigmaPlot (Systat Software, San Jose, CA, USA).

3. Results

3.1. Effect of dispersal evolution on aggression

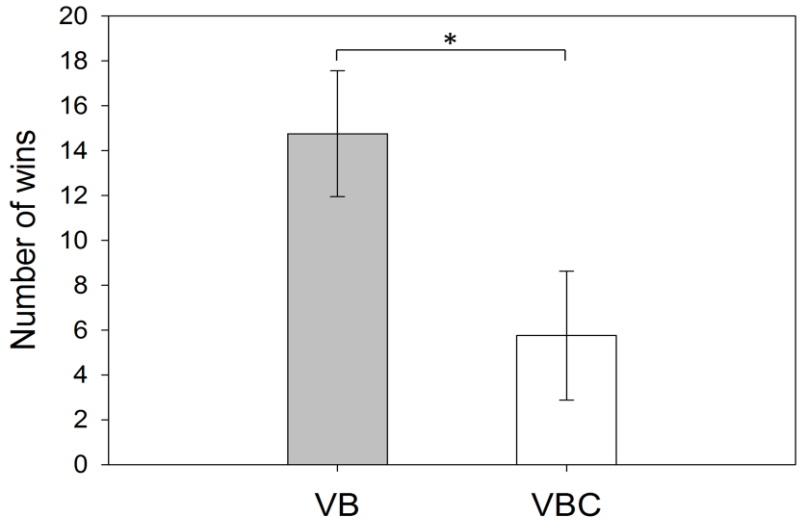

Figure 5. Effect of dispersal evolution on aggression. Average number of wins, defined as three consecutive chase-aways, of the dispersal-selected VBs and the control VBCs. Error bars represent standard error around the mean (SEM). * indicates significance at $p < 0.01$ level

The data indicate that the VBs, on average, win more fights than the VBC males (Fig. 5, $p<0.01$, $t_6=4.1026$). The effect size of the difference is also large (Cohen's $d = 2.0153$) indicating that this difference is biologically significant. Thus, the VB males are more aggressive than the VBC males which implies that the evolution of dispersal has led to simultaneous evolution of aggression as well.

