

UNIVERSITY OF BIRMINGHAM

NEURAL MECHANISMS UNDERLYING BEHAVIOURAL CHOICES IN *DROSOPHILA MELANOGASTER*

by

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Abstract

All animals are confronted with crucial behavioural choices on a daily basis, human and otherwise. These choices can range from the basic decision to feed or rest, to more complex decisions involving social interactions or finding a suitable mate. Behavioural choices are flexible and modulated by an animal's physiological needs and external environment. Despite the importance of behavioural choices in animal survival, very little is known about the underlying neural mechanisms that control them. How does the brain evaluate alternative options and determine the appropriate course of action? What influence do internal states, such as hunger or the health status of the animal, have on these pivotal decisions?

In my thesis, I aimed to uncover the neural basis of behavioural choices underlying various conflicts using *Drosophila melanogaster* as a model system. *Drosophila* exhibits a diverse range of innate behaviours such as courtship, feeding and escape, each displaying remarkable stereotypy. Its relatively simple brain structure, coupled with an array of available genetic tools, makes it an ideal model organism to address this question.

In Chapter 2, I delved into the trade-off between reproduction and immunity. Using several bacterial pathogens, I found that both infected male and female flies prioritised mating over survival suggesting that reproductive behaviours remain preserved despite the metabolic cost of infection.

Chapter 3 focused on the behavioural choice between feeding and courtship in male *Drosophila*. Starved males showed a preference for initiating feeding before courtship, while well-fed males prioritised courtship. Together with a master's student in our lab, we identified the tyramine signalling pathway as a key player in this decision-making process. Tyramine serves as a satiety signal, activating courtship-promoting neurons while simultaneously inhibiting feeding-promoting neurons. Conversely, the perception of food inhibits courtship-promoting neurons and activates feeding-promoting neurons. This antagonistic regulation of important neuronal modules enables flies to prioritise one behaviour over the other in a state-dependent manner.

In Chapter 4, I explored the choice between feeding and escape behaviours in flies. I observed that males starved for 24 hours exhibited a preference for escape over feeding. However, this behavioural prioritisation was reversed by the severity of starvation in a sex-specific manner. Employing techniques such as connectomics, neuroanatomy, and behavioural assays, I unravelled the neural mechanisms underlying this critical behavioural choice, specifically connecting sensory information to motor output.

These findings advance our understanding of how the brain resolves behavioural conflicts and incorporates the internal state of an animal to elicit the most appropriate behaviour. Through my research, I shed light on the neural mechanisms underlying behavioural choices in various contexts, using *Drosophila melanogaster* as a powerful model system.

Contributors

This thesis would not have been possible without the work and support of my colleagues and collaborators. Chapters 2 and 3 have been peer-reviewed and published in Proceedings B and Current Biology respectively (Manuscripts cited in Appendix 6). In Chapter 2, I conceived and performed the behavioural experiments under the guidance of my supervisor Dr Carolina Rezaval. Furthermore, I took the lead in writing and publishing the manuscript as the **first author**, as documented in Rose et al. 2022 [1]. Chapter 3 was a collaborative project, with the initial characterisation done by Sherry Cheriyamkunnel, a master's student at the Rezaval lab and imaging experiments were done by Dr Pedro Jacob from the University of Oxford. My role in this project included expanding the behavioural analysis, performing experiments for revision, writing the paper and addressing the reviewers' comments for which I was the **co-first author** (For more details, refer to Cheriyamkunnel, Rose et al. 2021 [2]). In Chapter 4, I performed all the behavioural experiments, while Anna Dmitsi and Youchong Zhang performed the confocal imaging.

The following authors contributed to Chapter 2:
Saloni Rose, Charlotte Burmester (Figure 2.9)

The following authors contributed to Chapter 3: Sherry J. Cheriyamkunnel (all preliminary work including Figure 3.1A-B, Figure 3.2A-D), Saloni Rose (Figure 3.1C-E, Figure 3.2E-H, Figure 3.3O and Figure 3.6A-D), Dr Pedro F. Jacob (Figure 3.3A-L) with the help of Lauren A. Blackburn, Shaleen Glasgow and Jacob Moorse.

The following authors contributed to Chapter 4: Saloni Rose, Anna Dmitsi (Figure 4.7A, 4.9A and 4.10A), Youchong Zhang (Figure 4.16A)

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List of Abbreviations

Ach: Acetylcholine

ECC15: *Erwinia carotovora carotovora*

CI: Courtship Index

CHC: Cuticular hydrocarbons

DA: Dopamine

DI: Defensive Index

DN: Descending Neuron

FI: Feeding Index

GABA: Gamma-aminobutyric acid

GFP: Green fluroscent protein

GF: Giant Fiber

GLUT: Glutamate

GRN: Gustatory Receptor Neuron

IR: Infrared

LPS: Lipopolysaccharide

MN: Motor neuron

OA: Octopamine

ORN: Olfactory receptor neuron

SEZ: Subesophageal Zone

VNC: Ventral nerve cord

VPN: Visual Projection Neuron

ZT: Zeitgeber Time

5-HT: 5-hydroxytryptamine

1 Introduction

1.1 Decision-making

1.1.1 In the oldest tale we know of

Paris made his choice [3]. He presented Aphrodite, the goddess of love with the golden apple. Aphrodite offered the most beautiful woman, Helen of Sparta in return. Thus, began the terrible Trojan War that lasted for ten years. Thousands of soldiers died, ships sank and villages were burnt. In the end, Paris lost everything: his life and Helen.

Needless to say, we can all agree that Paris made a terrible decision. Like Paris, we too are riddled with tough decisions throughout our lifetime. Should I eat an entire tub of Ben & Jerry's or a healthy avocado salad? Should I go to the gym today or skip it? Every decision has a consequence. However, decisions or choices are not unique to us humans. Even simple organisms need to make quick decisions on a minute-to-minute basis. For example, should a peacock show off his train to attract a female? Now, what happens when a predator is lurking around? Should it stop the courtship ritual and risk losing its potential mate? Or should it continue the courtship ritual and risk being vulnerable to predators? Decision-making is very complex. Indeed, how decisions are made remains an enigma. Despite significant contributions from various disciplines, including neuroscience, psychology, and economics, the mechanisms underlying decision-making processes are still poorly understood. So, what is decision-making?

