Behavioural correlates of chronic stressors in outbred and dispersal selected *Drosophila melanogaster*

Thesis submitted in partial fulfilment of the requirements of the Five Year BS-MS Dual Degree Program at

Indian Institute of Science Education and Research, Pune

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CERTIFICATE

This is to certify that this dissertation entitled "Behavioural correlates of chronic stressors in outbred and dispersal selected *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 Shraddha Lall at IISER Pune under the supervision of Dr. Sutirth Dey, Associate Professor, Biology Division, IISER Pune during the academic year 2018-2019.

Dey

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Date: 18/3/19

DECLARATION

I hereby declare that the matter embodied in the report entitled "Behavioural correlates of chronic stressors in outbred and dispersal selected *Drosophila melanogaster*" are the results of the work carried out by me at the Biology Division, IISER Pune, under the supervision of Dr. Sutirth Dey and the same has not been submitted elsewhere for any other degree.

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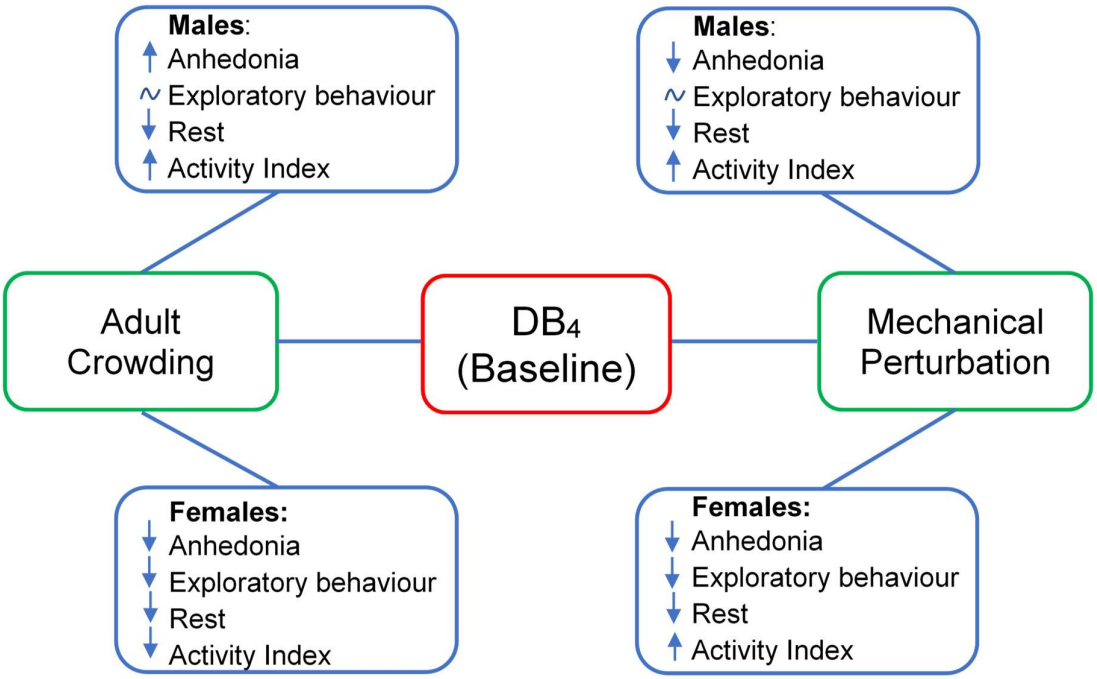
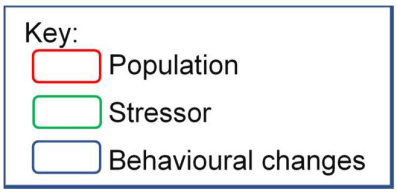
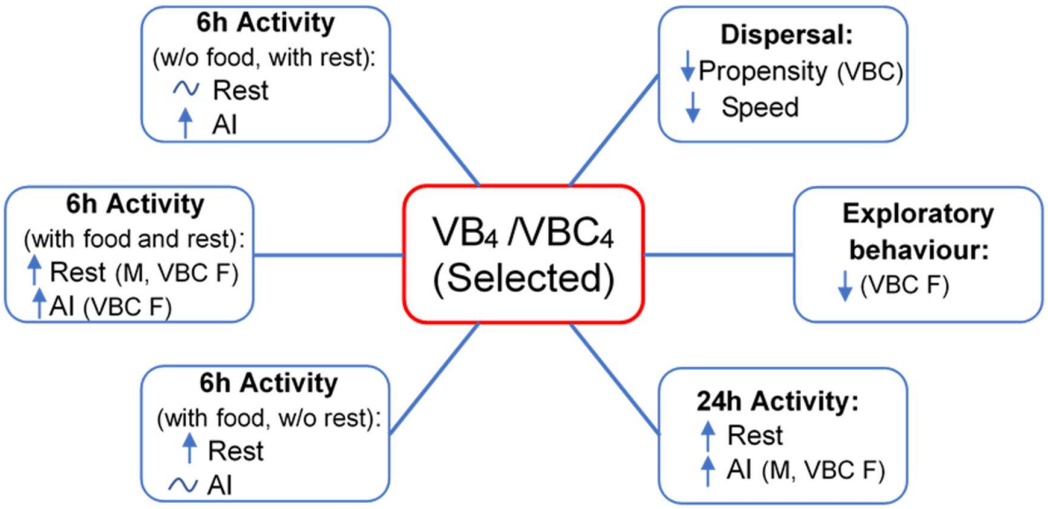
ABSTRACT

When faced with a stressful situation, an organism exhibits a behavioural stress response. Studying these responses can allow us to model and understand stress-induced disorders, such as depression and anxiety, in humans better. This study examines behavioural changes due to stress in *Drosophila melanogaster*, focussing on how sex and selection for increased dispersal can modulate this response. The behaviours studied, namely, anhedonia, motivation to explore and disperse, locomotor activity and sleep levels, have been well-investigated in human and rodent-based models of stress-disorders. These behaviours were studied in the context of two different stressors, namely mechanical perturbation and adult crowding. While mechanical perturbation caused anhedonia and made flies restless across sexes, the changes in these behaviours was sex-dependent after adult crowding. Further, evolutionary history of increased dispersal changed how flies responded to stress, with females selected for dispersal being highly resistant to stress as compared to controls. Changes in locomotor activity and rest levels after stress in the selected populations was crucially dependent on the presence or absence of food while recording and post-stress rest before recording. This study thus argues for a sexually dimorphic model of stress in the fruit fly, which can provide better appreciation of the sexual dimorphism in stress-induced mood disorders in humans. Additionally, it establishes that the environment of evolution can modulate stress-responses, furthering the argument for stress-induced disorders in humans to be due an evolutionary mismatch. This also paves the way for studies on how other evolutionary histories can shape this response.

GRAPHICAL ABSTRACT







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1. INTRODUCTION

The stress response can broadly be defined as the biological response of an individual when a threat to its homeostasis is perceived. The threat in question is termed to be the stress or stressor. In response to a stressor, an organism can display biological responses at any combination of these four levels – behavioural, neuroendocrine, autonomic or immunological (Moberg, 2000).

Studying stress can be difficult due to the complex ways in which the stress responses are generated, and the nature of the specificity of the response to the stressor (Rushen, 2000). For instance, in fruit flies, different stress paradigms have been shown to induce different stress responses (Neckameyer and Nieto-Romero, 2015). The utility of studying behavioural responses to stress, however, can be found in using stress to model mood-disorders in humans. The validity of these stressors and models can then be tested on the basis of three criteria – similar behavioural manifestation as the disorder (face validity), similar causation and physiological changes (construct validity) and comparable reversal of changes via drugs (predictive validity) (Abelaira et al., 2013).

Attempting to model human behaviours, in order to achieve face validity, involves drawing parallels of these behaviours in animals. Emotional states existing in animals has been postulated and studied since the time of Darwin, in his work *The Expressions of Emotions in Man and Animals,* wherein several examples of parallel emotional states and their evolutionary origins were analysed (Darwin, 1872). Mammals are often seen as natural models in which to study these, with rodent (Abelaira et al., 2013; Cryan and Holmes, 2005; Willner, 2017; Willner et al., 1992), dog (Seligman and Maier, 1967) and primate (Mendoza et al., 2000) models being very popular. Recently, it has been argued that invertebrates, specifically *Drosophila melanogaster* can be used as a system to model such behaviours (Iliadi, 2009), and basic behavioural tests can be used to test motivational states of the fly and potentially model stress-symptomatic behaviours in humans (Ries et al., 2017).

Stress and trauma are known to be major factors predisposing individuals to depression, and the disease is often characterised by behavioural symptoms which are manifestations of an inability to cope with stress (Cryan and Holmes, 2005; Krishnan and Nestler, 2008). Various studies, largely in rodents, have tried to model this disorder, by designing stressor paradigms that elicit behavioural changes in

stressed organisms which are similar to those expressed by depressed humans. (Abelaira et al., 2013; Cryan and Holmes, 2005; Willner, 2017). Stress has also been linked to and used in the modelling of disorders such as generalised anxiety disorder and post-traumatic stress disorder (Cryan and Holmes, 2005; Van Praag, 2004). Further, stress-induced neurotransmitter changes in model organisms have also been similar to those observed in humans with such disorders, with serotonin and dopamine reduction being implicated in both rodent (Holmes et al., 2003; Park et al., 2005) and fly (Araujo et al., 2018; Ries et al., 2017) models. Models based on chronic stress have been shown to have high face, construct and predictive validity in rodents (Abelaira et al., 2013).

The first behavioural response to stress could be to find ways to escape it or cope with it – when the stress becomes inescapable the organism develops helplessness, which is the basis of the learned helplessness (LH) paradigm to study stress. In this paradigm, an organism is subjected to an inescapable stress, and when later subjected to the same stress with an escape route, will show reduced tendency to escape or fail to escape the stress entirely (Yang et al., 2013). While changes to sleep patterns and neurotransmitter levels have been observed in rodents subjected to LH, these changes often do not persist after the stress is withdrawn (Abelaira et al., 2013). Additionally, studies in flies have found that this paradigm is environment specific, and no behavioural changes are observed upon transfer to a new environment (Batsching et al., 2016). Thus, in flies, this paradigm is very context specific for a single task or setting, and does not appear to be a reliable method to study persistent behavioural changes due to stress.

Another well-established chronic stress paradigm is that of Chronic Mild Stress (CMS), first studied by Katz and colleagues in the 1980s on rodents (Katz, 1982; Katz et al., 1981). In this, a series of mild, unpredictable stressors are given to the organism for several days, after which behaviours and neurobiological changes are recorded (Willner, 2017). Studies have also indicated the reversibility of symptoms of CMS by anti-depressants, such as fluoxetine and imipramine (Abelaira et al., 2013; Araujo et al., 2018). While long in duration and potentially difficult to implement practically, it has been shown to induce long-term changes in both rodents (Cryan and Holmes, 2005) and recently, fruit flies (Araujo et al., 2018).

While the CMS model involves using multiple stressors, a single abiotic stress model proposed by Ries et al., 2017*,* using only 3-day vibrational stress in male fruit flies has been shown to induce a serotonin-modulated depression-like state, responsive to lithium and fluoxetine treatment (Ries et al., 2017). Using a single stressor makes the protocol easy to replicate and modulate, circumventing the difficulty of the replication of the CMS model across laboratories (Abelaira et al., 2013). Single physical stressors of similar nature, specifically mechanical perturbation using a shaker or vibrational device, have also been used to induce learned helplessness (Brown et al., 1996) and sleep deprivation (Potdar et al., 2018) in fruit flies.

Stressors in humans are largely social in nature and contribute to the development of mood disorders including anxiety and depression (Palanza, 2001). Thus, social models of stress have also been studied in rodents. The social defeat model using aggressive conspecifics is commonly used in male rodents (Yan et al., 2010). Maternal stress has also been used as an early-life social stressor in rodents to mimic early life loss or neglect by parents in humans (Abelaira et al., 2013). Social isolation has been used to induce stress in fruit flies (Neckameyer and Nieto-Romero, 2015) as well as mice (Palanza, 2001). However, such social models are often sex-specific in rodents and when not, induce differential stress in males and females (Palanza, 2001). For instance, higher corticosterone levels – a biochemical indicator of stress – were observed in male rats subjected to adult crowding but not in females (Brown and Grunberg, 1995).

Adult crowding has been known to induce fitness-related effects in *D. melanogaster.* Increased adult densities have been shown to reduce both mortality during crowding and post-stress fecundity (Joshi et al., 1998). A reduction in lifespan is also observed, possibly due a reduction in stored energy reserves (Joshi and Mueller, 1997). This indicates an underlying physiological change due to this stress, but it also possibly changes the social environment of the fly, and can thus act as a biotic stressor.

Thus, in order to understand how sex and nature of stressor can impact behaviour in fruit flies, we studied how both male and female outbred flies respond to two different stressors – mechanical perturbation which is an abiotic stressor, and adult crowding, a biotic stressor.

Although encountering a stressful situation can have immediate negative consequences, the stress-response is believed to be advantageous over evolutionary time-scales. A primary stress-response is to activate fight-or-flight, allowing the organism to leave the stressful situation or gather resources in preparation for the crisis that triggered the response. Mismatches between our evolutionary environment and our modern lifestyle are believed to be responsible for a large number of adverse stress-reactions and stress-induced mood disorders (Brenner et al., 2015; Grinde, 2005; Nesse and Young, 2000). This is the basis of evolutionary medicine's mismatch hypothesis – when our current environment does not match the environment in which our behaviours, and genetic and physiological mechanisms have evolved, deleterious physiological effects are caused (Grinde, 2002). For depression, anxiety and other stress-induced disorders, the mismatches proposed range from our sedentary lifestyle as compared to the very active lifestyle of our Palaeolithic ancestors, to changes in our social structure – from close-knit, non-hierarchical communities to individualistic, discrimination-heavy societies – and changes in our sleep cycles, with large amount of artificial light (Brenner et al., 2015; Hidaka, 2012). This suggests that environmental conditions in which organisms have evolved could impact how they respond to stressors, and an empirical testing of this is possible with populations evolved under controlled, artificial settings. This could potentially allow verification for the hypotheses of depression, anxiety and other stress-induced disorders as maladaptive or dysfunctional responses to adversity (Hagen, 2011).