3.2. Effect of dispersal evolution on mating traits

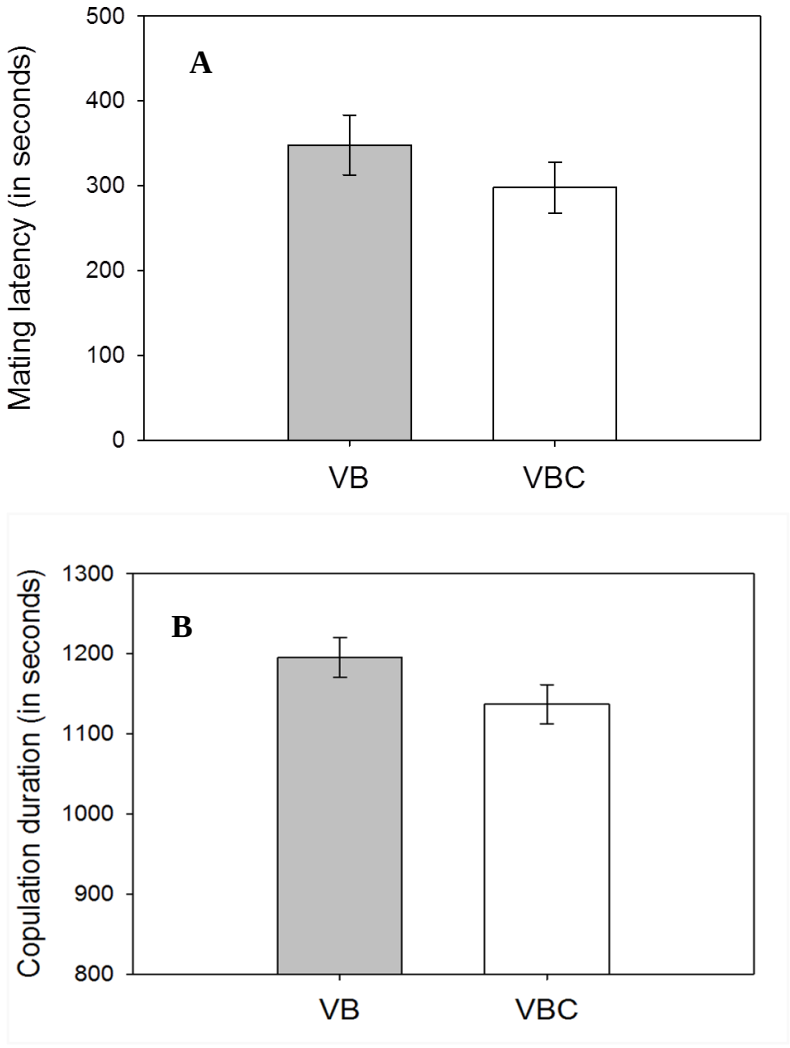

Figure 6. Effect of dispersal evolution on mating behaviours. A. Mating latency defined as time taken to initiate first copulation, given in seconds, of the dispersal-selected VBs and the control VBCs. Error bars represent SEM. **B.** The average duration of the copulation event, given in seconds, of the dispersal-selected VBs and the control VBCs. Error bars represent SEM.

The time taken by VB and VBC flies to initiate copulation are not significantly different from each other (Fig. 6A, p=0.3639, $F_{1,3}$=1.1400). The duration of copulation for the VB pairs and the VBC pairs are also not significantly different from each other (Fig. 6B, p=0.4093, $F_{1,3}$=0.9152). Thus, the selection for dispersal does not seem to have had any effect on the mating traits of mating latency and copulation duration in the flies. Additionally, the variances of the mating behaviours are not different between the VBs and the VBCs (ML-p=0.651 $F_{1,176}$=0.206, CD-p=0.523 $F_{1,176}$=0.409).

3.3. Effect of dispersal evolution on mating behaviours in each sex

3.3a. Male-specific differences (VB and VBC males with DB females)

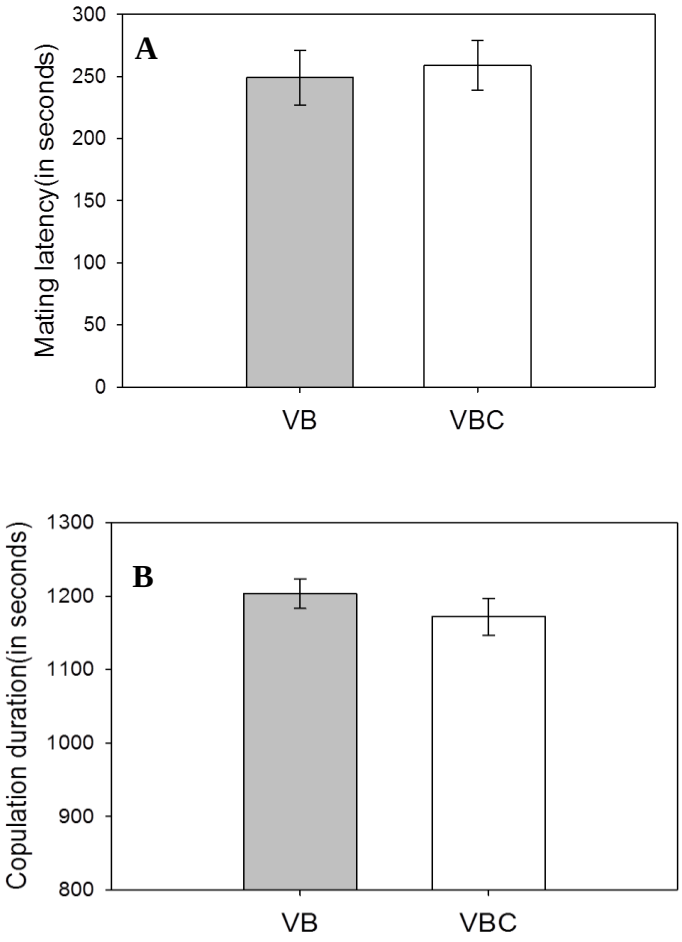

Figure 7. Effect of dispersal evolution on mating traits in males. A. Mating latency defined as time taken to initiate copulation, given in seconds, of the dispersal-selected VB and the control VBC males when paired with a DB female **B.** The average duration of the copulation event, given in seconds, of the dispersal-selected VB and the control VBC males with DB females. Error bars represent SEM.

There is no significant difference between the time taken by VB males and the VBC males to initiate copulation when paired with a DB female (Fig. 7A, p=0.8913, $F_{1,3}$=0.0221). The copulation duration of the VB male-DB female pairs and the VBC male-DB female pairs are also not significantly different from each other (Fig. 7B, p=0.3993, $F_{1,3}$=0.9607). There are no differences in the mating traits of mating latency and copulation duration due to dispersal evolution in male *D. melanogaster*.