1.1.2 What is decision-making?

Decision-making has been defined as the "process of selecting a behaviour or an action while the subject weighs the costs and benefits of other behaviours/actions" [4]. Two main variables influence a subject's decision: utility and subjective probability [5]. Utility refers to the assessment of the usefulness of the choices available to a subject. Subjective probability refers to the assessment of the consequences of the choice/decision. When an animal is given a choice between feeding

from two food sources that vary in their nutritional value, utility dictates the animal to choose the more nutritious food source. At the same time, the animal needs to assess the consequence of eating from a more nutritious food source. The animal needs to take into account other factors (called subjective probability) like competition and risk of predation before making the choice. If the predation risk is high near the nutritious food source, it might consider eating from the less nutritious food source.

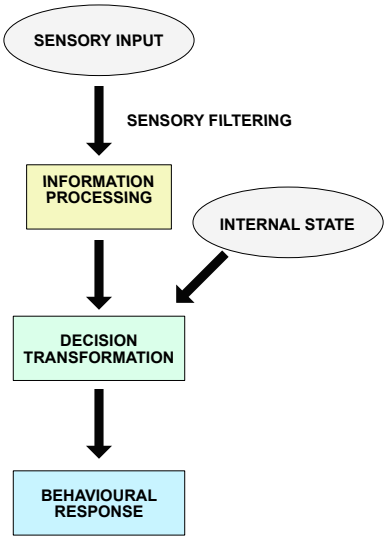


Figure 1.1: A simplified model of decision-making. Decision-making is a multi-step process: an animal acquires sensory information and filters it to eliminate the noise by which it is made aware of available options. Decision transformation enables the animal to check if any options satisfy the decision criterion and the animal carries out the optimal behaviour response.

Now, how are decisions made? Decision-making is a multi-step process that transforms sensory evidence into appropriate motor output [6]. An animal acquires information from its surroundings through its sensory systems, making it aware of the available alternatives (Fig 1.1). However, the incoming information is noisy, therefore there is a second process that integrates sensory information over time making it more reliable (Fig 1.1). The third step is decision transformation, which takes into account all alternatives available to the animal, their assigned utility, and the animal's internal state and checks if any of the alternatives fulfil the decision criterion (Fig 1.1. Finally, if the criterion is met, the animal executes the appropriate behavioural response.

In the next section, I will discuss the theoretical and practical approaches used to study decision-

making.

1.1.3 Decision Neuroscience: a theoretical framework

Mainly two kinds of theoretical models have been put forth to explain two-choice decisions: random walk models and recruitment models [7]. Both models assume that the incoming sensory information is noisy and must be sequentially sampled until the quality and quantity of evidence are strong enough to meet the decision threshold. However, they differ in the criterion used to implement the decision i.e. relative vs. absolute decision rules. Random walk models that use the relative decision rule assume the evidence for one choice can be used as evidence against the other choice. For example, imagine checking the weather every hour to decide whether to go outside. If it's sunny, it's reasonable to assume that it's not raining. So, the evidence for sunny weather can also be seen as evidence against rain, which helps us decide to go outside. Random-walk models can be further categorised into two different models depending on the time frames of evidence accumulation: diffusion models and non-diffusion models. For example, the Wiener diffusion model assumes the evidence accumulation is continuous and happens over a steady rate. Going back to the weather example, your decision to go outside isn't the same all the time. You would keep checking the weather continuously. If the conditions are good (sunny), you'll choose to go outside. But if it starts to rain heavily, you'll change your decision and choose to go back inside.

In contrast, recruitment models use absolute decision criteria. Here, the evidence for two alternatives is accumulated by two separate counters and the first alternative to reach the evidence threshold is chosen by the actor. More specially, the evidence against one option is not used as evidence against another option. Let's consider a situation where you're deciding between two movies to watch: Barbie and Oppenheimer. There are three factors you want to consider: show availability, previous reviews and your mood. Now, let's first gather evidence in favour of Barbie. You might discover that Barbie is readily available to watch in the cinema nearby. This availability is a positive factor in its favour. You also remember that you've enjoyed similar movies

featuring Margot Robie in the past, so you have a positive impression of her acting skills. Additionally, considering your current mood, you're up for something light and cheerful, and Barbie aligns with this preference.

Simultaneously, you're also considering Oppenheimer. While it's not as readily available as Barbie, you've heard excellent reviews about the cast and direction. The idea of expanding your understanding of the Manhattan Project is appealing. However, considering your current mood, you're not feeling particularly inclined towards a biographical drama style movie with darker undertones tonight. Next, based on this accumulation of evidence, you would ultimately choose the movie that meets or surpasses the predetermined evidence threshold. In this example, Barbie has surpassed the evidence counter and reached the threshold before Oppenheimer.

Recruitment models can take the form of an accumulator model, where varying amounts of evidence are collected at specific, evenly spaced time intervals, or a Poisson counter model where the timing of evidence accumulation is continuous but random. Returning to the movie example, you might repeatedly read movie reviews on Rotten Tomatoes, encountering different numbers of comments each time—a scenario similar to the accumulator model. In contrast, the fluctuations in your mood might follow a Poisson process. For instance, sudden conflicts with your parents or experiencing cramps during your period might influence your mood abruptly at different time points.

These theoretical models can complement the behavioural data and offer valuable insights into the underlying cognitive processes driving behavioural choices. In the next section, I will discuss the two different kinds of practical approaches used to study decision-making: the neuro-ethological approach and the cognitive approach.

1.1.4 Decision Neuroscience: the neuro-ethological approach

The neuro-ethological approach typically focuses on robust, yet simple behaviours executed by animals [8]. Often, this method employs invertebrate model systems that offer experimental

malleability including ease of behavioural, genetic and neuronal manipulation. All these features allow us to delve into a cellular or circuit-level dissection of decision-making.

In the early 1900s, Nobel Laureate Sir Charles Scott Sherrington proposed a simple model for decision-making. According to him, simple behaviours were executed when sensory cues triggered a fixed behavioural response [9]. At the peripheral level, environmental cues are detected by sensory neurons and the information is processed before reaching the decision-maker or "command" neurons (Fig 1.2)[8]. Command neurons connect to the pattern generators that control the temporal expression of behaviour (Fig 1.2). Finally, information reaches the motor neurons that execute the relevant behaviour (Fig 1.2). This process is very deterministic, a sensory cue always elicits the same behaviour. However, many of these simple behaviours are mutually exclusive i.e. two behaviours cannot be simultaneously performed by an animal. What neural mechanisms select between two alternative behaviours?