In our lab, populations of *D. melanogaster* have been artificially selected for increased dispersal. Dispersal can be defined as 'the movement of individuals or propagules with potential consequences for gene flow across space' (Ronce, 2007). It may be beneficial for an organism to disperse under stressful conditions (Wenny, 2001), such as those with less resources, and find habitats with better resource availability (Mathieu et al., 2010). However, dispersal is an energy-intensive process and comes with its costs, which can be incurred at various stages in organisms' life as well as during different stages of dispersal – departure from original habitat, transfer and settlement in new habitat (Bonte et al., 2012). Further, associated behavioural changes have occurred with selection for dispersal. Fly populations that are evolved to disperse have increased locomotor activity, are more exploratory in

novel environments, and are more aggressive (Tung et al., 2018a). Accompanying metabolic changes have also occurred, with dispersers having increased cellular respiration rates, which support their increased activity, and changed levels of neurotransmitters and their precursors. Increases in serotonin, dopamine and octopamine have been observed in dispersers (Tung et al., 2018a). Reduction in serotonin and dopamine have been observed in stressed fruit flies (Araujo et al., 2018; Ries et al., 2017). Additionally, Selective Serotonin Reuptake Inhibitors or SSRIs, which increase level of circulating serotonin, are commonly used to alleviate the symptoms of stress-induced disorders in humans and animal models (Abelaira et al., 2013; Cryan and Holmes, 2005; Ries et al., 2017). Thus, the evolutionary history for high dispersal could modulate how stress impacts these flies, both due to changes in behaviour such as an activity-heavy environment of evolution, as well as metabolic changes. To test this, we compared stress-responses between the VB population, selected for dispersal for over 100 generations, and the VBC population, their ancestry-matched controls.

First, we wished to check that the stressors we used did not cause any physical harm or injury to the flies, and that the behavioural responses, if any, were not indicative of lethargy, and only indicated the motivational state of the fly. Previous studies have shown that a 3-day vibrational stress protocol in male flies did not cause any changes to cue-based responses such as phototaxis and optomotor response (Ries et al., 2017). We studied the negative geotactic behaviour in flies, via the Rapid Iterative Negative Geotaxis (RING) assay (Gargano et al., 2005). Flies have an innate tendency to move upwards along the walls of a vial or container, against gravity. In the RING assay, measurement of negative geotaxis in response to a mechanical cue – being tapped to the bottom of the vial or channel – is measured for a group of flies at a time (Gargano et al., 2005). As this behaviour is cue-based, any reduction in this would indicate that the stressor could be causing physical harm to the flies, and hence the other behaviours would not be a reliable readout of motivational state.

In relation to stress, we studied anhedonia – a lack of interest in a normally pleasurable, rewarding activity. This is one of the core symptoms of depression in human beings (Krishnan and Nestler, 2008), and has been used as a measure of stress response in rodents in several studies (Abelaira et al., 2013; Cryan and

Holmes, 2005). It can be measured via a reduction in preference for feeding on a normally palatable solution such as sucrose in mice (Cryan and Holmes, 2005). Recent studies have also measured anhedonia as a response to vibrational stress in male *D. melanogaster* via glycerol feeding (Ries et al., 2017), and after CMS in male fruit flies via sucrose feeding (Araujo et al., 2018). Both sucrose and glycerol are sweet tastants in fruit flies (Gordesky-Gold et al., 2008).

The question of sex-differences in anhedonic responses has also been explored in rodent models. Historically, most studies of CMS in rodents were on males (Baker et al., 2006; Palanza, 2001). Recent studies on female rats using CMS model have had differing results – suggesting both higher anhedonic response in females than males (Lu et al., 2015) to no anhedonia in females (Baker et al., 2006). Further, it has been suggested that social stressors have sex specific hedonic responses in rodents. Isolation in female mice induces anhedonia, but not in males (Palanza, 2001). A social instability protocol – with periods of isolation and crowding – induced anhedonia in female rats (Herzog et al., 2009). Thus, different social stressors were found to be stressful for either sex, perhaps because of the dimorphism in how male and female animals behave in social contexts (Palanza, 2001).

Another behavioural measure was the exploration of a novel habitat. In rodent models, stress is often linked to a reduction in investigative behaviour (Abelaira et al., 2013; Yan et al., 2010). Stressed and anhedonic mice were shown to reduce exploration of a novel habitat (Strekalova et al., 2004). In fruit flies, this behaviour is related to centrophobism – a preference for edges over the centre – speculated to be shelter-seeking (Liu et al., 2007). Thus, a reduction in the exploration of a novel habitat could indicate increased fear or anxiety and reduced motivation to explore.

In flies, exploratory locomotion over 2 minutes in a novel arena is known to respond differently to starvation and oxidative stress for a 24-hour period, and also depends on the sex and sexual maturity of the fly (Neckameyer and Matsuo, 2008). While oxidative stress caused a decrease in exploratory locomotion across most ages and sexes, starvation both increased and decreased exploration depending upon the age and sex of the fly (Neckameyer and Matsuo, 2008). However, when the stressors were chronic and lasted for ten days, it was found that there is no change in exploratory behaviour in male flies (Araujo et al., 2018). Thus, the duration and

nature of the stressor, as well as the sex and age of the flies, modulates the effect on exploratory tendencies.

We further studied how stress changed locomotor activity patterns and rest/sleep levels. Locomotor activity in rodents has typically been investigated in an open field activity paradigm which measure locomotor behaviour over a small duration, ranging from 1 to 10 minutes. CMS under this paradigm can lead to a reduction in activity (Katz et al., 1981), hyperactivity (Grønli et al., 2005) or no change in activity (Lucca et al., 2009). Additionally, studies have shown that hyperactivity in rodents after stress is triggered by light (Strekalova et al., 2004). While altered psychomotor activity has been a diagnostic feature for depression in humans (Nelson and Charney, 1981), the lack of consistency in these measurements in the open-field paradigm in rodents has led to the belief that the degree of resemblance between these changes and human behaviour may be questionable (Willner et al., 1992). Further, changes in sleep patterns have been reported after learned helplessness in rodents (Abelaira et al., 2013). Both insomnia (or a lack of sleep) and hypersomnia (excessive sleeping) are criteria for diagnosing stress-related disorders in humans (Cryan and Holmes, 2005).

Vibrational stress in *D. melanogaster* over 3 days has been correlated with reduced activity over a 15-minute period in males (Ries et al., 2017). However, other studies using different stressors for different durations have found no change in short-term locomotor behaviour over 1-2 minutes (Araujo et al., 2018). These studies have, however, focussed on locomotion over very short durations. In order to understand the long-term changes in locomotor behaviour and sleep, we studied this over longer durations – with locomotor recordings over 6-hours and 24-hours. In order to reduce the effects of a novel environment in which the recording was made, we allowed 15-minutes of acclimatisation to the flies, before starting any recordings.

Finally, for the selected populations and their controls, we also quantified dispersal traits in response to stress. We characterised the propensity to disperse, which is indicative of the motivation to leave the natal habitat, and the speed of dispersal, which measures how quickly these flies complete their movement to the new habitat, potentially indicative of a continued motivation to reach the goal as well as a locomotor ability to do so (Mishra et al., 2018a).

Thus, our experiments have attempted to answer two broad questions – how sex and nature of stressor interact to change behavioural responses to stress in outbred fruit flies, and how selection for dispersal can modulate this response. The behavioural responses to stress were measured via motivation-based behaviours, aiming to parallel the behaviours in stress induced mood-disorders in humans and in rodent models. Through these studies, we have attempted to further understand the face-validity of a fruit fly-based stress-response model, across sexes. We have also attempted an evolutionary understanding of stress disorders, by attempting to understand whether evolutionary history can impact stress-induced behavioural responses, and how.

2. MATERIALS AND METHODS

2.1. Experimental populations

For the set of experiments on ancestral non-selected flies, a laboratory-bred baseline population of *Drosophila melanogaster* (DB_4) was used (breeding population of ~2400, 21-day discrete generation cycle). The detailed maintenance protocol of this population can be found elsewhere (Sah et al., 2013). For the experiments on flies selected for dispersal, two laboratory-bred populations derived from DB_4 were used – VB_4 which had been selected for increased dispersal for 104 – 109 generations at the time of various experiments, and VBC_4, their control population (breeding population of ~2400, 15-day discrete generation cycle). The selection protocol for these flies can be found elsewhere (Tung et al., 2018b) and is mentioned below in brief for the convenience of the readers. To avoid any non-genetic parental effects, the assays were performed after rearing both VB_4 and VBC_4 under common environmental conditions for one generation.

For each assay, age-matched flies were used for all treatment groups. Adult flies, between 11 and 13 days old, were separated by sex under light CO_2 anaesthesia. They were subjected to the experimental protocol after allowing them to recover overnight.

2.2. Selection for Dispersal

The set-up for dispersal consisted of a source, a path and a destination (Fig. 1). The path was clear plastic tube of ~1cm inner diameter connecting the source and the destination, which were cylindrical, clear plastic containers of volume ~1.5L. Every generation, on the 12^{th} day from egg-collection, flies from the VB_4 population were introduced into the source, which was kept devoid of food and moisture. The flies were allowed to disperse for 6 hours or till 50% of the initial population reached the destination. Only the flies which reached the destination were allowed to breed for the next generation, and the length of the path was incremented over generations, effectively selecting for increased dispersal. The control population VBC_4 was subjected to similar conditions of desiccation and starvation, and maintained identically to VB_4 (Tung et al., 2018b).

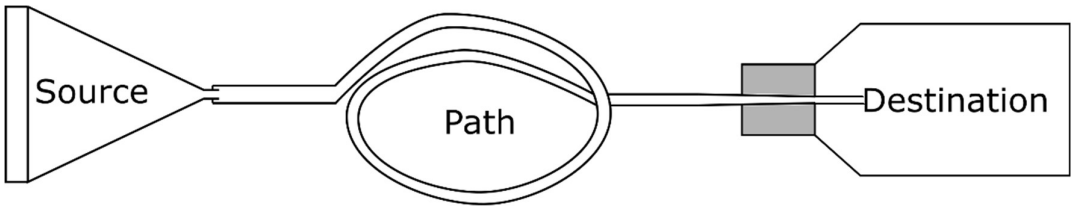

Fig. 1: Setup for dispersal selection and assay

2.3. Stressors:

2.3.1. Mechanical perturbation

This stress paradigm was modified from the vibrational stress protocol in Ries et al., 2017. 25-50 flies of either sex were kept in vials containing a sponge at the bottom, soaked with water, for the duration of the stress. The treatment vials were placed on a platform shaker, rotating at 400 RPM, while the control vials were placed on an undisturbed surface (Fig. 2A). The mechanical perturbation was provided for 15 minutes, followed by a period of rest for 15 minutes. This was repeated over the entire duration of the stress protocol, which was 8 hours for males and 10 hours for females. These durations were finalised on the basis of standardisations for both sexes. They were then transferred to vials containing food and allowed to recover overnight. The same protocol was carried out at the same time of the day for 3 days; on the 4^{th} day the flies were subjected to various assays.

2.3.2. Adult crowding

The protocol was modified from Joshi and Mueller, 1997. 150 flies of either sex were placed in a vial with ~6mL of food. A sponge plug was pushed into the vial such that there was 0.7cm distance between the food and the plug for males and 1cm for females (Fig. 2B). This stress was maintained for 72 hours, post which the flies were transferred to round-bottom fly bottles with food, relaxing the stress for 14 hours before the assays were conducted. Control vials had 50 flies of either sex, maintained in normal uncrowded conditions.

Fig. 2: Protocol for Stress Induction via A. mechanical perturbation and B. adult crowding

2.4. Assays

2.4.1. Rapid Iterative Negative Geotaxis (RING)

2.4.1.1. Setup and Protocol

The RING frame consisted of ~26 adjoining columns, ~1.2 cm wide and ~35 cm in height. The bottom of the frame was covered by doubled-over tape, to ensure a uniform base while ensuring that the surface is not sticky. This frame was loaded into a metallic support structure, consisting of two long rods to hold the frame in place, and a base covered by foam to absorb the shock, while maintaining it in a vertical position (Fig. 3).

In each frame, 25 flies of one treatment and one sex were loaded into one column, and alternate columns were filled. 8 columns were assayed at a time in one round. Each such round had replicates from all treatment groups from one sex. Once the flies were loaded into the columns, the top was closed using cotton plugs, and the frame was mounted on the support. The flies were allowed to settle. The assay was performed in a dark room, with diffused light from the back of the set-up, to facilitate contrast for recording with a video camera (Sony HDR-PJ410).

The frame was mechanically disturbed, and moved sharply to the base, to make all the flies fall to the bottom. Once the flies were at the bottom, video recording was started, and a timer was kept for 30 seconds, which constituted one trial. After 30 seconds, the frame was disturbed similarly, and the process was repeated for 10 consecutive trials.

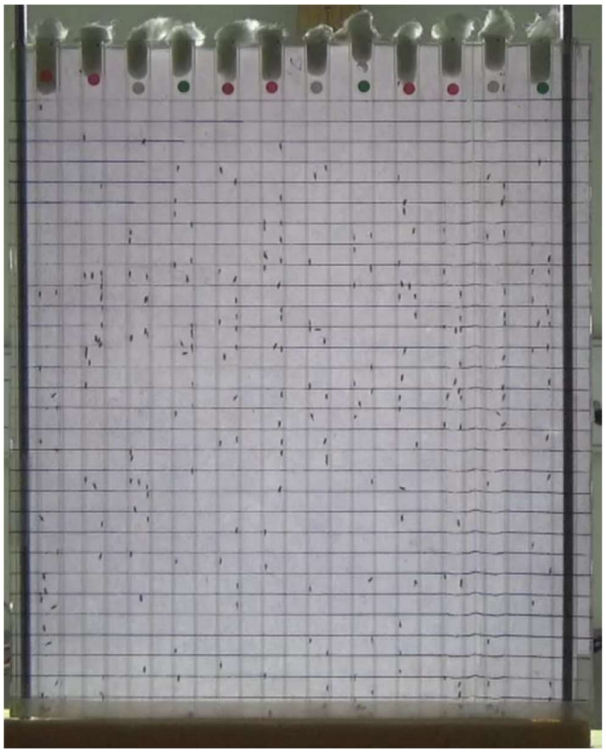

Fig. 3: Setup for RING assay

2.4.1.2. Scoring

For each round, both the 1st and the 10th trial were scored. Screenshots were taken from the video recorded, at a fixed time point in the trials. The time-points were selected such that the snapshots were taken when the flies were dispersed throughout the set-up, and a majority of them had not reached the top. For males, this fixed point was 10s, while it was 15s for females.

The length of the column was divided into 31 bins of 1 cm. The number of flies in each bin were counted. If a fly was halfway between bins, it was counted in the bin in which its lowermost tip was present. The distance travelled was measured as the distance crossed by the entire body of the fly, that is, the lower limit of the bin in which it was scored.

Two parameters were scored – the average distance travelled by the flies of each treatment, and the propensity to show negative geotactic mobility. The propensity was measured as the total number of flies in each treatment that left the base of the set-up and travelled at least 1 cm. Being a fraction, the propensity data was arcsine-square root transformed before analysis (Zar, 1999).

2.4.2. Stop-for-Sweet

2.4.2.1. Setup and Protocol

Mechanical perturbation:

After 3 days of stress (or control) treatment and recovery, on the 4th day the flies were subjected to the stress (or control) protocol for 4 hours, but in the absence of water (Ries et al., 2017).