3.3b. Female-specific differences (VB and VBC females with DB males)

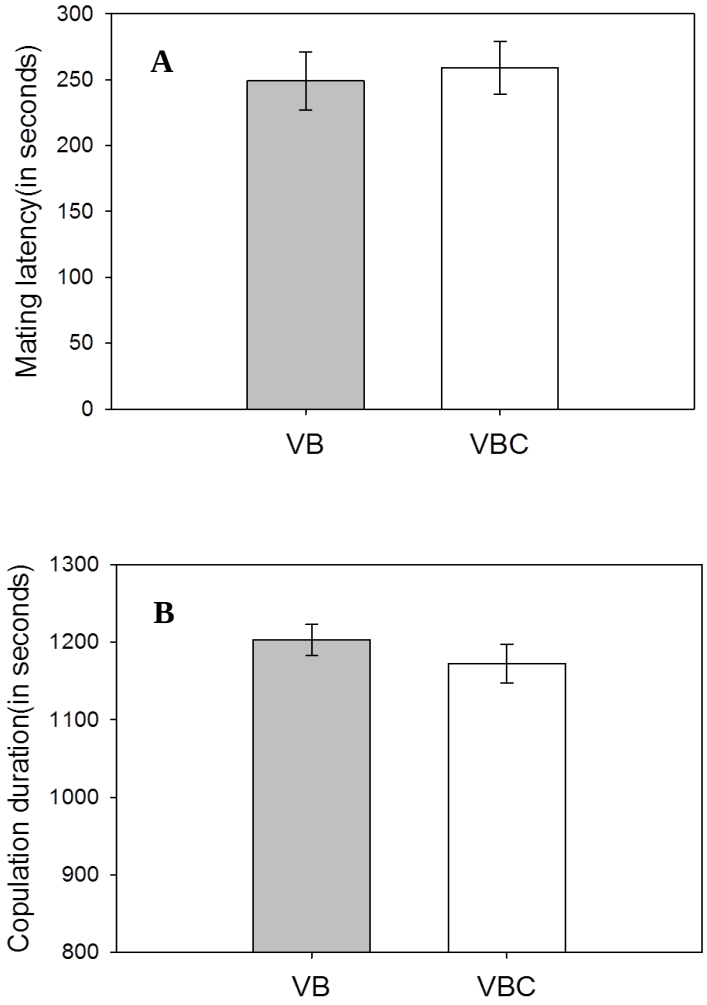

Figure 8. Effect of dispersal evolution on mating traits in females. A. Mating latency defined as time taken to initiate copulation, in seconds, of the dispersal-selected VB and the control VBC females when paired with a DB male. **B.** The average duration of the copulation event, in seconds, of the dispersal-selected VB and the control VBC females with DB males. Error bars represent SEM

There is a marginal significance in the difference between the time taken by DB males to initiate copulation when paired with either a VB or a VBC female (Fig. 8A, p=0.0654, $F_{1,3}$=9.0905, Cohen's d=0.2737). The copulation durations of the VB female-DB male pair and the VBC female-DB male pair are not significantly different from each other (Fig. 8B, p=0.2580, $F_{1,3}$=1.9390). There seem to be no differences in the mating trait of copulation duration due to dispersal evolution in the female flies. However, there exists a trend in the mating latency such that pairs with VB females show a higher latency than the pairs with VBC females.

3.4. Male mate choice assay

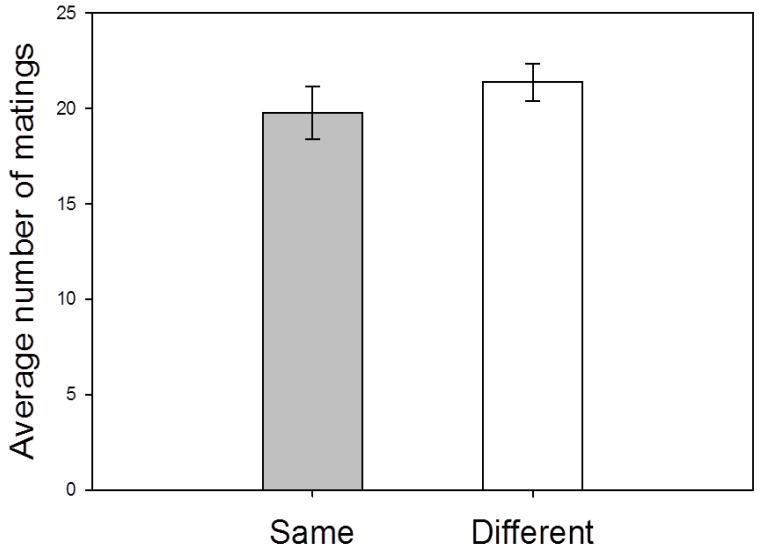

Figure 9. Comparison of choices made by males towards and against females of their type. The average (±SE) number of successful copulations of the 'Same' and 'Different' categories. The 'Same' category includes VB male-VB female and VBC male-VBC female mating, while 'Different' category includes VB male-VBC female mating and VBC male-VB female mating. Error bars denote SEM.