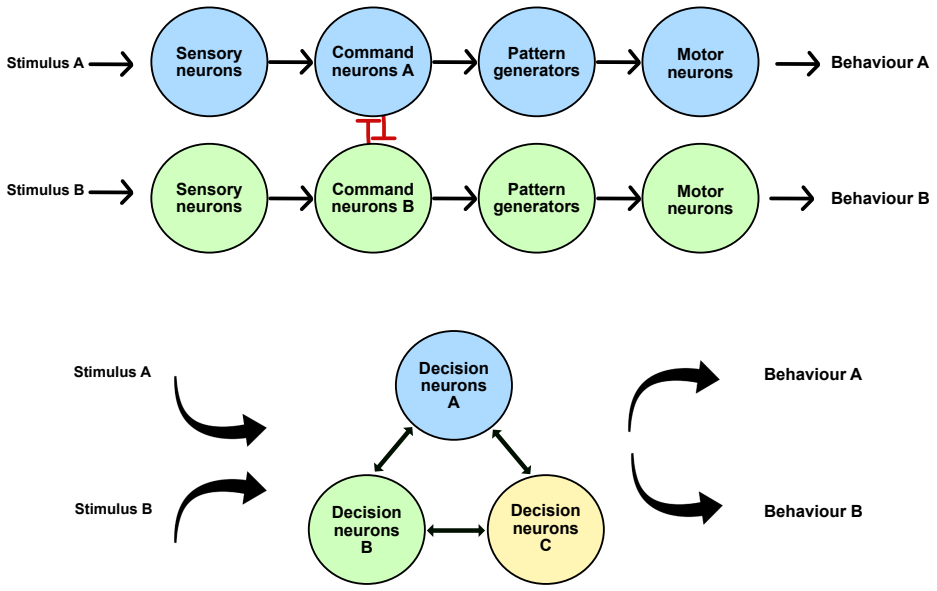


Figure 1.2: Neural mechanisms underlying behavioral choices: The reflexive model posits that inhibitory interactions between command neurons enable the selection of the right behaviour [8]. In contrast, the dynamical model suggests that activity patterns of the decision-maker neurons, called "attractor" states, enable behavioural choice [8]. The figure is adapted from Kristan, 2008 [8].

Pioneering studies in invertebrates have provided evidence for the role of command neurons in behavioural conflicts. For example, Jing and Gillette studied the choice between feeding and es-

cape behaviours in the predatory sea slug *Pleurobranchaea* [10]. They found that the presence of noxious mechanical stimulus triggered escape responses and suppressed feeding in the sea slug. Using electrophysiology and behavioural experiments, they identified the command neurons responsible for this behavioural choice [10]. A1 command neurons fire action potentials in response to the noxious stimuli and elicit escape behaviours. A1 command neurons in turn suppress PC_p neurons that trigger feeding. Therefore, inhibitory connections between the feeding and escape command neurons enable the sea slugs to elicit the appropriate behaviour and establish a behavioural hierarchy. This model is known as the reflexive model of decision-making wherein inhibitory connections between command neurons dictate the behavioural choice (Fig 1.2)[8]. However, Gaudry and Kristan found that inhibition between neurons does not happen necessarily at the level of command neurons [11]. Using the medicinal leech *Hirudo medicinalis*, they too looked into the competition between feeding and defensive behaviours. The authors found that the priorities of the medicinal leech are quite different, feeding behaviour took precedence over defensive behaviours like swimming, bending or shortening. The feeding behaviour suppressed the activity of the P cells, a group of mechano-sensory neurons that elicit defensive behaviours via serotonergic pre-synaptic inhibition, suggesting that the inhibition happens at the sensory level.

Another competing hypothesis for decision-making is the dynamical model [8]. Here, different sets of "decision-making" neurons are active during the same behaviours and respond to the same sensory stimulus. The difference in activity patterns of the neurons called ‘attractor states’ produces the appropriate behaviour. Unlike the reflexive model, there are no inhibitory interactions between the different subsets of decision-maker neurons. For example, Shaw and Kristan investigated the neural mechanism underlying the choice between swimming and shortening, a defensive behaviour. They found that command neurons for swimming (Tr1, SE1, and 61) in leeches were also active during a shortening [8]. This suggests that these command neurons are not dedicated to a single behaviour and indeed play multi-functional roles. Their activity patterns, not their connectivity dictate the type of behaviour executed (Fig 1.2).

The neuro-ethological approaches using invertebrate models in the studies mentioned above have been helpful in deciphering neural mechanisms and their underlying cellular components. Generalising these neural mechanisms to mammalian systems may not always be straightforward due to inherent differences between invertebrate and vertebrate neural circuits and behaviours. Nonetheless, these findings contribute significantly to our understanding of decision-making processes and provide a framework to investigate and test hypotheses in vertebrates.

1.1.5 Decision Neuroscience: the cognitive approach

The cognitive approach common to psychologists and cognitive neurobiologists traditionally involves studying human or primate behaviour whilst simultaneously recording their brain activity [8]. The participants are provided with a wide variety of decision tasks that might take the form of perceptual discrimination, target tracking or economic choice behaviour. For example, Padoa-Schioppa and Assad performed an economic choice task where they provided Rhesus monkeys with a choice between two drinks that differed in quality [12]. Then, they recorded the brains of the monkeys using EEG as the monkeys were performing the choice task. The authors found that orbitofrontal cortex neurons assign activity correlated with value assignment of the provided drinks, but not visual input and motor responses suggesting that these neurons might be crucial for economic choice.

Similarly, Newsome et al. performed a visual discrimination task where monkeys had to make a binary choice about the direction of a visual stimulus that was interspersed with random dots (that act as visual distractors) [13]. They altered the difficulty of the task by changing the direction of a fraction of random dots. If the random dots moved in the same direction as the visual stimulus, the motion strength was 100% and it was easy for the monkey to follow the visual stimulus. However, when the direction of the random dots was altered, the task became harder and the monkeys took a longer time to make the decision ($\sim$ 400ms longer). The study found that the activity of lateral intraparietal area neurons spiked as the monkeys made saccadic eye movements to follow the stimulus. LIP neurons acted as neural integrators that fired irrespective of the difficulty of the

task. Other brain areas like the ventral intraparietal (VIP) area have also been implicated in perpetual decision-making tasks like these.