A cotton strip soaked in 99% glycerol was stuck across the middle of a 35mm petri plate of thickness 1.5cm (Setup modified from Ries et al., 2017). The plate was covered by a lid and sealed (Fig. 4). Individual flies were aspirated into clean 5mm transparent glass tubes right before the assay. They were introduced into the set-up via a small hole drilled into the side of the lid, with the help of a glass tube and an aspirator. The fly was then shaken down to the bottom of the plate and allowed to wander around in the setup. For each fly, it was scored whether during a cross-over of the strip, it overran the glycerol or stopped to eat. Care was taken to only count the stops where the fly was eating, and not grooming. After each time the fly ran over the glycerol or stopped to eat, the setup was shaken again to let the fly start from the bottom of the plate. This process was repeated for 10 cross-overs for each fly.

Adult crowding:

After 72 hours of crowded (or control) conditions and 14 hours of recovery, both the treatment and control groups were subjected to 4 hours of starvation and desiccation.

The assay was performed similarly as described above.

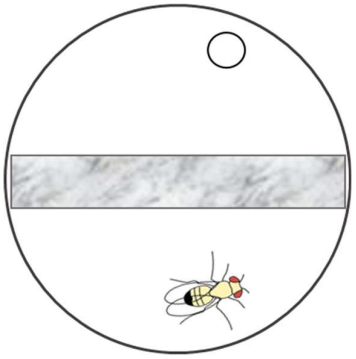

Fig. 4: Schematic for Stop-for-Sweet assay

2.4.2.2. Scoring

Each set-up was scored at the time of the assay, by observers trained to identify the behaviours of stopping and feeding versus not-stopping, but blind to the nature of the treatment. The proportion of stops by each fly was calculated, given by:

(Number of times each fly stopped to eat) / (Total number of cross-overs monitored)

This value was arcsine-square root transformed for further analysis (Zar, 1999).

2.4.3. Exploratory behaviour

2.4.3.1. Setup and Protocol

To measure exploratory tendency in flies, an established experimental arena was used (modified from Soibam et al., 2012) and the activity was recorded using a video camera (Sony HDR-PJ410, Sony DCR-SR20E) for scoring later. The experimental arena consisted of a clear polycarbonate petri dish lid, with an inner diameter of 10 cm. The lid was placed over a blank sheet of paper having two concentric circles. The outer circle was of the same diameter as the lid, while the inner circle was such that it divided the arena into two zones – the zone between the outer and inner circle constituted 1/3rd of the total area, and the zone inside the inner circle constituted 2/3rd of the total area (Fig. 5). Immediately before the assay, individual flies were aspirated into clean 5mm transparent glass tubes. They were introduced into the set-up via a small hole drilled into the centre of the lid, with the help of the tube and an aspirator. They were given 1 minute to acclimatize to their environment, and observed for the next 10 minutes.

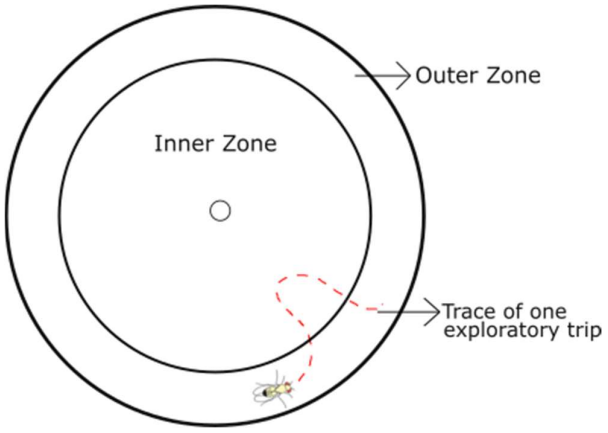

Fig. 5: Arena for exploratory behaviour

2.4.3.2. Scoring

As the flies tend to stay towards the outer edge of the arena, each time they entered the inner zone and came back was counted as one exploratory trip. The parameter scored was the total number of trips made by each fly within the 10-minute period.

2.4.4. Locomotor Activity and Rest

2.4.4.1. Setup and Protocol

Locomotor activity of the flies was measured using *Drosophila* Activity Monitor (DAM2) data collection systems (Trikinetcs Inc., USA) (Fig. 6A) using standard protocol (Chiu et al., 2010). This system measures the activity of an individual fly in a glass tube as the number of times it crosses an infrared beam which bisects each channel in the DAM, perpendicular to the axis of the tube (Fig. 6B). Activity readings were taken every 5 minutes or 1 minute for a period of 6 hours or 24 hours respectively.

After 3 days of stress and overnight recovery, flies were aspirated into transparent glass DAM tubes (5-mm diameter), devoid of any food, and plugged with cotton on each side, for analysis over 6 hours. For analysis over 24 hours, DAM tubes were prepared with banana-jaggery medium on one side of the tube, sealed with paraffin, while the other side was plugged with cotton, and flies were aspirated into the tubes immediately after the stress protocol ended on the 3rd day. Aspiration was preferred over CO_2 anaesthetisation as the latter could affect their activity levels if the readings are taken without sufficient time for recovery from anaesthesia. The DAM tubes were loaded onto the monitors, with 32 flies in each monitor, and placed undisturbed in an incubator at 25^{o}C at constant light.

A

B

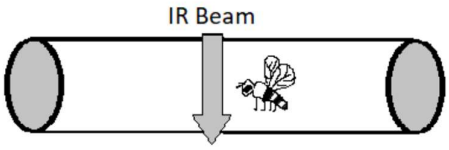

Fig.6: DAM assay A. Drosophila *activity monitor and B. DAM tube*

2.4.4.2. Scoring

The first 15 minutes of the data recorded was not scored to allow for acclimatisation of the fly to the environment. Two parameters were scored for each fly – activity index and proportion of rest. Activity Index (AI) was measured as the total activity counts of a fly divided by the duration that the fly spent awake or not resting (Gilestro, 2012; Kayser et al., 2014). No activity for a period of 5 minutes was scored as rest (Chiu et al., 2010; Hendricks et al., 2000); the fraction of the assay duration spent resting was scored as the proportion of rest. This value was arcsine-square root transformed for further analysis (Zar, 1999). The two parameters are independent, as a fly that spends a larger proportion of time resting does not necessarily have higher or lower activity when awake.

2.4.5. Dispersal Traits

2.4.5.1. Setup and Protocol

A source-path-destination set-up was used for this assay (Fig. 1), with the source being a 100-mL conical glass flask, connected via the path – a transparent 2-m-long plastic pipe – to the destination, which was a 250-mL plastic fly bottle, devoid of food and moisture. The mouth of the bottle was plugged using a sponge plug with a hole in the middle, through which the path pipe could pass. The pipe protruded via a plastic nozzle ~1.5 inches into the destination as this prevents backtracking of flies into the path (Tung et al., 2018b). The assay was performed in two conditions – with or without food in the source. For the former, the source was supplied with 35-mL of banana-jaggery food.

100-120 flies of either sex were placed into individual sources. The destination was replaced every 10 minutes, and the assay was continued for 1.5 hours. The number of dispersers in the destination was recorded at each 10-minute interval.

2.4.5.2. Scoring

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

- Dispersal Propensity – the proportion of flies that left the source and initiated dispersal (Friedenberg, 2003). This value was arcsine-square root transformed for further analysis (Zar, 1999).

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

- Dispersal Speed – the average speed at which the dispersers completed their source-to-destination movement.

$$Dispersal\ Speed = \frac{\sum_i (\frac{d}{T_i} \times n_i)}{\sum_i n_i}$$

Where, d = path length (i.e. 2m in this case), N is the total number of flies introduced 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 dispersal assay, and T_i is the total time in hours since the setup at the end of the i^{th} time interval.

2.4.6. Starvation Resistance

2.4.6.1. Setup and Protocol

Following the recovery period after stress (or control) treatment, groups of 10 flies of each treatment and sex were made under light CO_2 anaesthesia. They were transferred to vials containing 1.24% agar, which allowed for an environment of starvation but not desiccation. They were placed in an incubator at 25^0C at constant light. At intervals of 4 hours following the set-up, the total number of flies alive in each vial were counted. This was continued till there were no flies alive in any vial.

2.4.6.2. Scoring

Two parameters were scored – The Kaplan-Meier (KM) estimate (Kaplan and Meier, 1958) and the time point at which 50% of the flies in each vial died. The KM estimate for survival $S(t)$ at time t was given by:

$$S(t) = \prod_{t_i < t} \left(1 - \frac{d_i}{n_i}\right)$$

where d_i is the number of flies that died at the time point t_i and n_i is the total number of flies which are at risk till just before the point t_i.

2.5. Statistical Analysis

Males and females were analysed separately for all the assays, because the stress treatment differed with sex.

2.5.1. For DB_4 (baseline) experiments

For RING, replicates of treatment and control groups on which the assay was performed together were analysed together as one round. Two-factor mixed-model ANOVA was performed with treatment (stress or control) as a fixed factor, and round as a random factor. For all other assays, Mann-Whitney U (MWU) tests were performed with treatment (stress or control) as the factor, as the data failed Shapiro-Wilk normality tests. However, there were no major changes in significance levels of data when MWU test results were compared to ANOVA results for the same datasets and all interpretations remain essentially unchanged, which demonstrates the robustness of our results. Therefore, here we report only the results of the non-parametric MWU tests.

2.5.2. For $VB(C)_4$ (selected) experiments

For all assays, two-factor mixed-model ANOVA was performed with treatment (stress or control) and selection (VB or VBC) as fixed factors. Round was added as a random factor for RING. For significant main effects, pair-wise differences were analysed using Tukey's HSD test.

For all experiments (DB_4 and $VB(C)_4$), Cohen's *d* effect sizes were estimated to compare between groups. The value of effect size was interpreted as large ($d > 0.8$), medium ($0.8 > d > 0.5$) or small ($d < 0.5$) following standard recommendations (Cohen, 1988). MWU tests were performed using Past3 and ANOVAs were performed using STATISTICA ver. 5 (StatSoft Inc). All graphs were plotted in R version 3.1.3 (R Core Team, 2015).

3. RESULTS

3.1. Baseline (DB_4) population

For all experiments, the statistical data has been reported in Table 1. In all the figures, 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 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. The points beyond this are indicated as outliers (solid black circles).

3.1.1. No change in innate response

Compared to their controls, neither male (Fig. 7A) nor female (Fig. 7B) flies subjected to mechanical perturbation showed any significant change in their propensity of negative geotaxis measured in the 1st trial of the RING assay. Similar results were obtained for males (Fig. 7C) and females (Fig. 7D) subjected to adult crowding.

Similarly, neither males (Fig. 8A) nor females (Fig. 8B) showed a change in the average distance travelled during negative geotaxis after mechanical perturbation. These trends were also retained when male (Fig. 8C) and female (Fig. 8D) flies were subjected to adult crowding.

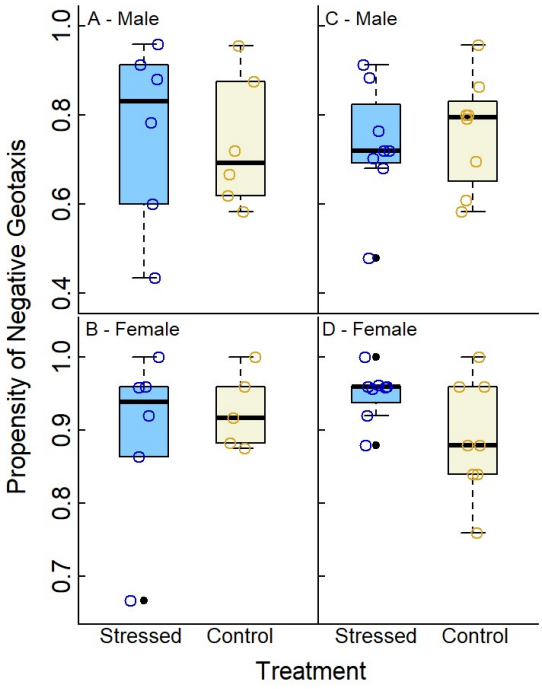

Fig. 7: Propensity of negative geotaxis after the 1st trial in A. males and B. females after mechanical perturbation; C. males and D. females after adult crowding vs their respective controls

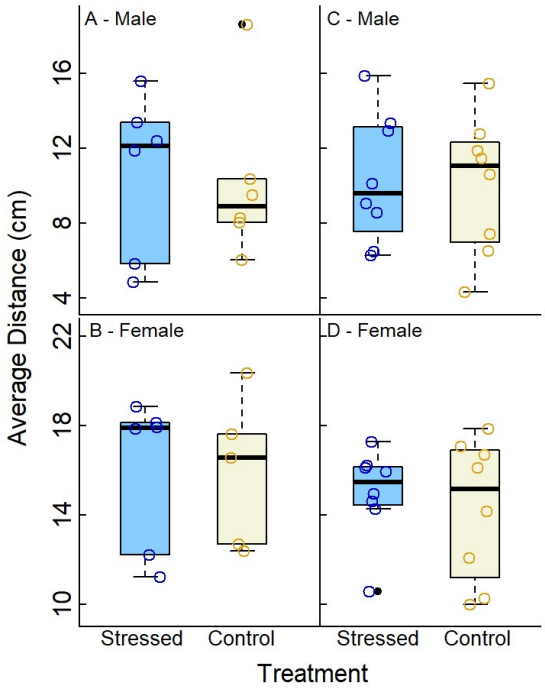

Fig. 8: Average distance travelled after the 1st trial in A. males and B. females after mechanical perturbation; C. males and D. females after adult crowding vs their respective controls

When measured after 10 trials, no change in propensity of negative geotaxis was observed across both sexes after mechanical perturbation (Fig. 9A & 9B) or after adult crowding (Fig. 9C & 9D), and neither were any changes observed in the average distance travelled by males (Fig. 10A & 10C) or females (Fig. 10B & 10D) after either stressor.