There is no difference between the preferences of the males, pooled over both the VBs and the VBCs (Fig. 9, U=22, $p > 0.05$). Males of both the dispersal-selected and the control populations had no bias either for or against females of their own population type. Thus, even after 60 generations of selection, males of the selected (or the control) type do not have a preference between females of their own type and the other type. This could be either because of an inability to recognise females of their own type or because they do not care to mate preferentially even after recognising females of their own type. Additionally, there is a marginally significant effect of colour (p=0.053, $t_{12.159}$ =-2.141) with females coloured green being preferred over females coloured pink (See discussion for more details).

3.5a. Effect of dispersal selection on exploration

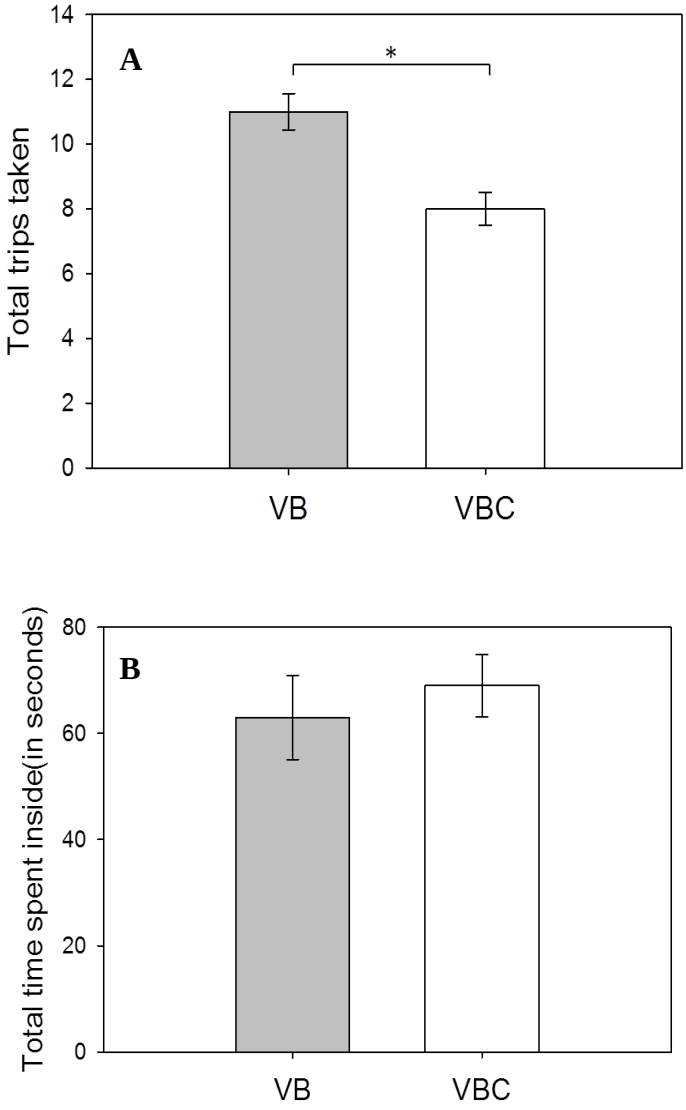

Figure 10. Effect of dispersal evolution on exploratory behaviour. A. Mean number of exploratory trips made by VBs and VBCs. VBs make significantly more exploratory trips than VBCs. **B.** Total time spent inside the arena by VBs and VBCs. Error bars represent SEM. * indicates significance at $p<0.05$ level

The frequency of exploratory trips made by the VBs is significantly greater than that of the VBCs (Fig. 10A, $p=0.0384$, $F_{1,3}=12.5169$). The effect size of the difference is medium (Cohen's $d= 0.3523$). On the other hand, the total time spent in the inside arena is not significantly different between the VBs and the VBCs (Fig. 10B, $p=0.4852$, $F_{1,3}=0.6306$). Thus, while the selected flies make more exploratory trips into the inner area, the amount of time that both the dispersal-selected flies and the control flies spend in the inner area is fairly similar.