The above-mentioned studies identified broad brain areas underlying the decision, how is the decision made at a network level? Wang and colleagues came up with a network model to explain the choice [13]. The model consisted of two populations of excitatory pyramidal neurons (groups A and B) and a third group of inhibitory interneurons (group C) (Fig 1.3)[13]. Each subpopulation of the excitatory neurons was selective to one direction (i.e. left or right) and showed strong recurrent activation (Fig 1.3)[13]. In addition, they competed through feedback inhibition from interneurons (group C). When the monkey perceives the stimulus, the activity of both Group A and B neurons ramps up. As the activity of one group increases, the activity of the other group of neurons decreases [13]. When one group of neurons wins the competition, the choice is made. Cognitive studies and theoretical models like these provide insights into complex decisions we encounter, however, these studies lack resolution at a cellular level and might not reveal detailed mechanistic aspects of neural circuits.

While neuro-ethological studies have parsed out cellular mechanisms and high-resolution neuronal connectivity, the cognitive approach has produced complex behavioural data along with strong theoretical models. By integrating the strengths of both cognitive and neuro-ethological approaches, we will achieve a more comprehensive understanding of the intricate processes governing behavioural decision-making.

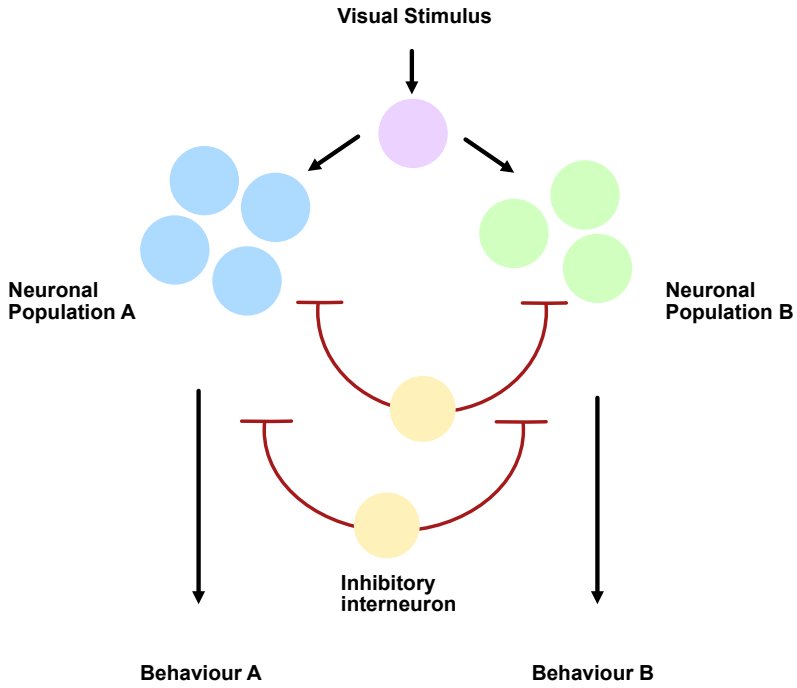


Figure 1.3: Recurrent neural circuit model underlying perpetual decisions in monkeys. Two populations of excitatory neurons selective for one behaviour (A and B) compete using feedback inhibition through from interneurons (group C). These inhibitory interneurons selectively inhibit either population A or B, thereby influencing the decision-making process in favour of behaviour A or B, depending upon the visual input received. The figure is adapted from Wang, 2008 [13].

1.2 *Drosophila melanogaster* as a model system to study decision-making

1.2.1 Why *Drosophila*?

"This animal has been extremely cooperative in our hands - and has revealed to us some of its innermost secrets and tricks for developing from a single-celled egg to a complex living being of great beauty and harmony"

Christiane Nusslein-Volhard, speech at the Nobel Banquet, Dec 1995

The 20th century saw remarkable breakthroughs in genetics, and it is the humble fruit fly (*Drosophila melanogaster*) that helped us with this revolution. *D. melanogaster* is a cosmopolitan member of the family *Drosophilidae* (vinegar flies) [14]. Initially described by Johann Meigen in 1830, it was Thomas Hunt Morgan who popularised the fly to study the principles of heredity [15]. *Drosophila* offers several advantages including short generation time (it only takes 10 days for the fly to develop into an adult at 25°C), small size and easy maintenance [15]. It has a small genome (175Mb) with around 14,000 genes [16]. The fly genome is 60% homologous to that of humans and about 75% of the genes responsible for human diseases have homologues in flies [17, 18]. In addition, the *Drosophila* brain is small and extremely tractable, as it only has $\sim 130,000$ neurons [19].

Despite being a small insect, *Drosophila* displays a variety of complex behaviours [20]. For example, upon encountering a female, the *Drosophila* male performs a complex sequence of actions as a part of an innate courtship ritual [20]. Flies also show other innate behaviours like aggression, escape, sleep, chemotaxis, and thermotaxis [20]. Furthermore, they display a wide variety of cognitive abilities including olfactory conditioning, and visual and spatial learning [21,

22]. All these behavioural phenotypes are robust and easily quantifiable in the laboratory.

A century of research into flies has led to the development of sophisticated tools. For example, a bi-partite GAL4-UAS system can be used to regulate gene expression in a specific set of tissues (Fig 1.4A)[23]. Combined with optogenetic and thermogenetic tools, the GAL4-UAS system enables us to activate or inactivate specific subsets of neurons, or even individual neurons, and assess the effects on behaviour (Fig 1.4A)[24]. In addition, genes can be knocked down in neurons using RNA interference (RNAi) tools in a time-specific manner [25]. The activity of specific neurons can be monitored in live flies performing behaviours under the microscope, using techniques like electrophysiology or live imaging (Fig 1.4C)[26]. The connectome of the adult fly brain published in 2018, has mapped all synaptic connections at a high resolution and will prove helpful in parsing out neural circuits responsible for different behaviours (Fig 1.4B)[19, 27, 28]. All these features and available tools make *D. melanogaster* an attractive model organism for uncovering neurons and circuits underlying behavioural choices with great resolution.