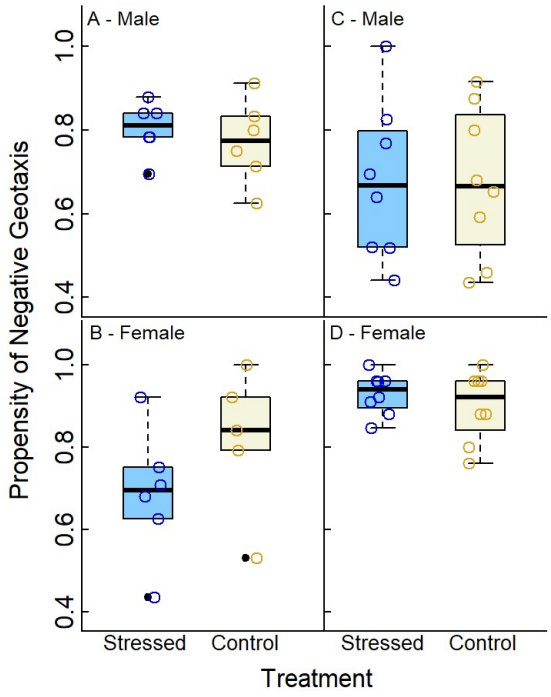

Fig. 9: Propensity of negative geotaxis after 10 trials in A. males and B. females after mechanical perturbation; C. males and D. females after adult crowding vs their respective controls

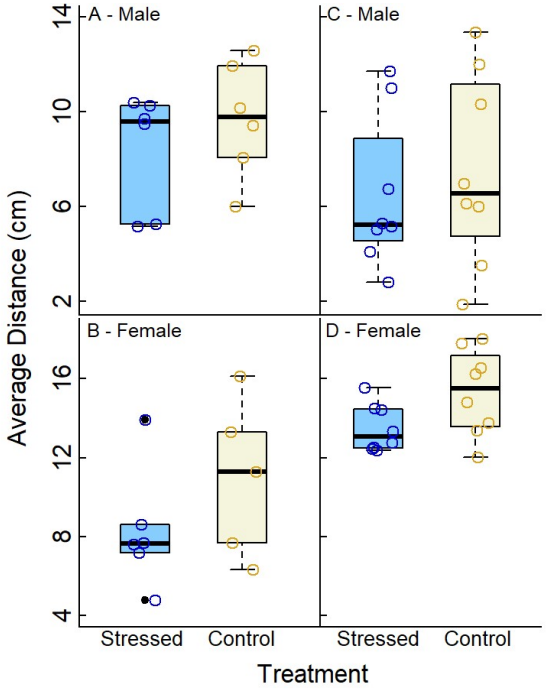

Fig. 10: Average distance travelled after 10 trials in A. males and B. females after mechanical perturbation; C. males and D. females after adult crowding vs their respective controls

Thus, to summarize, there were no significant differences between the stressed flies and the controls in either their propensity of negative geotaxis, or their ability to climb the walls of the RING setup. This indicates that neither stressor injured or caused physical harm to the flies.

3.1.2. Lesser interest in pleasurable activities

Both male (Fig. 11A) and female (Fig. 11B) flies subjected to mechanical perturbation showed significantly reduced tendency to feed on glycerol as compared to their controls. This indicates that mechanical perturbation induced anhedonia, i.e. a reduction of interest in pleasurable activities.

When subjected to adult crowding, female flies showed anhedonia and fed lesser on glycerol (Fig. 11D). However, male flies showed an increased tendency to feed on glycerol (Fig. 11C). This suggests that adult crowding induces sexually dimorphic effects on anhedonic behaviour.

3.1.3. Reduced exploration of novel habitat in females

Male flies showed no significant change in the tendency to explore their habitat after mechanical perturbation (Fig. 12A). However, there was a significant reduction in the number of exploratory trips made by female flies (Fig. 12B) subjected to this stressor.

There was no significant change in exploratory tendency after adult crowding in males (Fig. 12C). There was a marginally insignificant reduction in the number of exploratory trips in females subjected to crowding (Fig. 12D) (where $0.05 < p < 0.1$; when the effect size is medium to high and the p-value is marginally insignificant, the difference between the two groups was interpreted as significant).

Thus, female flies after being subjected to stress showed a reduced motivation to explore their habitat, indicating sexually dimorphic effects in exploratory behaviour in response to stress.

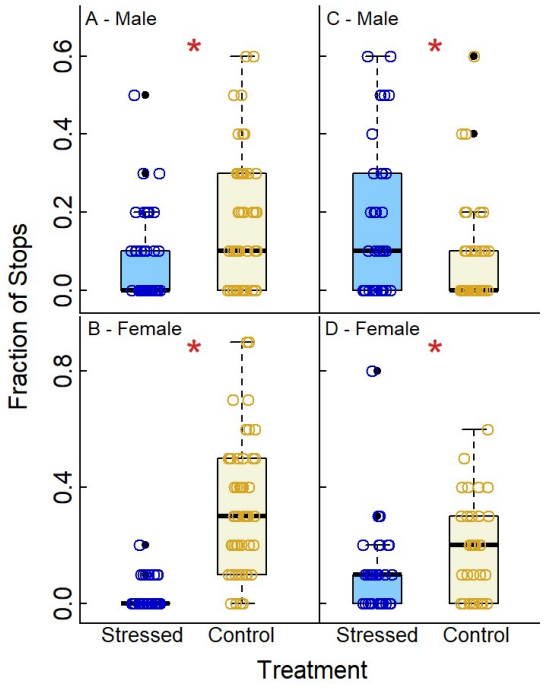

Fig. 11: Fraction of stops to feed on glycerol in A. males and B. females after mechanical perturbation; C. males and D. females after adult crowding vs their respective controls.

* *indicates MWU $p < 0.05$*

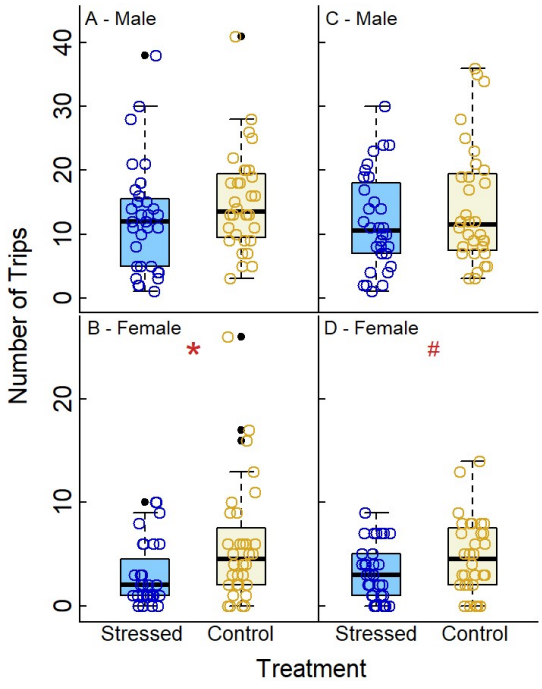

Fig. 12: Number of exploratory trips in A. males and B. females after mechanical perturbation; C. males and D. females after adult crowding vs their respective controls. * *indicates MWU $p < 0.05$;* # *indicates $p < 0.1$*

3.1.4. Increased locomotor activity and restlessness

The proportion of time spent resting was significantly lowered after mechanical perturbation in both males (Fig. 13A) and females (Fig. 13B). Similar reduction was also observed across both sexes after adult crowding (Fig. 13C & 13D).

However, when the Activity Index (AI) was compared for these stressors, crowding again induced a sexual dimorphism. While both males (Fig. 14A) and females (Fig. 14B) showed increased AI after mechanical perturbation, after crowding, males showed a marginally insignificant increase in AI (Fig. 14C), while females showed a reduction in AI (Fig. 14D).

Thus, while stress makes flies rest less across sexes, the nature of stressor modulates sexual dimorphism in AI levels.

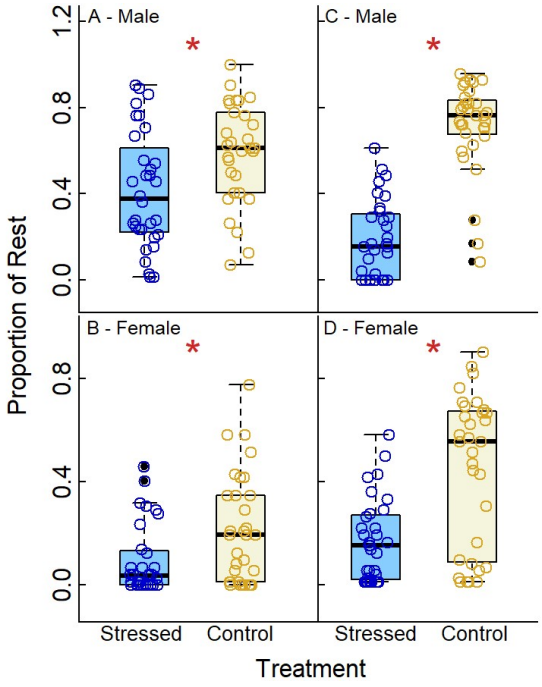

*Fig. 13: Proportion of time spent resting over 6 hours in A. males and B. females after mechanical perturbation; C. males and D. females after adult crowding vs their respective controls. * indicates MWU $p < 0.05$*

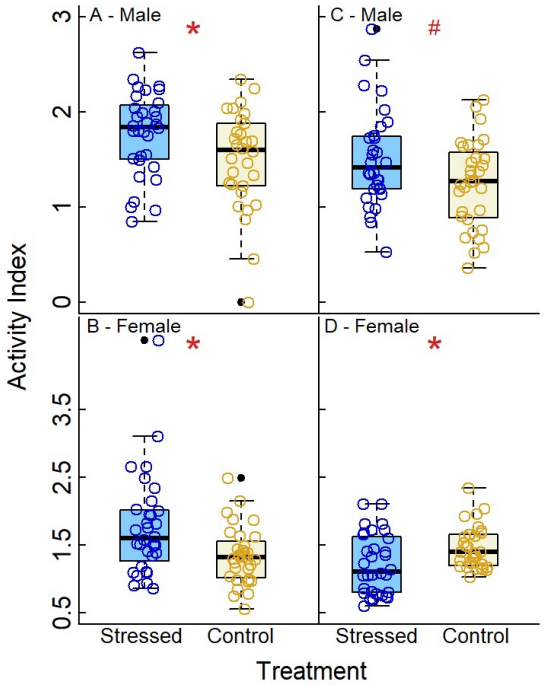

*Fig. 14: Activity Index over 6 hours in A. males and B. females after mechanical perturbation; C. males and D. females after adult crowding vs their respective controls. * indicates MWU $p < 0.05$, # indicates $p < 0.1$*

3.1.5. No change in starvation resistance

When the starvation resistance of flies which had been subjected to adult crowding was compared to their controls, there was no difference in the time taken for 50% mortality in the vial across treatment and control groups for both males and females (Table 1). This is congruent with the observation that the KM survivorship curves almost superimpose in both cases (Fig. 15).

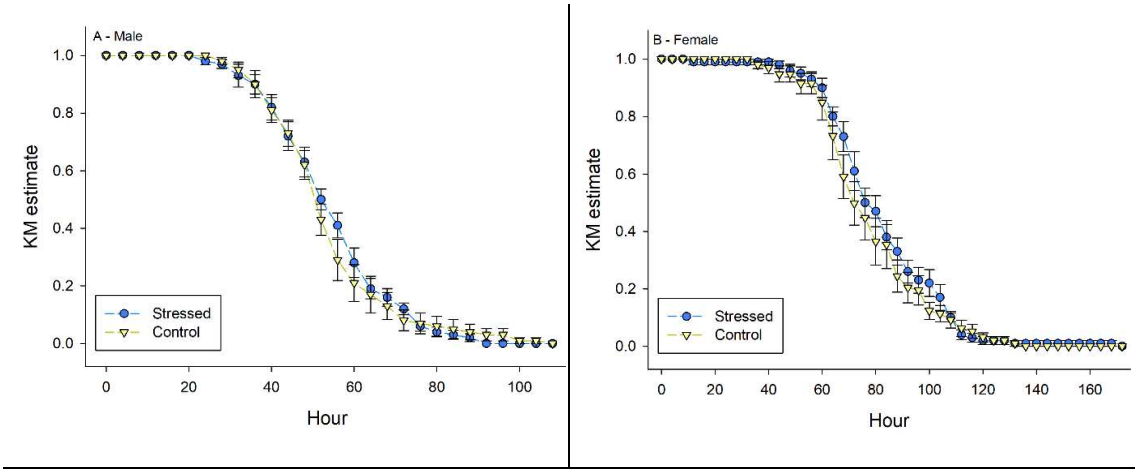

Fig. 15 : Survivorship curve under starvation conditions based on KM estimates for A. males and B. females after adult crowding compared to their respective controls

Assay:	Sex	p-value		Test statistic		Cohen's *d*		Sample size (n)	
		M.P.	*A.C.*	*M.P.*	*A.C.*	*M.P.*	*A.C.*	*M.P.*	*A.C.*
RING Propensity (Trial 1)	*M*	0.512	0.663	$F_{(1,2)} = 0.62$	$F_{(1,2)} = 0.23$	0.140	0.227	6	8
	F	0.670	0.335	$F_{(1,2)} = 0.24$	$F_{(1,2)} = 2.96$	0.343	0.970	6	8
RING Average Distance (Trial 1)	*M*	0.637	0.893	$F_{(1,2)} = 0.30$	$F_{(1,2)} = 0.02$	0.117	0.079	6	8
	F	0.924	0.386	$F_{(1,2)} = 0.01$	$F_{(1,2)} = 2.08$	0.033	0.268	6	8
RING Propensity (Trial 10)	*M*	0.302	0.577	$F_{(1,2)} = 1.90$	$F_{(1,2)} = 0.39$	0.367	0.001	6	8
	F	0.123	0.203	$F_{(1,2)} = 6.68$	$F_{(1,2)} = 9.18$	0.768	0.417	6	8
RING Average Distance (Trial 10)	*M*	0.165	0.204	$F_{(1,2)} = 4.61$	$F_{(1,2)} = 2.62$	0.537	0.285	6	8
	F	0.194	0.192	$F_{(1,2)} = 3.72$	$F_{(1,2)} = 10.34$	0.745	1.045	6	8
Fraction of Stops on Glycerol	*M*	1.26E-05	3.39E-02	U = 636	U = 441	0.876	0.518	50	35
	F	3.43E-16	5.85E-03	U = 125.5	U = 386	1.928	0.594	50	35
Number of Exploratory Trips	*M*	1.02E-01	4.08E-01	U = 390	U = 450	0.350	0.287	32	32
	F	3.25E-02	7.18E-02	U = 353.5	U = 378.5	0.601	0.509	32	32
Proportion of Rest	*M*	9.92E-03	4.47E-10	U = 296.5	U = 48	0.694	2.723	32	32
	F	2.22E-02	3.13E-04	U = 343.5	U = 244	0.656	1.161	32	32
Activity Index	*M*	3.52E-02	6.89E-02	U = 330	U = 376	0.573	0.543	32	32
	F	8.33E-03	2.21E-02	U = 315	U = 341	0.700	0.630	32	32
Starvation Resistance - 50% mortality time	*M*	--------	0.88	--------	U = 47.5	-------	0.118	------	10
	F	--------	0.59	--------	U = 42.5	-------	0.265	------	10

Table 1: p-values, test statistics, Cohen's d and sample sizes for various assays conducted on baseline fly populations. For p-value: Red p<0.05 (significant); blue p<0.1 (marginally insignificant); for Cohen's d: Red d>0.8 (high); blue 0.8>d>0.5 (medium); M: Male; F: Female; M.P: Mechanical Perturbation; A.C.: Adult Crowding.

3.2. Selected ($VB(C)_4$) population

For all experiments, the statistical data for the main effects has been reported in Table 2, and that of pairwise interactions has been reported in Table 3. In all the figures, the points represent the data for all replicates of the particular group with small random jitter on the x-axis, the edges of the box denote the 25th and 75th percentiles, the black solid line represents the median and the red triangles represent the mean. 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. The points beyond this are indicated as outliers (solid black circles).