3.5b. Sex comparison of exploratory behaviour

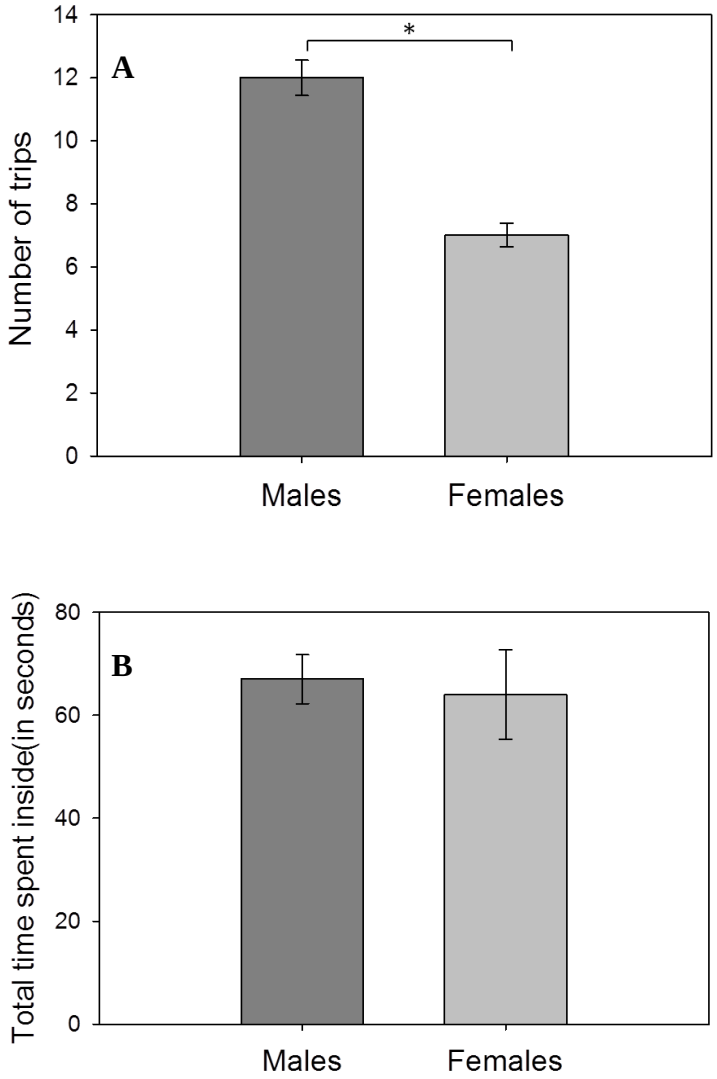

Figure 11. Comparison of exploratory behaviours between the sexes. A. Mean number of exploratory trips made by males and females. **B.** Total time spent inside the arena by males and females. Error bars represent SEM.

Males make significantly more exploratory trips than females (Fig. 11A, p=0.0144, $F_{1,3}$=26.228, Cohen's d = 0.65372, large effect). On the other hand, the total time spent in the inside arena is not significantly different between the males and females (Fig. 11B, p=0.4914, $F_{1,3}$=0.6112). The interaction terms of Selection x Sex did not have a significant effect on both the number of trips (p=0.7686, $F_{1,3}$=0.1036) as well as the total time spent inside (p=0.3580, $F_{1,3}$=1.1733).

4. Discussion

4.1. Dispersal evolution in *Drosophila melanogaster*

Evolution of dispersal is a complex phenomenon since it involves a large number of interacting factors that act together to cause changes in many physiological, morphological and behavioural traits of an organism. Further, other traits that are functionally related to these dispersal traits are also bound to evolve as correlated responses. Additionally, constraints that are imposed on traits in an organism, either by evolutionary history or by current ecology, will also play a substantial role in determining the way evolution alters a trait or a set of traits. Our dispersal-selection setup requires individuals to leave the source, thus selecting for their dispersal propensity. Since the length of the path increased over generations, there was selection for the capability to cover the entire distance, thus leading to increased dispersal ability. Assays performed after 33 generations of selection, revealed that the selected flies (VB) had a significantly greater dispersal propensity and dispersal ability than the control flies (VBC) (Tung et al, 2016). These results were observed even in the presence of a source of nutrition, the lack of which probably served as a proximate cause of dispersal during selection. Thus, some constitutive changes appear to have taken place which are causing the flies to be more dispersive even in the absence of the presumable proximate driver for dispersal. Now, it is of interest to see how various traits have changed (or stayed the same) as a consequence of the evolution of dispersal. Studies in the past year in the lab have been directed at doing that, looking at the effect of evolution of dispersal on different traits. My thesis constitutes one part of this big study, focussing on changes in certain behavioural traits due to this selection for dispersal.