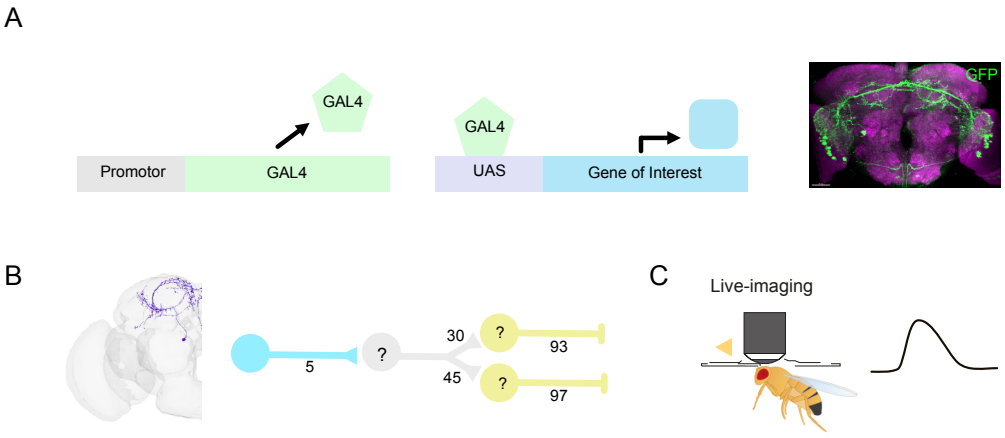


Figure 1.4: Schematic of tools available to study decision-making in *Drosophila melanogaster*. (A) GAL4/UAS Bipartite system can be used to express genes of interest in specific tissues/cells. GAL4-driven GFP expression in lateral horn input neurons in the adult brain (image by Youchong Zhang). (B) The hemi-brain connectome has mapped all synaptic connections in the fly brain [19, 27, 28]. (C) Live imaging experiments can be used to measure the neural activity in freely behaving flies. The fly schematics are designed by Gil Costa.

1.2.2 Reproductive behaviours

After reaching sexual maturity, flies divert their attention mainly towards reproduction. In order to achieve copulation, flies engage in a series of progressive innate courtship behaviours [29, 30]. During this process, male and female flies exchange a wide range of visual, olfactory, auditory and gustatory cues that allow them to determine their species type and fitness as reviewed in [30]. The courtship ritual begins with the male orienting towards the female and tapping her cuticle/abdomen to assess her pheromones (Fig 1.5)[30, 31]. As courtship progresses, the male chases the female and produces a courtship song by unilaterally extending a wing (Fig 1.5)[30].

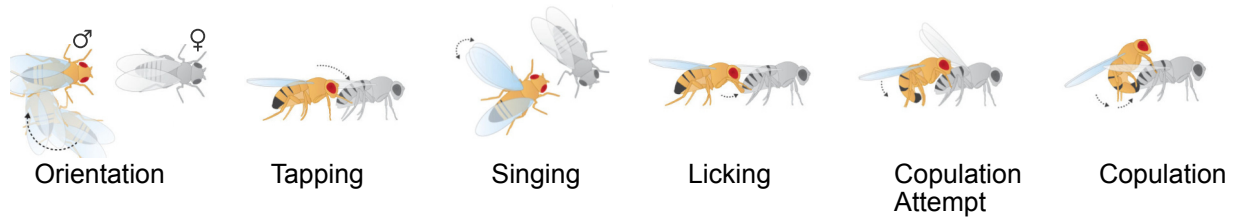


Figure 1.5: Schematic of reproductive behaviours in *Drosophila melanogaster*. During courtship, the male performs a stereotypical sequence of innate behaviours [29]. The male orients towards the female and taps her. He chases her whilst performing a courtship song. As the courtship progresses, the female slows down and he licks her. The males attempt to mount her and copulation takes place. In response to male courtship, female flies either show acceptance or rejection behaviours. The figure is adapted from Von Philipsborn et al. [32].

Although females do not show any of these courtship steps, they are able to judge the courtship display and decide whether to copulate or not [30]. If the female decides to accept, she slows down, starts grooming and opens her vaginal plates (Fig 1.5)[30]. The males attempt to mount her and copulation takes place for around 20 minutes [30]. On the other hand, if she deems the male unworthy, she displays a variety of rejection behaviours including decamping, kicking and ovipositor extrusion [30].

The female, upon mating, becomes unreceptive to future mating attempts temporarily and instead focuses on producing a large number of eggs [30]. This decrease in receptivity lasts for days and is mediated by sex peptide, a component of male seminal fluid (Fig 1.5)[30]. However, females can remate and this effect depends on the social environment and presence of the food [33].

Other post-mating responses include changes in feeding, increased attraction to yeast, salt and polyamines, decrease in sleep, and increased aggression [34]. It is important to realise that the reproductive biology of flies in the wild differs from that of those in laboratory conditions. Most of the courtship experiments in the lab are done in small chambers, however in nature, females get ample space to show rejection behaviours like decamping [35].

Methods to measure courtship behaviour have been described in detail in [32, 36]. Traditionally, in a single-pair courtship assay, a virgin male is presented with a virgin female and their behaviour is recorded for 1 hour. Following the recording, the courtship behaviour is manually quantified using software like BORIS [37]. Here, the total time spent by the male performing courtship is measured to give rise to the courtship index. Furthermore, the courtship index can be broken down into parameters focusing on each courtship step (e.g. singing index or wing extension index).

The female sexual receptivity can be simultaneously measured. This includes behavioural indices measuring the time the female spends showing rejection behaviours. Additionally, two parameters that are indirect readouts of female receptivity can be measured: mating success and copulation latency. Mating success is based on the binary decision of a female fly to mate with a male (or not) and calculated as % of flies that have mated within one hour. Copulation latency measures the time it takes for the female to accept the male for mating, with lower latency indicating greater receptivity. All of these parameters can be annotated manually. To measure the behaviour in an automated fashion, the physical attributes of the flies (including the location of the centroid, wings, and legs) and their movement can be quantified using a tracking software. These data can then be fed into machine learning algorithms like JAABA, in order to automatically classify different courtship stages as done in [38].