3.2.1. No change in innate response

When analysed after the 1st trial of the RING assay, males did not show any significant change in the propensity to show negative geotaxis (Fig. 16A), or in the average distance travelled (Fig. 17A).

However, in females, there was a significant effect of the treatment x selection interaction for propensity of negative geotaxis, with VBC females subjected to stress showing a higher propensity than their controls (Fig. 16B). While the average distance travelled by females also showed a significant interaction effect, there were no pair-wise significant effects among treatment groups within either VB or VBC (Fig. 17B).

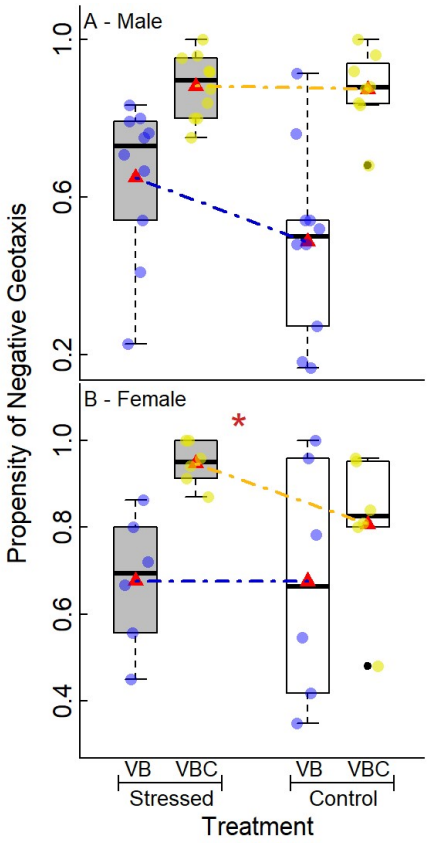

*Fig. 16: Propensity of negative geotaxis after the 1st trial in A. males and B. females after mechanical perturbation vs their respective controls. * indicates p < 0.05 for the selection x treatment interaction.*

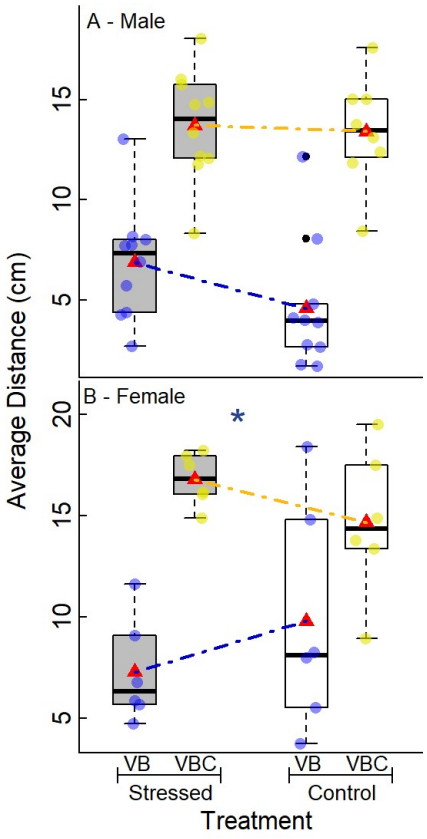

*Fig. 17: Average distance travelled after the 1st trial in A. males and B. females after mechanical perturbation vs their respective controls. * indicates p < 0.1 for the selection x treatment interaction.*

When measured after 10 trials, neither males nor females showed a significant change in the propensity of negative geotaxis (Fig. 18). No change in the average distance travelled was observed in either sex (Fig. 19).

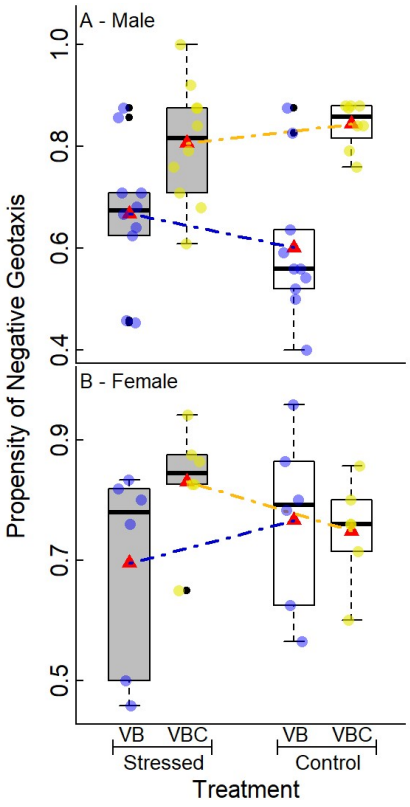

Fig. 18: Propensity of negative geotaxis after 10 trials in A. males and B. females after mechanical perturbation vs their respective controls.

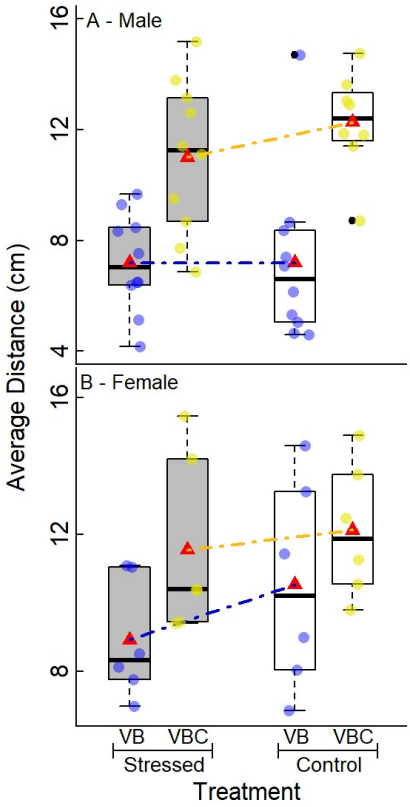

Fig. 19: Average distance travelled after 10 trials in A. males and B. females after mechanical perturbation vs their respective controls.

Interestingly, across both 1st and 10th trials, a significant main effect of selection was observed in male flies in both negative geotactic propensity and the average distance travelled, with VBC males showing significantly higher levels of both than VB males (Table 2).

Thus, while male flies subjected to stress did not show any changes in negative geotactic behaviour, female VBC flies showed an increase in the immediate propensity of negative geotaxis after stress. This indicates that stress did not diminish the cue-based responses of the flies of either sex, and thus did not cause any physical harm or injury to the flies.

3.2.2. Reduced dispersal after stress

Across experiments and sexes, dispersal propensity and speed were higher for VB flies than for VBC flies, in keeping with previous results (Tung et al., 2018b; Table 2).

In both males and females, when dispersal propensity was measured without food in the source, selection showed a significant interaction with treatment (Fig. 20). VBC flies across sexes showed significantly reduced propensity to disperse after stress as compared to their controls, while no change was observed among VB flies (Table 3).

In the presence of food, the treatment x selection interaction was marginally insignificant in males (Fig. 21A). However, due to a high value of the partial-eta squared (= 0.289), post-hoc analysis was carried out, and similar results as above were observed. In females, the interaction was significant (Fig. 21B), with stressed VBC flies showing lower propensity than their controls.

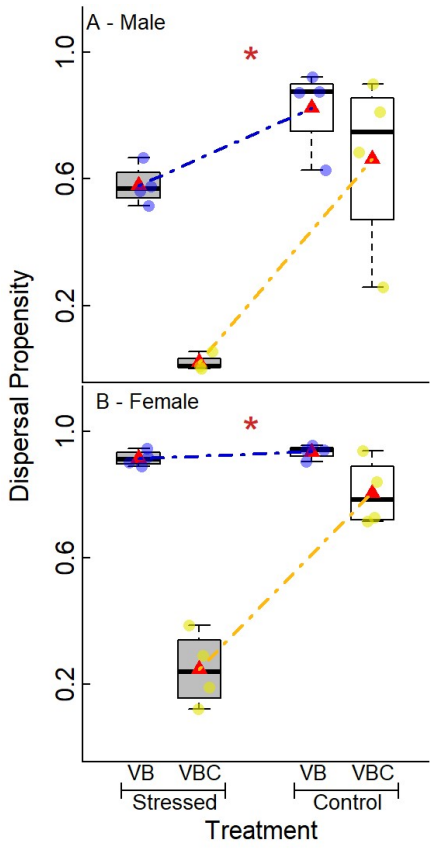

*Fig. 20: Propensity to disperse in the absence of food in the source in A. males and B. females after mechanical perturbation vs their respective controls. * indicates $p < 0.05$ for the selection x treatment interaction.*

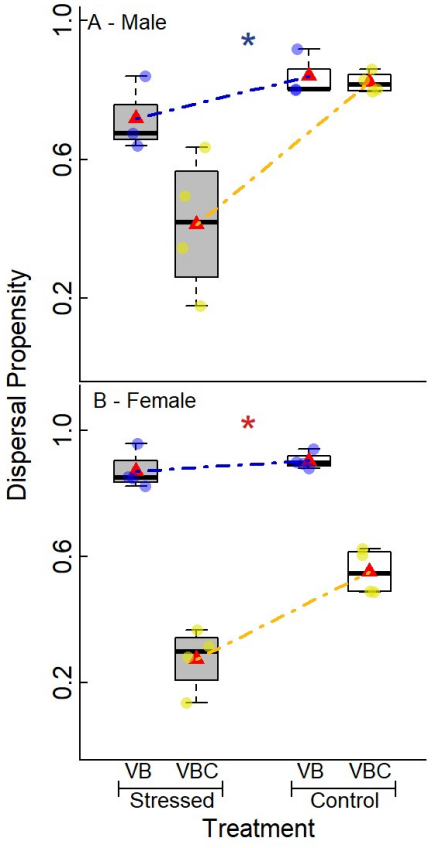

*Fig. 21: Propensity to disperse in the presence of food in the source in A. males and B. females after mechanical perturbation vs their respective controls. * indicates $p < 0.05$ for the selection x treatment interaction; * indicates $p < 0.1$ for the selection x treatment interaction.*

There was no interaction of treatment x selection across sexes for dispersal speed in the absence of food in the source. However, a main effect of treatment was observed in both males and females, with stressed flies showing reduced speed of dispersal (Fig. 22). Similar results were observed when food was present in the source (Fig. 23).

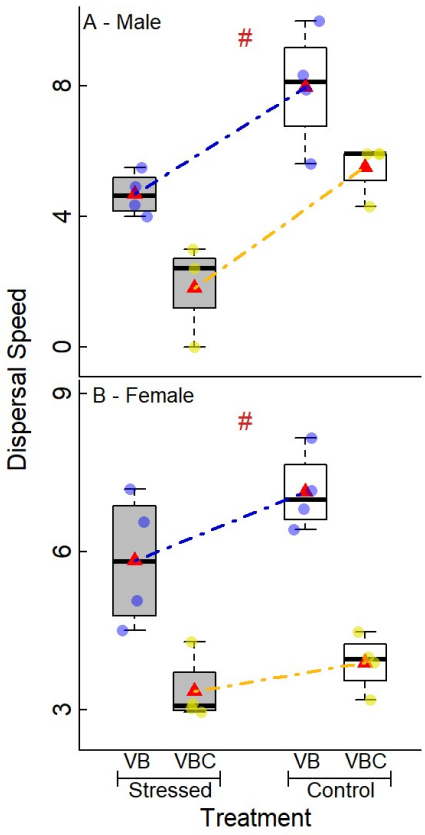

Fig. 22: Dispersal speed in the absence of food in the source in A. males and B. females after mechanical perturbation vs their respective controls. # indicates $p < 0.05$ for the main effect of treatment.

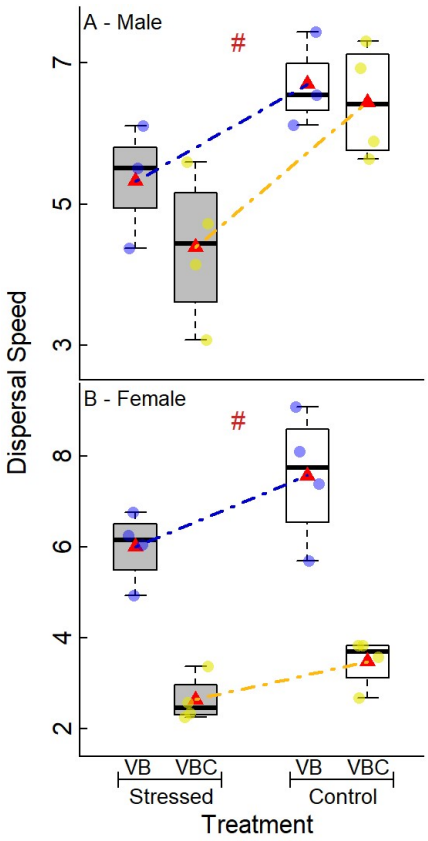

Fig. 23: Dispersal speed in the presence of food in the source in A. males and B. females after mechanical perturbation vs their respective controls. # indicates $p < 0.05$ for the main effect of treatment.

Thus, for both sexes, while stress reduced the speed of dispersal across selection regimes, flies selected for increased dispersal do not show a change in dispersal propensity after being subjected to stress. Non-selected flies show a reduced propensity to disperse from the source after being subjected to stress, irrespective of the presence or absence of food in the source.

3.2.3. Reduced exploration of novel habitat in females

Male flies subjected to stress showed no change in exploratory tendencies as compared to their controls (Fig 24A). A significant effect of selection, with VB males showing greater exploratory behaviour than VBCs, was observed, in keeping with previous results from the lab (Tung et al., 2018a).

In females, the interaction of selection with stress was significant - stressed VBC females showed a reduction in the number of exploratory trips as compared to their controls (Fig. 24B). Thus, both selection for increased dispersal and sex interact to modulate the response of exploratory behaviour to stress.

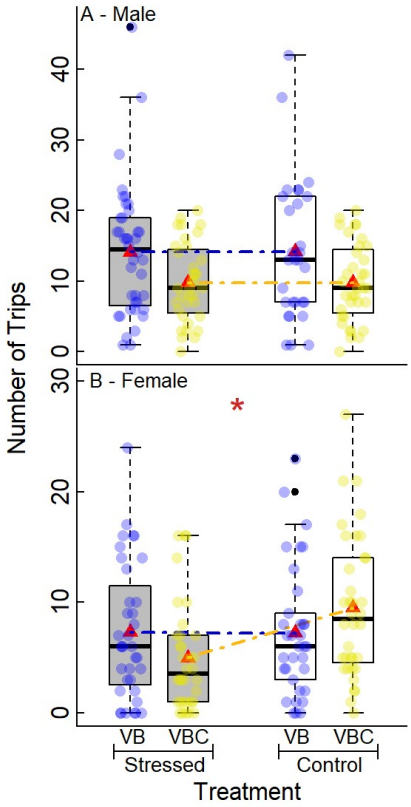

Fig 24. Number of exploratory trips in A. males and B. females after mechanical perturbation vs their respective controls. * *indicates p < 0.05 for the selection x treatment interaction.*

3.2.4. Changes in locomotor activity and rest

VB flies rested lesser and higher activity index (AI) as compared to VBCs, in keeping with previous results (Tung et al., 2018a), across sexes (Table 2).