4.2. Flies selected for dispersal are more aggressive

As a part of my study, the first trait that I looked at was aggression. Aggression is an important trait that influences an individual's ability to retain resources and mates or gain new ones. Studies that have been conducted across many animal taxa have shown that the relationship between dispersal traits and aggression can be complicated. The relationship is dependent on how and when aggression is required

in the dispersal process. A major influence on this relationship is when an individual leaves the source patch. Firstly, aggressive individuals could force submissive individuals away from the territory causing the dispersers to be the less aggressive individuals (as in Schradin and Lambert, 2002). Alternatively, the individuals who are more prone to taking risks could also be more aggressive (Reaney and Backwell, 2007) and if the bold individuals disperse from their territory, the dispersers would be more aggressive. Another major factor that decides how aggression and dispersal are related is how aggression helps a disperser when it reaches a new habitat. Since the dispersers are the ones who have to colonise new territory, competing against extant residents of the territory, it would be essential for them to be more aggressive (as in Duckworth, 2008). My study reveals that with the evolution of dispersal, aggression has also evolved such that the dispersers were more aggressive. In the case of the bluebirds (Duckworth, 2008) aggression was an ecological demand and it was important for the displacing bluebirds to replace the closely related mountain bluebirds. In our system, on the other hand, aggression seems to have evolved, as a by-product of dispersal evolution, even without a direct 'need' for the trait. This is because, in the source in our selection, all flies were getting desiccated and there were no territories or resources to guard and defend. Similarly, there was no need to colonise the sink and compete with native individuals, since all flies that reached the sink were allowed to contribute to the progeny. On the other hand, it is possible that the dispersers were the bold ones who may also have been more aggressive.

Since *Drosophila melanogaster* is used widely as a study organism in many fields across biology, a lot is known about mechanisms and molecular players for various behaviours and pathways. For example, octopamine is a molecule that is similar to noradrenaline in humans, and is a neurotransmitter and neuromodulator in many invertebrates including *D. melanogaster* (Pfluger and Stevenson, 2005). Studies have shown that in *D. melanogaster*, octopamine is a key molecule that is required for aggression towards other males and increasing octopamine expression also increases initiation of aggression (Zhou et al, 2008). Apart from its effects on aggression, octopamine, along with serotonin and dopamine, have also been shown to increase activity in flies (Yellman et al, 1997). Interestingly, flies with elevated serotonin levels (either pharmacologically induced or by genetic modifications) are known to exhibit increased aggression (Dierick and Greenspan, 2007). Dopamine is

also known to affect aggression in flies but the relation is not as straightforward as for the other molecules. Inactivation of dopaminergic neurons resulted in flies that are hyperactive but do not engage in social interactions (Alekseyenko et al, 2010). Other molecules like choline (Zwarts et al, 2012), insulin (Luo et al 2014, Belgacem and Martin, 2005), tachykinin (Asahina et al, 2014, Winther et al, 2006) etc, either affect aggression or activity or sometimes both, but in opposite directions. Thus, octopamine and serotonin are very strong contenders for a molecular basis to the increased aggression as well as activity that is observed in the VBs over the VBCs.

4.3. Dispersal evolution has had no effect on mating traits

The aggression assay was a study of male-male encounters and how these seem to have changed between the VBs and the VBCs. Though flies were given both a nutritional resource and a mating resource in the aggression assays, it was mostly the dead female that the flies fought over and many flies attempted to mate with the dead female too. I then wanted to see if this trait of aggression continued to be expressed when a male encounters a live female as well. In *D. melanogaster*, the mating latency or how long it takes for flies to initiate copulation is dependent both upon the time it takes for the male to recognise the female and court it, as well as the time taken for the female to become receptive (O'Dell, 2003). Many factors can influence the mating latency and copulation duration in the flies including their activity levels. This is because greater activity increases the chances of an encounter (O'Dell, 2003) which in turn could potentially reduce mating latency in the VBs. However, this may not have been a problem since the chamber that we used was not so big that males and females never met each other. In fact, in all the replicates across both the VBs and the VBCs, the male and the female always encountered each other, though cases of no-copulation were recorded in both VBs and VBCs. I saw no differences in the average mating latency and copulation duration between the VBs and the VBCs. Thus, we can conclude that the evolution of dispersal traits has had no effect on these mating traits. However, one must note that these assays were performed after 50-60 generations of selection, and it is entirely possible that these traits might show some response in the longer run.