1.2.3 Judging a potential mate during courtship

The female perspective

As in most animals, *Drosophila* females are choosier than males, and assess the male's quality by

integrating a variety of cues during the courtship ritual [30]. The male cues evaluated by females are as follows:

- **Courtship Song:** The male flies produce a species-specific courtship song by vibrating their wings [39]. The courtship song consists of two parts: the sine song and the pulse song [39]. The core structure of the song, for example, the inter-pulse interval is invariant within a species, but the intensity of the song varies between individuals [30]. Female flies are less receptive towards males without wings that are unable to produce songs [30]. Females exhibit a preference for males who produce longer pulse songs [30].

- **Pheromones:** Females assess cuticular hydrocarbons (CHCs) in the male's abdomen which act as pheromones [40]. In particular, females show a preference for males with high levels of 7-tricosene (7-T) [30]. cVA(cis-Vaccenyl Acetate), a compound produced by the ejaculatory bulb acts as an aphrodisiac for females, increasing short-range and long-range attraction [30]. In addition, methylated pheromones like methyl myristate and methyl palmitate have been shown to be important for male mating success [41].

The male perspective

Male flies are more promiscuous and can even court inappropriate targets, such as dead females and inanimate objects [42, 43]. However, they still have the ability to assess their mates on visual and chemical cues [30]. The female cues evaluated by males are as follows:

- **Pheromones:** Female flies emit 30-50 CHCs, which are used by males to assess their quality [40]. Perfuming flies with certain CHCs can induce courtship behaviour in males [30]. For example, adding 7,11-heptacosadiene (7,11-HD) to females induces male courtship. Other CHCs that influence courtship behaviour include 7,11-nonacosadiene (7,11-ND) and 7-pentacosene (7-P); however, they are less potent at inducing courtship [30].

- **Visual cues**: This sensory modality plays a less important role in courtship, as flies can mate in the dark [44]. However, male flies use body size as a reliable indicator of mate

quality [30]. When given a choice between females that vary in body size, males show a preference for bigger females [30].

1.2.4 Neural control of reproductive behaviours

Reproductive behaviours in flies are under the control of two important sex-determining transcription factors, *doublesex* (*dsx*) and *fruitless* (*fru*) [45]. Expression of sex-specific isoforms of *fru* and *dsx* orchestrates the development of neurons at multiple levels i.e. sensory-, inter- and motor neurons [45]. These neurons are responsible for sensing and integrating critical cues during courtship and generating motor output in the form of sexual behaviour [45].

Fruitless (fru) expressing neurons are critical regulators of courtship behaviour in males [45]. A subset of these neurons, called the P1 neurons act as the central courtship node in the male fly brain [45]. P1 neurons integrate sensory cues and send signals to downstream motor neurons to produce courtship behaviours [45]. Major inputs to P1 neurons include gustatory information i.e. female pheromones that are sensed by pickpocket (ppk23) sensory neurons and further processed by PPN1 and vAB3 neurons [46]. Visual projection neurons LC10 sense fly-like targets also feed onto P1 neurons and help in the pursuit of female targets [46]. P1 receives inhibitory input from neurons that sense male cues including DC1, LC1 and mAL, thus inhibiting courtship towards unfavourable targets [46].

Information from P1 neurons is sent to the ventral nerve cord (VNC) via pIP10 descending neurons. Subsequently, these descending neurons contact thoracic neurons dPR1 and vPR6, that control the production of courtship song [46]. The neurons responsible for copulation are located in the VNC but it is not known how they communicate with P1 neurons [46]. Glutamatergic motor neurons control the joining of genitalia while inhibitory GABAergic inter-neurons control the release [46]. Finally, corazonine-expressing neurons control the duration of copulation and ejaculation [46]. Zhang et al. showed that a male's courtship drive decreases after multiple copulations. This courtship drive is under the control of dopaminergic neurons of the SMPa cluster

that also connect to P1 neurons [47].

Doublesex has a key role in determining sex-specific circuitry underlying sexual receptivity [45]. In females, *dsx* is expressed in several clusters in the central brain including pC1, pC2 and PC3 [48]. pC1 neurons respond to courtship song and pheromones (cVA) while pCd neurons only respond to cVA [49]. Silencing these neurons leads to a decrease in female receptivity [49]. Surprisingly, activation of pC1 neurons leads to male-like courtship behaviour in female flies [50]. In addition, pC1 receives inhibitory neuro-modulatory input from serotonergic neurons, but very little is known about the mechanistic aspects [34]. Ishimoto and Kamikouchi identified a feed-forward ellipsoid body circuitry, independent of *fru* and *dsx*, could mediate rejection response in females. [51]

Doublesex is also differentially expressed in the ventral nerve cord where it controls different post-mating responses [49, 52, 53]. For example, the post-mating decrease in receptivity is mediated by the binding of sex peptide to sex peptide sensory neurons (SPSN) [54]. The binding leads to the dampening of the activity of SPSN and its downstream neuron SAG that relays information to the neurons in the central brain including pC1 neurons [54]. Similarly, the inactivation of the SPSN-SAG axis leads to the disinhibition of oviDNs (oviposition descending neurons) that promote egg-laying in female flies [55]. However, not all post-mating responses are mediated by sex-peptide. For example, increased attraction to polyamines in mated females is regulated by the binding of myo-inhibitory peptides to the sex-peptide receptor [56].

1.2.5 Feeding behaviour in *Drosophila*

Drosophila feeding behaviour is a robust innate behaviour consisting of several modules or behavioural sub-programs. As reviewed in [57], starved flies usually start with foraging i.e. looking for a palatable food source (Fig 1.6). After finding a suitable food source, flies will cease locomotion and initiate feeding (Fig 1.6). The feeding motor program is rhythmic and occurs as a series of proboscis extensions and withdrawals (Fig 1.6)[58]. After satiation, flies terminate feeding and disengage from the food source (Fig 1.6)[57]. The expression of these behavioural modules is flexible and depends on the internal state of the fly, the available food source etc [57].