Following the stress/control treatment and overnight rest in food vials, activity patterns of flies were measured for 6 hours without food in the DAM tube. There was no change in rest levels after stress across sexes (Fig. 25). Male flies subjected to stress had a significantly higher AI than the controls (Fig. 26A). For female flies, treatment had a marginally insignificant effect, with stressed flies having higher AI than the controls (Fig. 26B)

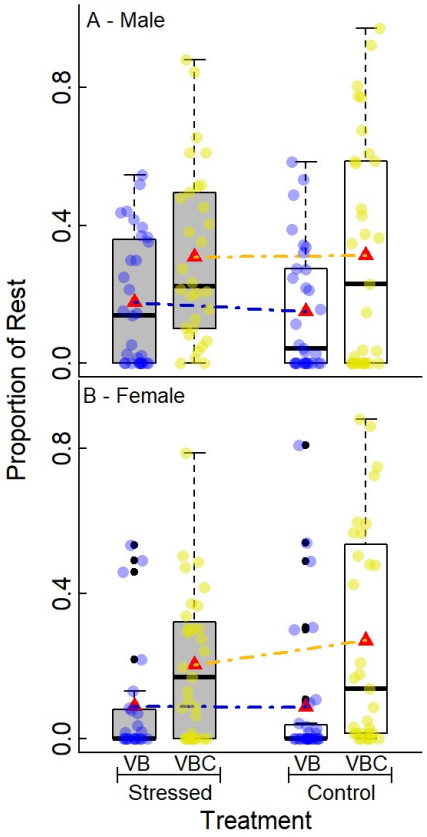

Fig. 25: Proportion of time spent resting over 6 hours in the absence of food in A. males and B. females after mechanical perturbation vs their respective controls.

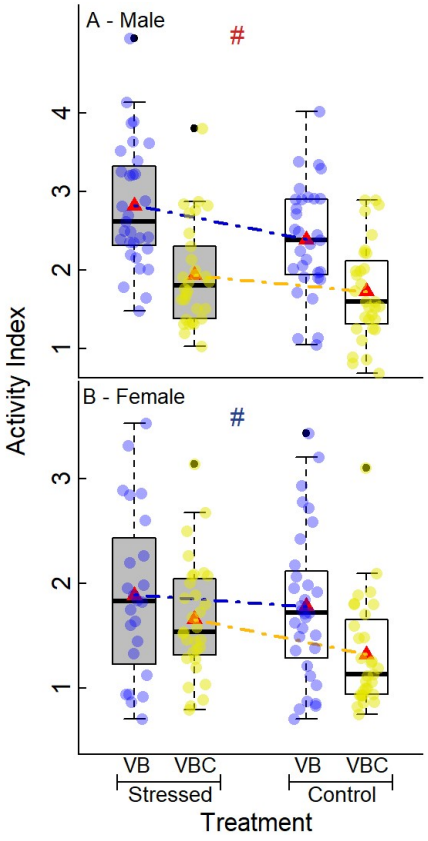

Fig. 26: Activity Index over 6 hours in the absence of food in A. males and B. females after mechanical perturbation vs their respective controls. # indicates $p < 0.05$ for the main effect of treatment; # indicates $p < 0.1$ for the main effect of treatment.

When activity patterns were measured over 6 hours with food in the DAM tube, after overnight rest in the DAM tubes on food, male flies subjected to stress were found to rest significantly more (Fig. 27A). In females, the treatment x selection interaction was significant, and stressed VBC flies rested more than their controls, while the VB flies rested comparably across treatments (Fig. 27B).

The AI of stressed male flies were comparable to the controls, with no significant effect of treatment or interaction (Fig. 28A). Selection interacted significantly with treatment in females, with stressed VBC flies showing higher AI than their controls, indicating that while they were awake, they were more active (Fig. 28B).

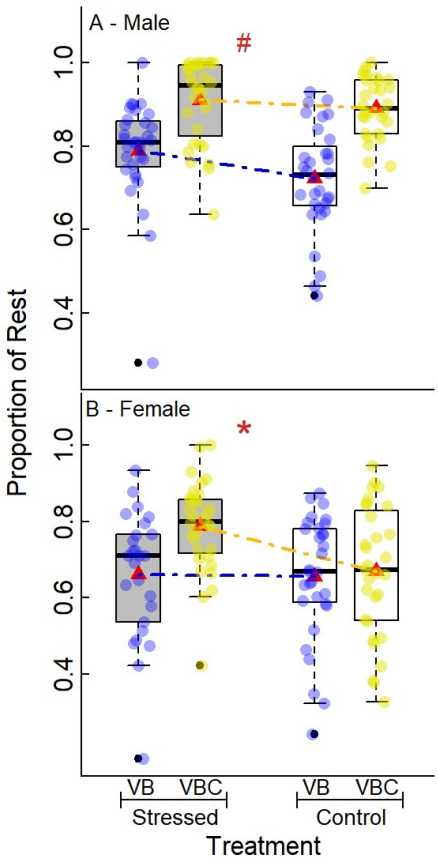

*Fig. 27: Proportion of time spent resting over 6 hours in the presence of food in A. males and B. females after mechanical perturbation vs their respective controls . * indicates p < 0.05 for the selection x treatment interaction; # indicates p < 0.05 for the main effect of treatment*

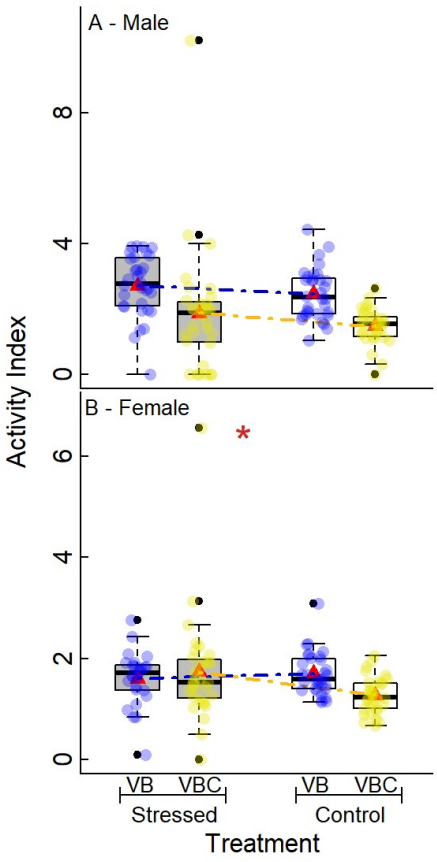

*Fig. 28: Activity Index over 6 hours in the presence of food in A. males and B. females after mechanical perturbation vs their respective controls. * indicates p < 0.05 for the selection x treatment interaction.*

Further, to study the immediate effects of stress on activity, the measurements were taken for 6 hours immediately after the stress, in DAM tubes with food. Stressed male flies across selection regimes rested significantly higher amounts immediately after stress (Fig. 29A), while females also showed a similar trend, with marginally insignificant effect of treatment (Fig. 29B). There was no interaction observed between selection and treatment.

Stress did not have any effect on the AI of male flies (Fig. 30A). While selection x treatment was significant in females, there were no pair-wise differences between treatment and control groups across selection (Fig. 30B, Table 3).

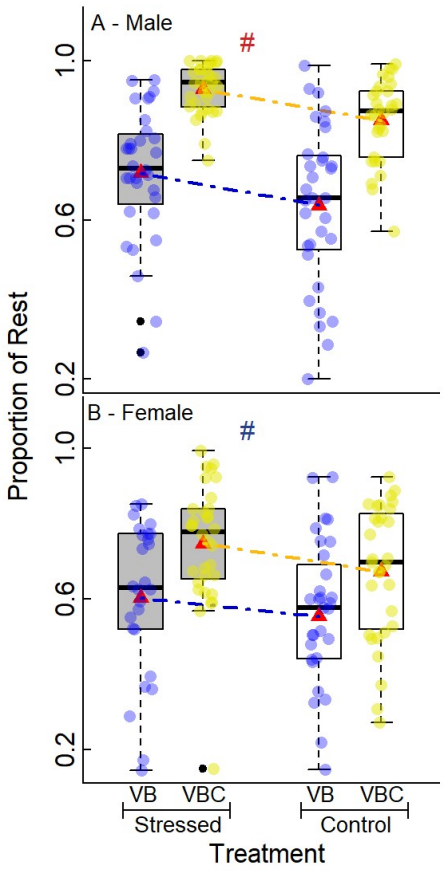

Fig. 29: Proportion of time spent resting over 6 hours immediately after stress in the presence of food in A. males and B. females after mechanical perturbation vs their respective controls; # indicates p < .05 for the main effect of treatment; # indicates p < 0.1 for the main effect of treatment.

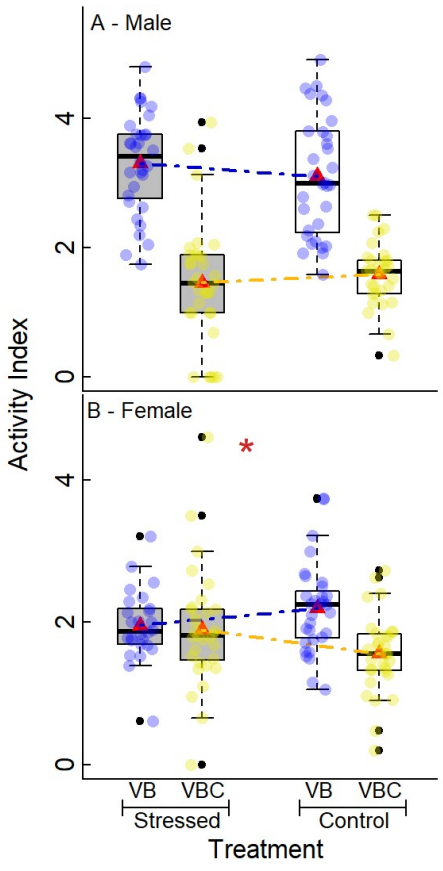

*Fig. 30: Activity Index over 6 hours immediately after stress in the presence of food in A. males and B. females after mechanical perturbation vs their respective controls. * indicates p < 0.05 for the selection x treatment interaction.*

Finally, these parameters were measured over 24 hours with food, starting immediately after the stress. Stressed flies across sexes rested significantly more than their controls (Fig. 31). There was no interaction of selection with treatment. The AI of stressed male flies was significantly higher than the controls, with no interaction of selection (Fig. 32A). Again, selection interacted with stress in females – stressed VBC flies had significantly higher AI (Fig. 32B) as compared to controls.

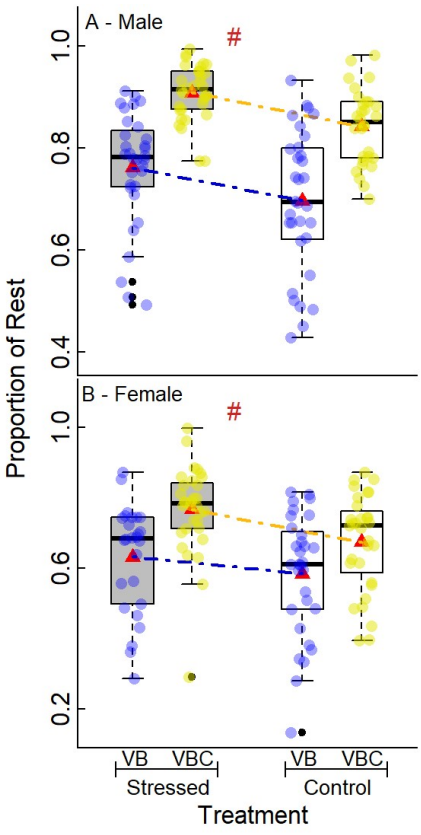

Fig. 31: Proportion of time spent resting over 24 hours immediately after stress in the presence of food in A. males and B. females after mechanical perturbation vs their respective controls; # indicates $p < 0.05$ for the main effect of treatment.

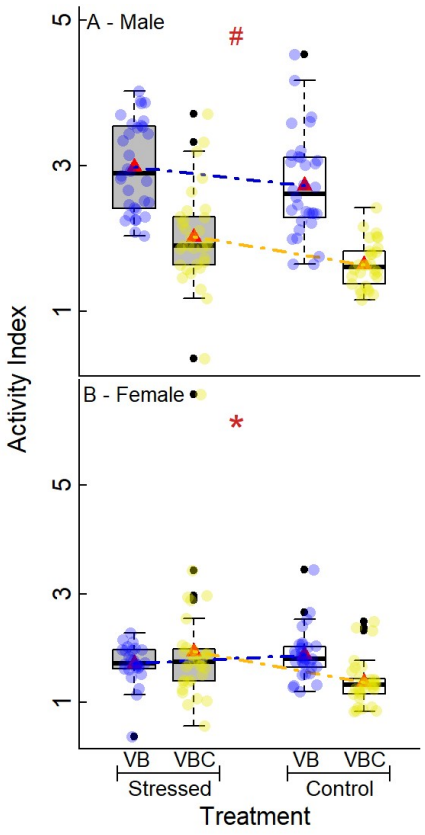

*Fig. 32: Activity Index over 24 hours immediately after stress in the presence of food in A. males and B. females after mechanical perturbation vs their respective controls. * indicates $p < 0.05$ for the selection x treatment interaction; ; # indicates $p < 0.05$ for the main effect of treatment.*

To summarise, the stress-induced changes in activity and rest patterns were modulated by food, and in females, impacted by selection for dispersal.