4.4 Presence of dissimilar selection pressures on the two sexes did not result in evolution of sex-specific differences in mating traits

Simultaneous sex-specific changes in both the males and females for behaviours could also explain this lack of difference between the mating latency and copulation duration in the VBs and the VBCs. For example, if the female were to become more evasive along with the male becoming more aggressive, it would result in a similar mating latency as that in the control case. In our system, male and female flies have different options to gain reproductive fitness. When the flies are introduced into the selection setup, they are non-virgins. A male in the source can gain reproductive fitness in two ways. One would be to disperse and mate with the females that have reached the destination as well. Additionally, a male can also gain reproductive fitness by mating with a female which disperses and reaches the destination. In the latter case, a male would only have to mate with as many females as possible while still in the source and still gain reproductive fitness without any physical movement. However, assays revealed no differences in the variance of the mating latency and the copulation duration of the VBs and the VBCs. This eliminates the existence of two different strategies within the males in the VBs. For a female, on the other hand, the only way to ensure that she can contribute to the next generation would be to reach the destination. This asymmetry between the two sexes could lead to different strategies getting selected in the males and the females during dispersal evolution. My next experiment was designed to check for these sex-specific differences in mating behaviours. For this, I made pairs of VB and VBC males with DB females and vice versa (VB and VBC females with DB males) and studied their mating behaviours.

Sex-specific differences in dispersal are widely observed in many animals, including birds and mammals (Pusey, 1987). In many cases, these sex biases in dispersal are often explained by a combination of factors including inbreeding avoidance, mate and resource competition (Handley and Perrin, 2007). In lions, females are philopatric (i.e., females stay in their natal site) while males disperse away from their natal site for breeding. This difference in lions has been attributed to the observation that costs of leaving for males being much lower than the cost of leaving for females, a consequence of their polygynous mating system (Pusey and Packer, 1987). As mentioned above, the selection pressures on males and females in our selection for

dispersal is slightly different and this could have led to evolution of different behaviours in both the males and females. To check for sex-specific differences in mating behaviours, I then assayed for these behaviours in the presence of a common mate. Thus, VB and VBC males were individually paired with the baseline population, DB females and similarly VB and VBC females with DB males. The DB populations have not been selected for any trait and have been maintained under regular lab conditions. Hence, it is not expected that they will bias the mating behaviours of just one of the VBs or the VBCs. I observed that there were no sex-specific effects of dispersal evolution on the mating traits of mating latency and copulation duration. However, there appears to be a trend in the mating latency in VB and VBC females with the former having a greater latency than the latter. This result, though not statistically significant, does not deny the existence of a trend. This trend can possibly be explained by the higher activity of VB females, leading to a longer duration before a successful copulation is initiated by the male. The lack of appreciable differences between the mating traits in the VBs and VBCs show that despite the existence of different pressures for the two sexes in our selection for dispersal, the mating traits themselves have not evolved.

4.5. Dispersal evolution did not lead to the evolution of male mate choice

Even if mating traits themselves do not evolve, it is possible that mate recognition or mate compatibility might be greater within the VBs/VBCs than across them. The next assay that I performed was to check if males of one type (VB/VBC) had a greater preference for females of their own type when given a choice. Male choice assays are performed to check if the male has a preference for a particular type of female. The existence of a mate preference is a composite of both the choice the male makes as well as the receptivity of the female (O'Dell, 2003). In my assay, I saw that males made no differentiation between females of their own type and females of the other type. There was also a near-significant colour preference, with males preferring the female coloured green over the female that was coloured in pink. However, the colour preference does not affect the mate preference overall since half the time VB females were coloured in pink and in the other half VBC females were coloured in pink. Hence, we can conclude that the selection pressures that have been acting on

the VBs and the VBCs are not strong enough to result in the evolution of strong preferences. Prior studies of male mate choice have shown that males prefer females that are larger (Byrne and Rice, 2006). Additionally, in *D. melanogaster*, studies have shown that males and females of a particular natural population have a greater preference for sexual characters of their own populations (Hollocher et al, 1997). There is a lack of a difference in the mate choice behaviour in my experiment thus suggesting that either male flies do not recognise flies of their own type, or that the distinction, even if it can be made, does not really matter to them. Our selection is perhaps not strong enough to expect reproductive isolation, but a difference in mate choice would have revealed the evolution of preferences, which is an interesting observation in its own right.