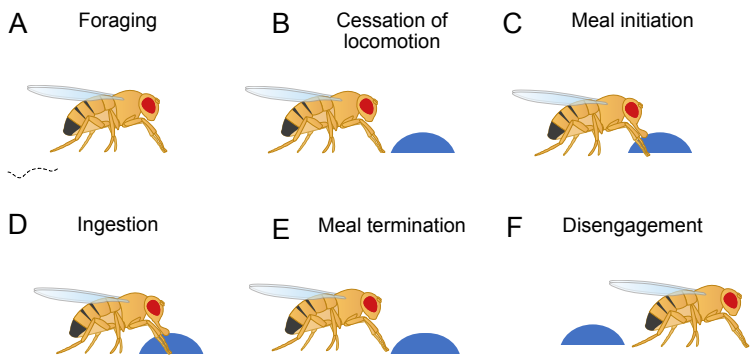


Figure 1.6: Schematic of feeding behaviour in *Drosophila melanogaster*. Feeding can be broken down into several behavioural sub-programs: foraging (looking for food), termination of locomotion, feeding initiation, ingestion, termination and disengagement. The figure is adapted from Pool and Scott, 2014 and the fly schematics are designed by Gil Costa [57].

Multiple assays have been developed to study feeding behaviour in a systematic fashion. One commonly used assay is the Proboscis Extension Response (PER), which measures the probability of proboscis extension when flies are stimulated with a tastant, such as sucrose [59]. However, PER assays do not measure the total volume of food consumed by the fly. Another assay, known as the Capillary Feeding (CAFE) assay, measures the change in food volume in capillaries presented to flies, providing an indirect measure of food consumption [59]. Additionally, the flyPAD, developed by Itsokov et al. (2014), utilises automated capacitance-based measurements to quantify fly-food interactions reliably [58]. The authors observed a distinctive square wave-like pattern in the capacitance signal when flies rhythmically fed from the food source. They were

able to classify putative "sips" based on the shape of the signal, providing a readout of food intake and detailed information about the micro-structure of feeding events [58]. Among these methods, the flyPAD is considered the most reliable and sensitive tool for measuring food intake as it measures the strength of the direct physical interaction with the food patch. However, none of these techniques allow us to measure other parameters such as locomotion of the fly or location during the feeding process which might provide insights about foraging strategies.

1.2.6 Neural control of feeding behaviour in *Drosophila*

Detection of food happens at two different sensory levels: olfactory and gustatory. Volatile substances are often detected by olfactory receptor neurons (ORNs) on the antennae and maxillary palps [59]. From the olfactory sensory neurons, the information is passed to higher processing centres like the mushroom body and lateral horn via the antennal lobe [59] to trigger approach or avoidance. For example, OR42b neurons are necessary for attraction towards vinegar while OR56a neurons drive avoidance towards the harmful compound geosmin, a fungal metabolite [59].

Gustatory receptor neurons (GRNs) present in the proboscis and tarsi detect non-volatile compounds [59]. Sweet-sensitive gustatory receptors include GR5a which detects trehalose and GR64A which detects maltose, sucrose, and glucose (Fig 1.7)[59]. These receptors work in conjunction with GR64f, which acts as a co-receptor [57]. Activation of these sugar-sensitive gustatory receptor neurons (GRN) has been shown to increase proboscis extension reflex in flies. In contrast, bitter compounds like quinine and caffeine are detected by GR32a, GR33a, and GR66f receptors which have been shown to inhibit feeding (Fig 1.7)[57].

Shiu et al. 2022 showed that information from the sugar GRNs is processed by a local circuit in the sub-esophageal zone (SEZ) to trigger feeding initiation [60]. In particular, there are three to four neuronal relays between GRNs and the motor neurons that control proboscis extension namely second-order, third-order, and pre-motor neurons (Fig 1.7)[60]. Activation of neurons at each level triggers feeding initiation, while only inhibition of second-order neurons decreases

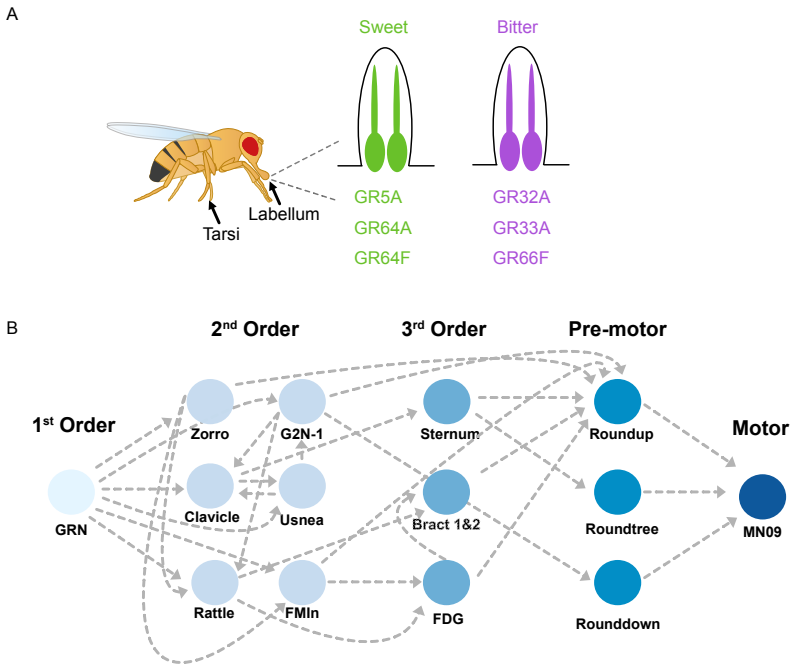


Figure 1.7: (A) Schematic of gustatory sensillia and receptors in *Drosophila*. (B) Schematic of feeding circuitry in *Drosophila melanogaster* [60]. Information from sugar-sensitive gustatory neurons is transmitted to motor neurons via 3-4 layers of inter-neurons. The figure is adapted from Shiu et al. [60] and the fly schematics are designed by Gil Costa.

feeding behaviour. Interestingly, second-order neurons G2N-1 and Clavicle might be modulated by hunger state along with sugar GRNs [60]. Third-order neurons mainly synapse onto three pre-motor neurons i.e. Roundup, Roundtree, and Rounddown which also receive input from bitter sensitive GRNs (Fig 1.7)[60]. These three pre-motor neurons feed onto the MN9 motor neuron (Fig 1.7). Schrawz et al. 2017 identified the motor neurons underlying proboscis movements during feeding. While MN9 neurons trigger the lifting of the rostrum, MN1 neurons cause proboscis retraction [61].

Along with the feeding motor circuit, previous studies have looked into the role of neuropeptides and neuro-modulators in the central brain encoding the motivational drive to feed. Many of these neuro-modulators are evolutionarily conserved and play a similar role in mammals [59]. Given below is a list of some neuro-modulators and their role in feeding behaviour (Table 1).