Assay:	Sex	p-value			df (effect,error)			F			Sample size (n)
		Treatment	*Selection*	*Treatment x Selection*	*Treat-ment*	*Selec-tion*	*Treatment x Selection*	*Treat-ment*	*Selec-tion*	*Treatment x Selection*	
RING Propensity (Trial 1)	*M*	0.116	8.49E-03	0.205	(1,4)	(1,4)	(1,4)	3.993	23.281	2.282	10
	F	0.723	0.096	3.57E-02	(1,2)	(1,2)	(1,2)	0.166	8.931	26.516	6
RING Average Distance (Trial 1)	*M*	0.106	1.70E-03	0.431	(1,4)	(1,4)	(1,4)	4.329	56.137	0.767	10
	F	0.943	1.55E-02	6.32E-02	(1,2)	(1,2)	(1,2)	0.007	63.187	14.337	6
RING Propensity (Trial 10)	*M*	0.673	4.87E-03	0.270	(1,4)	(1,4)	(1,4)	0.206	31.782	1.637	10
	F	0.855	0.649	0.303	(1,2)	(1,2)	(1,2)	0.043	0.280	1.892	6
RING Average Distance (Trial 10)	*M*	0.571	7.05E-03	0.602	(1,4)	(1,4)	(1,4)	0.380	25.863	0.320	10
	F	0.157	0.229	0.723	(1,2)	(1,2)	(1,2)	4.911	2.938	0.167	6
Dispersal Propensity (without food)	*M*	1.43E-04	6.91E-04	1.68E-02	(1,11)	(1,11)	(1,11)	32.247	21.741	7.932	4
	F	4.34E-05	1.01E-06	1.36E-04	(1,12)	(1,12)	(1,12)	38.896	82.308	30.252	4
Dispersal Speed (without food)	*M*	2.77E-04	2.12E-03	0.749	(1,11)	(1,11)	(1,11)	27.451	15.933	0.107	4
	F	4.71E-02	1.83E-05	0.384	(1,12)	(1,12)	(1,12)	4.893	46.589	0.818	4
Dispersal Propensity (with food)	*M*	2.38E-03	3.65E-02	7.14E-02	(1,10)	(1,10)	(1,10)	16.283	5.825	4.067	4
	F	2.76E-03	4.40E-08	1.78E-02	(1,12)	(1,12)	(1,12)	14.075	146.485	7.523	4
Dispersal Speed (with food)	*M*	4.77E-03	0.234	0.488	(1,10)	(1,10)	(1,10)	4.827	5.180	0.738	4
	F	1.94E-02	2.45E-06	0.433	(1,12)	(1,12)	(1,12)	7.274	69.552	0.659	4
Number of Exploratory Trips	*M*	0.655	7.73E-03	0.698	(1,139)	(1,139)	(1,139)	0.200	7.306	0.151	40
	F	2.58E-02	0.972	2.31E-02	(1,136)	(1,136)	(1,136)	5.081	0.001	5.276	36

Table 2: p-values and test statistics for main effects and interaction in 2-way ANOVA; and sample sizes for various assays conducted on selected fly populations. For p-value: Red p<0.05 (significant); blue p<0.1 (marginally insignificant); M: Male; F: Female

Assay:	Sex	p-value			df (effect,error)			F			Sample size (n)
		Treatment	*Selection*	*Treatment x Selection*	*Treat-ment*	*Selec-tion*	*Treatment x Selection*	*Treat-ment*	*Selec-tion*	*Treatment x Selection*	
Proportion of Rest (6h, with rest, w/o food)	*M*	0.441	1.76E-03	0.912	(1,119)	(1,119)	(1,119)	0.598	10.237	0.012	32
	F	0.685	9.98E-05	0.459	(1,114)	(1,114)	(1,114)	0.165	16.266	0.552	32
Activity Index (6h, with rest, w/o food)	*M*	1.26E-02	8.93E-09	0.371	(1,119)	(1,119)	(1,119)	6.413	38.291	0.807	32
	F	7.04E-02	4.65E-03	0.337	(1,114)	(1,114)	(1,114)	3.335	8.338	0.930	32
Proportion of Rest (6h, with rest, with food)	*M*	8.89E-03	2.12E-12	0.774	(1,122)	(1,122)	(1,122)	7.070	61.151	0.083	32
	F	2.69E-02	8.68E-03	4.54E-02	(1,116)	(1,116)	(1,116)	5.022	7.127	4.091	32
Activity Index (6h, with rest, with food)	*M*	0.120	2.32E-05	0.748	(1,122)	(1,122)	(1,122)	2.454	19.369	0.104	32
	F	0.141	0.235	2.60E-02	(1,116)	(1,116)	(1,116)	2.191	1.425	5.084	32
Proportion of Rest (6h, w/o rest, with food)	*M*	7.38E-04	2.29E-14	0.426	(1,122)	(1,122)	(1,122)	11.990	75.050	0.638	32
	F	7.41E-02	2.01E-04	0.628	(1,116)	(1,116)	(1,116)	3.248	14.746	0.236	32
Activity Index (6h, w/o rest, with food)	*M*	0.812	9.14E-22	0.260	(1,122)	(1,122)	(1,122)	0.057	137.863	1.280	32
	F	0.677	3.79E-03	1.58E-02	(1,116)	(1,116)	(1,116)	0.175	8.732	6.004	32
Proportion of Rest (24h, w/o rest, with food)	*M*	1.41E-04	4.55E-14	0.484	(1,122)	(1,122)	(1,122)	15.457	72.881	0.492	32
	F	8.28E-03	4.80E-05	0.326	(1,116)	(1,116)	(1,116)	7.218	17.841	0.972	32
Activity Index (24h, w/o rest, with food)	*M*	3.31E-03	1.01E-16	0.558	(1,122)	(1,122)	(1,122)	8.979	93.164	0.345	32
	F	8.99E-02	0.242	3.16E-03	(1,116)	(1,116)	(1,116)	2.926	1.384	9.087	32

Table 2 (Contd.): p-values and test statistics for main effects and interaction in 2-way ANOVA; and sample sizes for various assays conducted on selected fly populations. For p-value: Red p<0.05 (significant); blue p<0.1 (marginally insignificant); M: Male; F: Female

Assay:	Sex	p-value (inter-action)	Pairwise Tukey's HSD p-value		Effect size	
			VB (S) - VB (C)	*VBC (S) - VBC (C)*	*VB (S) - VB (C)*	*VBC (S) - VBC (C)*
RING Propensity (Trial 1)	*M*	0.205	------		0.749	0.086
	F	3.57E-02	0.883	4.40E-02	0.002	1.088
RING Average Distance (Trial 1)	*M*	0.431	------		0.744	0.114
	F	6.32E-02	0.138	0.241	0.569	0.774
RING Propensity (Trial 10)	*M*	0.270	------		0.467	0.415
	F	0.303	------		0.446	0.882
RING Average Distance (Trial 10)	*M*	0.602	------		0.000	0.542
	F	0.723	------		0.647	0.248
Dispersal Propensity (without food)	*M*	1.68E-02	0.210	7.59E-04	2.351	3.169
	F	1.36E-04	0.953	2.05E-04	0.894	5.034
Dispersal Speed (without food)	*M*	0.749	------		2.412	2.947
	F	0.384	------		1.268	0.933
Dispersal Propensity (with food)	*M*	7.14E-02	0.563	4.51E-03	1.367	2.908
	F	1.78E-02	0.890	3.13E-03	0.715	3.177
Dispersal Speed (with food)	*M*	0.488	------		1.752	2.192
	F	0.433	------		1.364	1.587
Number of Exploratory Trips	*M*	0.698	------		0.008	0.173
	F	2.31E-02	1.000	7.07E-03	0.007	0.793
Proportion of Rest (6h, with rest, w/o food)	*M*	0.912	------		0.142	0.016
	F	0.459	------		0.011	0.260
Activity Index (6h, with rest, w/o food)	*M*	0.371	------		0.571	0.325
	F	0.337	------		0.136	0.632
Proportion of Rest (6h, with rest, with food)	*M*	0.774	------		0.539	0.224
	F	4.54E-02	0.999	1.29E-02	0.033	0.796
Activity Index (6h, with rest, with food)	*M*	0.748	------		0.298	0.283
	F	2.60E-02	0.951	3.79E-02	0.197	0.573
Proportion of Rest (6h, w/o rest, with food)	*M*	0.426	------		0.438	0.920
	F	0.628	------		0.236	0.433
Activity Index (6h, w/o rest, with food)	*M*	0.260	------		0.232	0.171
	F	1.58E-02	0.502	0.164	0.421	0.485
Proportion of Rest (24h, w/o rest, with food)	*M*	0.484	------		0.518	1.032
	F	0.326	------		0.289	0.693
Activity Index (24h, w/o rest, with food)	*M*	0.558	------		0.386	0.741
	F	3.16E-03	0.806	4.61E-03	0.378	0.702

Table 3: Tukey's HSD p-values and Cohen's d effect sizes for pairwise comparison between VB/VBC stressed and control flies for various assays conducted on selected fly populations. For p-value: Red p<0.05 (significant); blue p<0.1 (marginally insignificant); for Cohen's d: Red d>0.8 (high); blue 0.8>d>0.5 (medium); M: Male; F: Female; S: Stressed; C: Control

4. DISCUSSION

4.1. Inferences from studies on baseline populations

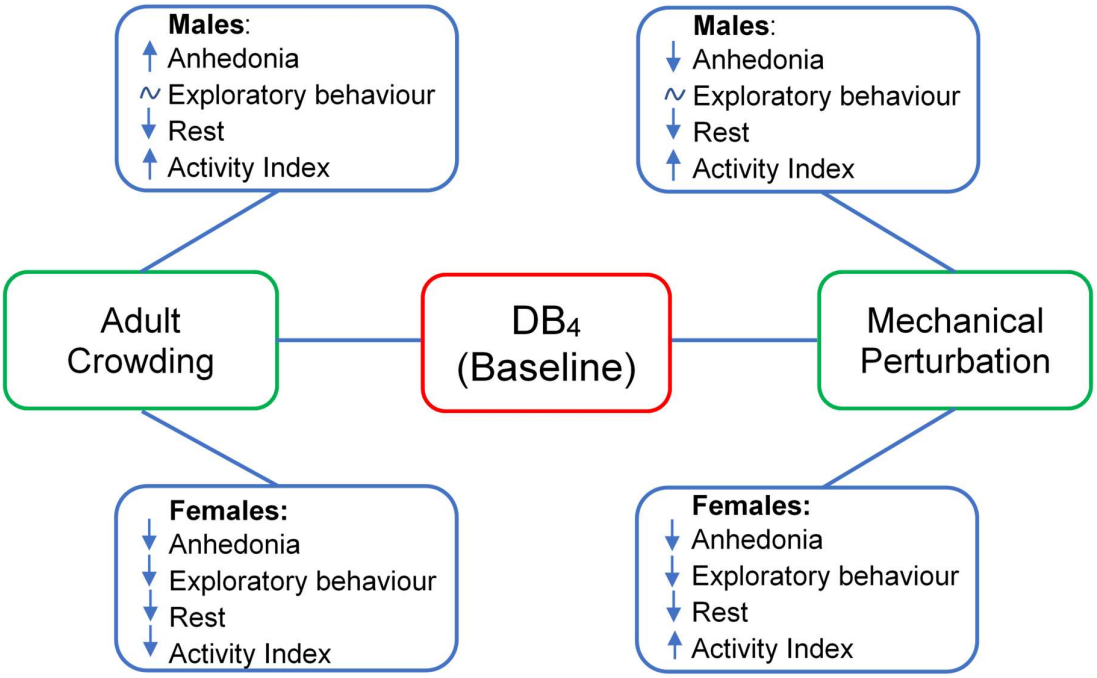

Fig. 33: Behavioural changes due to different stressors in male and female baseline flies

4.1.1. Sexual dimorphism in stress-induced anhedonic behaviour depends on the nature of the stressor

Hedonic behaviours as measures of stress response have been prevalent in rodent models of chronic mild stress (CMS), in which a series of unpredictable, mild, largely abiotic stressors are provided to rodents over several weeks (Willner, 2017). A reduced preference to feed on sucrose in rats is considered anhedonic – indicating a lack of interest in a pleasurable activity (Katz, 1982; Willner et al., 1987). An abiotic stressor - vibrational stress - in male *D. melanogaster* has been shown to induce anhedonia – measured as a reduction in feeding on glycerol (Ries et al., 2017). Further, CMS in male *D. melanogaster* – via a series of mild abiotic stressors over 10 days – also induced anhedonia, measured as a decreased preference for feeding on sucrose (Araujo et al., 2018).

In our experiments, the abiotic mechanical perturbation stress paradigm led to a reduction in glycerol feeding in both male and female flies, thus indicating a lack of motivation to partake in pleasurable activities. However, the biotic stressor – adult crowding – induced anhedonia only in stressed females. Males subjected to this

stressor showed a surprising increase in glycerol feeding (Fig. 11). To the best of our knowledge, this is the first demonstration of a sexually dimorphic anhedonic response to stress in *D.melanogaster.*

In rodents, different social stressors, such as isolation and crowding, have been found to have sex-specific effects (Herzog et al., 2009; Palanza, 2001). In line with these prior observations, we found that adult crowding, which is a social or biotic stressor, seems to effect male and female flies differently, inducing anhedonia only in females (Fig. 11C & 11D). Surprisingly, male flies show an increase in glycerol feeding after crowding. A possible reason for this could have been that crowding was leading to a competition for resources (Joshi and Mueller, 1997) and thus reducing the availability of food to the flies. Since male flies are smaller in size, they could have been affected more severely by starvation under crowded conditions. This starvation could then be providing an immediate impetus for the male flies to feed. For the females, which are larger, the starvation possibly was not as strong a factor, and their anhedonic response was thus only an indication of their motivational state. To investigate this possibility, we assayed the starvation resistance of the stressed and unstressed flies. We found that adult crowding does not have an effect on the starvation resistance of either males or females (Fig. 15), thus overruling this possibility. Thus, the physiological reason for this dimorphism remains unclear.

Summarily, it can be stated that the nature of the stressor plays a crucial role in anhedonic responses to stress, and sexual dimorphism in sex response seems to be modulated by the nature of the stressor.

4.1.2. Stress reduces motivation to explore novel habitat in females

Our paradigm of non-lethal 3-day stressors revealed a sexual dimorphism in exploratory behaviour in response to stress. Male flies showed no change in the number of exploratory trips, while female flies explored significantly lesser. This dimorphism was consistent across both the biotic and abiotic stressor (Fig. 12). This is in keeping with previous results of dimorphism in this behaviour across sexes in flies after 24-hour long starvation and oxidative stress. It also supports the finding that the neuronal circuitry affected by stress could depend upon the hormonal environment of the brain, which is modulated by sex (Neckameyer and Matsuo, 2008).

The basis of exploratory measurements in fruit flies is centrophobism – the tendency to stay away from the centre of an arena. Females fruit flies show higher centrophobism and thus reduced exploratory tendencies as compared to males at basal levels (Besson and Martin, 2005). In our experiments, when stressed, this centrophobism is further increased in females, and is not as pronounced in males.

The tendency to explore is related to seeking out novel habitats (Cote et al., 2010) and is also energy intensive. This decrease in exploratory tendencies of female flies after stress could indicate both a physical inability to explore due to exhaustion or injury from the stressor, as well as a lack of motivation to explore new surroundings. However, it is crucial to note that the cue-based response of negative geotaxis is not affected across sexes by either stressor (Fig. 7-10), indicating that the changes are not likely due to physical harm, fatigue or injury to the fly. Thus, we conclude that these flies lack motivation to explore after being stressed. Additionally, preference for edges in flies is postulated to be a marker of seeking shelter (Liu et al., 2007), and the increase in this behaviour could possibly represent increased fear or anxiety-like behaviour due to stress.

Further, exploratory behaviour is related to locomotor activity levels in rats (Willig et al., 1987). In fruit flies, exploration is characterised by an initial elevated level of activity (Liu et al., 2007). Hence, we next investigated the impact of stress on locomotor activity.