4.6. The evolution of dispersal led to simultaneous evolution of exploratory behaviour

Aggression and mating behaviours help organisms in their interactions with other individuals and both are important for maintaining a hold on resources as well as successful reproduction. Survival is mandatory for an organism to realise its Darwinian fitness. In the context of dispersal, the trait of exploration and exploratory behaviour becomes extremely important for survival as individuals have to survey their surrounding when they leave their patch/territory and assess the quality of the new habitat patch before making a decision about settling. Thus, it is not a surprise that many studies have reported a correlation between exploratory behaviours and dispersal (Korsten et al, 2013, Fraser et al, 2001, Dingenmanse, 2003). In my study, I see that the dispersal-selected VBs make more exploratory trips than the VBCs. However, the time they spend away from their zone of comfort is the same for both the VBs and VBCs. This can be explained by the observation that the VBCs are far more sedentary than the VBs. In many instances, it was observed that the VBC flies tended to initiate an exploratory trip and then remain motionless for the entire duration of the recording, while the VBs were constantly moving. The selection for dispersal has thus seemed to result in the evolution of exploratory behaviour as well.

4.7. Associations of other traits with dispersal as it evolves helps predict the behaviour of invasive species, among other uses

Apart from associations between exploratory behaviour and dispersal, some studies show that individuals that are more exploratory are also more aggressive (Verbeek et al, 1996, Bergmuller and Taborsky, 2007) and more active (Wilson and Godin, 2012). Putting this together with the results that I have obtained, it appears that the traits of exploration, aggression and dispersal are a suite of traits that may be linked with each other, the first time a strong association has been show between all these traits in a single organism. Establishing trait relationships between dispersal and other traits allow us to predict the dispersal and spread of many species and the properties that can be associated with this spread (Clobert et al, 2012). This allows us to predict properties such as the rate of spread of invasive species, which are known to have high dispersal rates (Sih et al, 2004). For example, in the cane toad *Bufo marinus*, the rate at which invasion front has been progressing has increased five-fold in ~50 years (Philips et al, 2006). Invasive species, apart from greater dispersal, are also known to reproduce faster (O'Connor, 1986) and compete better (Vila and Weiner, 2004). Invasive species are thus posing a big threat to many indigenous species (Gurevitch and Padilla, 2004) which are already suffering from the ill effects of climate change. If invasive species are not kept in check, loss of biodiversity is imminent. Understanding the different characteristics that allow for these invasive species to spread, such as their dispersal, allows us to control their spread in a better manner. Studying dispersal along with its costs and correlates helps us understand several tenets of its mechanistic underpinnings. This knowledge enables us to control the spread of non-native and invasive species and help prevent biodiversity loss and maintain ecological balance.

5. Conclusions and future work

The nature of the environment of organisms is changing significantly due to major habitat loss, fragmentation, and climate change which are making vast swatches of habitat inhospitable. Dispersal thus becomes a beneficial strategy for them as it allows them to escape these unfavourable habitats and track favourable environmental conditions. Another important concern in the field of ecology currently is invasive species and the expansion of their ranges. They are known to pose a great threat to indigenous species. Further, invasive species are known to have greater rates of dispersal. Understanding the evolution of dispersal, its costs and consequences and its interactions with the biotic as well as the abiotic environment helps us arrive at an understanding of the mechanisms that govern these natural processes. However, to arrive at such an understanding it is important to study how dispersal is associated with other traits.

In this study, I have shown that the evolution of dispersal can also lead to the evolution of some but not other behavioural traits. The evolution of dispersal has led to the evolution of aggression and exploratory behaviour such that the dispersers are more exploratory as well as aggressive. Exploration and aggression are two behaviours that are of immense consequence for dispersers to both move through an inhospitable matrix during dispersal as well as for colonisation after reaching new territory. The associations that exist between dispersal, aggression and exploration, in the literature points to molecular mechanisms that might be responsible for these changes. Potential players include molecules like serotonin and octopamine, among others. The influence of these molecules can be ascertained by performing assays which compare the levels of these molecules across the dispersal-selected and the control lines. On the other hand, there has been no considerable change in the sex-specific mating behaviours between the selected and control lines, despite the existence of dissimilar pressures on the male and females during selection. Currently, work in our lab is directed at arriving at associations between dispersal and other traits that may be very important in realising the Darwinian fitness of an individual. Such studies, along with studies on the ecology of dispersal will then contribute to an understanding of dispersal which, as mentioned above, is a trait of great significance in ecology.

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