4.1.3. Stress causes restlessness across sexes and changes locomotor activity

Long-term changes in rest and activity patterns is indicative of a lasting effect of stress on the organism. We found that even when the first 15-minute period of acclimatization to a new environment is excluded, over the next 6-hours, stress causes a change in locomotor activity and rest levels. Both mechanical perturbation and crowding caused the flies to spend lesser time resting or sleeping across sexes (Fig. 13). These flies thus show a marked lack of sleep, in keeping with previous results in mice that suggest disruption of sleep patterns after stress with lighter and more fragmented sleep (Cryan and Holmes, 2005). Analysis of rest levels for longer periods such as 24 hours or several days after stress may help understand the changes in sleep patterns in stressed flies in greater detail.

Activity Index is a measure of the flies' activity in the DAM tube during their period of wakefulness (Gilestro, 2012; Kayser et al., 2014). Mechanical perturbation resulted in increased locomotor activity during wakefulness in both males and females (Fig. 14A & 14B). This, coupled with lower rest levels, indicate that this stressor induces hyperactivity in flies. However, adult crowding brings about sexual dimorphism in activity indices of flies. While male flies subjected to this stressor showed hyperactivity, female crowded flies were less active than their controls when awake (Fig. 14C & 14D). This reduction in activity could be due to a reduced motivational state in female flies subjected to crowding, such that while their sleep is disturbed, when awake, they are not motivated to move around as much in the tube. The increased activity in male flies over 6 hours in in contrast to previous studies in flies subjected to vibrational stress, which found a reduction in locomotor activity in stressed males over a 15-minute period (Ries et al., 2017). This could, however, be due to the vastly different durations over which activity has been measured.

While exploration and locomotor activity seem to be correlated (Liu et al., 2007; Willig et al., 1987), our results suggest that stress can impact these two behaviours in very different ways. Higher activity levels and reduced rest over 6 hours after stress does not cause a concomitant increase in exploratory activity. Rather, a decrease in exploratory behaviour in females occurs, which can be interpreted as a measure of a reduced motivational state.

4.2. Inferences from studies on selected populations

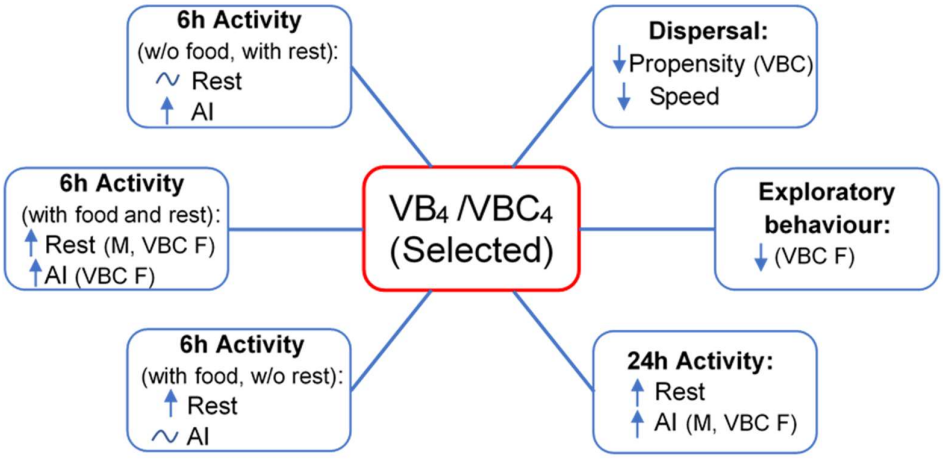

Fig 34. Behavioural changes due to mechanical perturbation in flies selected for dispersal and their controls. When indicated in brackets, changes are significant for those groups only; otherwise they are consistent across selection regimes and sex. M: Male; F: Female; AI: Activity Index

4.2.1. Injury or fatigue does not impact observed behaviours

Our experiments aimed to measure the motivational state of the fly, and it was crucial to first establish that any changes we observed were not due to tiredness, physical harm or injury. We measured the negative geotactic propensity and ability of the flies, which, being cue-based responses, are not posited to be related to the motivational state (Ries et al., 2017). We found that across trials, males showed no changes in these behaviours (Fig. 16A, 17A, 18A & 19A). While after 10 trials in the setup, there were no changes in females either (Fig. 18B & 19B), there was a change in propensity of negative geotaxis after the 1st trial in female VBCs, but with stressed flies showing higher propensity than controls (Fig. 16B). Thus, while this indicates that some cue-based response is affected, it also reaffirms that no physical harm or fatigue has been induced in the flies.

The difference between the 1st and the 10th trial in female VBC flies could be potentially attributed to two factors. First, the flies have not had enough time to acclimatise to the environment in the 1st trial, which could be impacting the measurements. However, the multiple iterations of moving the frame up and down could also be a proximate stressor, and the measurements in the 10th trial could be impacted by this.

4.2.2. Evolutionary history interacts with stress to impact dispersal-related traits

Dispersal is an energy-intensive process and comes with its costs (Bonte et al., 2012). But it can be beneficial for an organism to disperse in stressful environments (Wenny, 2001). Furthermore, dispersal is linked to the boldness of an organism (Fraser et al., 2001) and invasion of a novel habitat (Rehage and Sih, 2004), and thus may be linked to the motivational state of the organism.

We found that when subjected to stress, the speed of dispersal decreased across selection regimes in the absence of food in the source for both males and females (Fig. 22). This indicates that those flies which leave the source travel at a slower speed after stress, which could be due to

both a reduction in motivation to disperse or a change in their locomotor activity (See section 4.2.4).

Further, selection interacted with stress to affect dispersal propensity and only the populations not selected for increased dispersal showed a decrease here (Fig. 20). This measure could be indicative of motivation to disperse. However, the absence of food in the source can serve as a proximate motivator for dispersal (Tung et al., 2018b). Hence, we also checked these behaviours in the presence of food in the source and found similar results across sexes (Fig. 21 & 23).

Thus, regardless of a proximate reason to disperse, VBC flies showed a decreased motivation to leave the natal habitat, and a reduced speed while dispersing. VB flies, while being equally motivated to disperse after stress, showed a decrease in speed in their source-to-destination movement. The difference in how these populations respond to stress could be because of both behavioural and neuroendocrine changes that selection has had. In addition to increased dispersal related traits, the selected flies also show increased exploratory behaviour and aggression (Tung et al., 2018a) – thus, they may have higher basal motivational tendencies to disperse. Adding to this, the changes in neurotransmitter profiles of VB flies – with increased octopamine and precursors of serotonin and dopamine (Tung et al., 2018a) – could modulate the impact of stress. This would be in keeping with studies in flies that suggest that hormonal environment of the brain (Neckameyer and Matsuo, 2008) and neurotransmitter levels (Ries et al., 2017) can change behavioural response to stress.

4.2.3. Stress, modulated by selection regime, lowers female exploratory behaviour

Exploration of novel habitats is closely related to dispersal (Dingemanse et al., 2003; Korsten et al., 2013), potentially because it can help dispersing organisms to both find and survey new habitats. VB flies selected for dispersal have higher exploratory tendencies across sexes

(Tung et al., 2018a), which was also what we observed in our experiments (Table 2).

We found that stress induces both sexual dimorphism as well as a selection-based bias in exploratory behaviour. While male flies of either selection regime are unaffected in their exploratory tendencies after stress (Fig. 24A), there is an interaction of selection with treatment in females. Only VBC females show a drop in the number of exploratory trips (Fig. 24B, Table 3). The sexual dimorphism can be interpreted in a similar manner as in the baseline populations (see section 4.1.2).

Further, the impact of selection is in the same direction as seen in dispersal propensity – VBC females show a reduced motivation to explore novel habitat and increased centrophobism. The co-evolved response of increased exploration with selection for dispersal could have made the basal motivation to explore higher in VB females. This could be furthered by the neurotransmitter levels of VBC flies being different (Tung et al., 2018a), possibly making them more susceptible to stress, and causing them to be more shelter-seeking and exhibiting anxiety-like behaviours in response.

Interestingly, while stressed VBC males showed a marked decrease in dispersal propensity, they did not show a correlated reduction in exploratory tendencies. Thus, while these behaviours may be implicated together, the motivation to engage in them – and how stress changes these motivations – differs, and this difference is modulated by sex.

4.2.4. Food and rest modulate short term (6-hour) locomotor behaviour in selected populations after stress

Presence or absence of food at the time of locomotor recording has been shown to influence activity levels – starvation during recording increases the locomotor activity of flies (Martin, 2003). Further, activity recordings both in the presence and absence of food have found that VB flies are more active than the VBC flies (Tung et al., 2018a, Table 2). Moreover, stress can potentially have immediate consequences, such as sleep rebound experienced by sleep-deprived flies (Hendricks et al., 2000).

Therefore, we incorporated both, the presence/absence of food as well as the amount of rest before recording, in our experimental design to investigate locomotor activity and rest over 6 hours.

4.2.4.1. Effects of presence/absence of food when rest is available post stress

When rest was available after stress, we found that the presence or absence of food gave rise to sexual and selection-based dimorphism. In the absence of food, sexes behaved alike, and showed no change in the rest levels (Fig. 25). The wakefulness activity levels, measured via the AI, were higher for the stressed flies in both the VB and VBC populations (Fig. 26). This indicates that while there is no insomnia or hypersomnia in the absence of food, hyperactivity is induced due to stress. However, in the presence of food, male flies had increased rest levels after stress (Fig. 27A), although their AI was comparable to the controls (Fig. 28A). Thus, in males, absence of food could be the proximate driver for increased AI in stressed flies. Additionally, when a substrate is present, it perhaps allows stressed flies to rest and recuperate, leading to hypersomnia.

Interestingly, presence of food in the DAM tube affected female flies differently, modulated by their selection history. VB females were comparable to their controls in both parameters, indicating that the effect of stress was completely absent in the presence of food. VBC females, however, spent longer amount of time resting than their controls on food, and also showed higher AI (Fig. 27B and 28B). Thus, while in VBC females, food allowed the stressed flies to rest (possibly allowing for some recovery after the stress), it did not modulate the hyperactivity that stress seemingly induced.

4.2.4.2. Effects of presence/absence of post-stress rest, when food is available

When the flies are not rested after stress, and their immediate locomotor activity is measured on food, there is no sexual or selection-based dimorphism. Stressed flies rest more than controls (Fig. 29), and do not

show any difference in activity levels from their respective controls during wakefulness (Fig. 30, Table 30).

Comparing this to when post-stress rest is provided, detailed above, it is interesting to note that when food is present, resting after stress causes no changes in the pattern of how stress effects male locomotor activity and rest. Thus, presence or absence of food seems to be the major proximate factor modulating stress-response of activity and rest patterns in males.

In females, the effect of selection is highlighted in this comparison. While VB females rest more immediately after stress as compared to controls, it is likely that they recuperate faster, and thus their rest levels become comparable after overnight (14 hours) rest. They are not hyperactive in the presence of food. In contrast, stressed VBC females continue to display hypersomnia after stress throughout. While they aren't hyperactive immediately after stress, their restlessness when they are awake becomes pronounced over time. This dimorphism due to selection could be due to the basal difference in activity levels between VBs and VBCs, and potentially due to the differing neurotransmitter levels, evidently making VB females more resistant to stress.

In conclusion, both food and rest modulate stress effects differently across sexes. Male responses to stress are largely driven by a proximate source of food, while female responses are affected by rest, food and evolutionary history.

4.2.5. Changes in locomotor activity and sleep patterns in selected populations over 24 hours after stress

To study the long-term effects of stress, we recorded the locomotor activity in the presence of food for 24 hours, starting immediately after stress. Over this duration, we found that both male and female flies slept more through the day across selection regimes (Fig. 31). While stressed male flies were hyperactive on average through the day (Fig. 32A), selection yet again modulated this response in females. Only stressed VBC flies showed restlessness when awake over the entire day (Fig. 32B,

Table 3), furthering the evidence for heightened stress resistance in VB females.

Octopamine, dopamine and serotonin, all three of which are implicated in locomotor activity (Yellman et al., 1997), have increased in male VB flies (Tung et al., 2018a). Both serotonin and dopamine reduce after stress in male fruit flies (Araujo et al., 2018; Ries et al., 2017). We postulate that similar changes in female fruit flies could have allowed the VB females to be more resistant to stress. Additionally, different subsets of neurons have been implicated in the stress response circuitry in males and females (Neckameyer and Matsuo, 2008), which could explain the sex-biased nature of the effect of selection on locomotory sex response.

Sexual dimorphism in locomotor activity in flies has previously been linked to a few neurons in the mid-anterior region of the *pars intercerebralis* (Gatti et al., 2000). It is possible these dimorphic brain structures are responding differently to stress, and further investigation into the neurophysiology of stress-response can help understand this.

5. CONCLUSIONS AND FUTURE DIRECTIONS

Organisms face rapidly changing environments, which can be a potential cause for stress. Responses to stress can be multi-faceted, and the effected traits in organisms can range from physiological – such as changes in body size (Araujo et al., 2018) – to purely behavioural – such as anhedonia or a decreased motivation to mate (Ries et al., 2017).

Understanding stress-induced changes in model systems, and using behaviours which parallel human response to stress, can allow us to understand stress-induced behaviours and disorders in human beings, such as depression, anxiety and post-traumatic stress disorder.

In our experiments with baseline populations, we established that stressors of differing nature can cause varying behavioural responses, and these can be modulated by the sex of the fly. Sex specific hormones could interact with the response of the brain circuitry to stress (Neckameyer and Matsuo, 2008), leading to sexual dimorphism.

The sex-biased nature of stress responses is a crucial finding, considering that in humans, a sexual dimorphism exists in reactivity to stressful situations, and in the prevalence of stress-induced mood disorders (Palanza, 2001). This highlights the importance of using appropriate and if needed, separate systems to understand the male and female stress-responses. For a mechanistic basis of how different stressors are affecting both sexes, experiments to understand the underlying neurobiological changes in response to the stressors we used could be conducted. This could be done via studying metabolic profiles or imaging the fly brain before and after stress, or via using genetic mutants with appropriate neurobiological modifications.

Moreover, we found that evolutionary history can impact behavioural changes and motivational states after stress. Selection for dispersal can make the flies more resistant to stress, especially in females. The neurotransmitters serotonin and dopamine have been implicated in stress response, and have been found to increase after selection for dispersal in males (Tung et al., 2018a). Similar changes in females could render them

more resilient to stress. In addition to hormonal environments of the brain causing sexual dimorphism, here we must also consider that the dispersal syndrome itself is sex-biased (Mishra et al., 2018b) leading to sex-based differences in the traits that have evolved with dispersal. Thus, while both male and female VB flies have evolved to disperse more, the underlying physiochemical or neurotransmitter changes that have occurred, may not be the same. These changes, if any, could also impact the sex-differential response to stress in selected populations. Further investigation into metabolic changes in VB and VBC females after stress can help us understand the underlying mechanism for this difference. These findings can further an evolutionary medicine-based understanding of stress-induced mood disorders in humans. We can employ similar behavioural tests after stress-protocols in populations selected for different traits, such as larval crowding or malnourishment. We could then understand how the environment or stressors faced by a population during evolution changes their resistance to stress, potentially allowing us to extrapolate this understanding to how humans respond to stress.

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