Serotonin

Serotonin (5-HT) is an important bio-amine that controls various behaviours in flies such as sleep,

courtship and feeding (reviewed in [62]). Activation of 5-HT neurons has been shown to decrease food intake in adult flies [63]. Conversely, inhibition of 5-HT neurons increases food intake [64]. Yao and Scott identified 3 pairs of 5-HT neurons in the SELs that were critical for satiety [65]. These 5-HT were activated upon sugar consumption and promoted insulin release to restrict food intake [65]. In contrast, Albin et al. showed that a subset of 5-HT neurons (belonging to the clusters AMP, LP, and PLP) induced feeding behaviour in sated flies [66]. Therefore, different 5-HT clusters may play different roles in regulating appetite in flies.

Dopamine

Dopamine is an important biogenic amine that controls cognitive processes like motivation as well as locomotion in flies (reviewed in [62]). A study by the Scott group showed that inhibition of all dopaminergic neurons labelled by TH GAL4 decreases feeding [67]. They narrowed down the effect to one dopaminergic cluster TH-VUM in the subesophageal ganglion, which upon activation enhances PER [67]. Eriksson et al. confirmed this effects by inhibiting TH neurons using another tool [64]. Inagaki et al. showed that dopamine modulates the sensitivity of GR5A gustatory receptor neurons in starved animals, thereby increasing the probability of proboscis extension [59].

DILPS

DILPs (*Drosophila* Insulin-like peptides) play a key role in nutrition and development. Eight DILPs have been identified in *Drosophila* which are produced by the insulin cells in the gut and the brain. They act as key regulators of satiety i.e. suppress food intake when an adequate amount of food is ingested [59].

Leucokinin

Leucokinin is a neuro-peptide/neuro-hormone expressed in the neurons in the central brain and abdominal ganglion that modulates feeding in flies [59]. Al Anzi et al. showed that the mutants for Leucokinin and its receptor displayed over-eating phenotypes [68]. Ablation of Leucokinin receptor neurons showed the same phenotype, suggesting the neuronal expression of Leucokinin

is sufficient to control feeding [68]. Interestingly, Zandawala et al. showed that LK mutants behaved differently when compared to LKR. LK mutants showed a decrease in proboscis extension index while LKR mutants showed an increase in PER [69].

Neuropeptide F

Neuropeptide F (NPF), the homolog of mammalian Neuropeptide Y promotes feeding behaviour in flies. Activation for NPFR neurons in larvae increases feeding behaviour even when presented with unpalatable food sources [59]. These NPFR neurons are under the control of negative regulation of DILPs. In adult flies, inhibition of NPF neurons led to a decrease in feeding [64].

Tyramine and Octopamine

Octopamine, the homologue of noradrenaline and its precursor tyramine play a crucial role in starvation-related behaviours. Starvation increases octopamine levels while decreasing tyramine levels [70]. Concomitantly, octopamine promotes feeding and locomotion in starved flies while tyramine inhibits both behaviours in fed flies [70]. Youn et al. identified an octopaminergic cluster VMP4 that triggers proboscis extension [71]. These neurons modulate the response of sugar-sensitive gustatory neurons, thereby promoting food intake [71].

Hugin

Hugin, the homologue of Neuromedin U has been shown to suppress feeding in both larvae and adult flies [59].

Neuromodulator	Experiment	Effect	References
Serotonin	Inhibition of 5-HT neurons using Kir2.1 in adult flies	Increase in feeding	[64]
Serotonin	Activation of 5-HT neurons in the lateral subesophageal ganglion (SEL) using TrPA1 and Chrismon	Decrease in feeding	[65]
Serotonin	Activation of 5-HT clusters (SE1, ALP, LP2, PLP, and PMP) using TrPA1	Increase in feeding	[66]
Dopamine	Activation of all dopaminergic neurons using TrPA1	Increase in proboscis extension reflex	[67]
Leucokinin	Inhibition of leucokinin and leucokinin receptor neurons using Reaper	Increase in feeding	[68]
Leucokinin	Mutants for LKR	Increase in proboscis extension reflex	[69]
Leucokinin	Mutants for LK	Decrease in proboscis extension reflex	[69]
Neuropeptide F (NPF)	Inhibition of NPF neurons using Kir2.1	Decrease in feeding	[64]
Octopamine	Activation of OA-VPM4 neurons using TrPA1	Increase in food intake	[71]

Table 1: Summary of the effect of some neurotransmitters and neuromodulators on Drosophila feeding behaviour

1.2.7 Defensive behaviours in *Drosophila*

Animals encounter life-threatening situations on a day-to-day basis. Avoiding life-threatening dangers like predators is central to an animal's survival and has huge consequences on its fitness. Therefore, animals have evolved "defensive behaviours" to minimise the harm from predators. Le Doux and Daw broadly categorised defensive behaviours into two groups: innate reaction patterns and instrumental behaviours (learned) [72]. Innate reaction patterns include innate reflexes and fixed action patterns that take the form of stimulus-reaction outcomes. For example, a blink of an eye when confronted with a poke. Instrumental behaviours, in turn, rely on an animal's past experience. Animals can learn to avoid odours or places previously associated with noxious stimuli like electric shocks. In the next section, I will mainly focus on innate reflexes in flies.

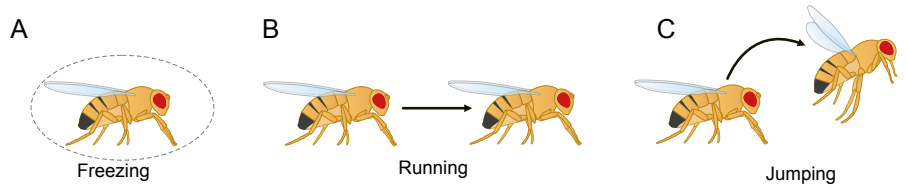


Figure 1.8: Schematic of defensive behaviours in *Drosophila melanogaster*. Flies mainly exhibit three kinds of defensive behaviours in response to visual threats. They freeze (sustained immobility), run (increase their walking speed) or jump. The fly schematics are designed by Gil Costa.

Like many other animals, *Drosophila* have evolved defensive behaviours to protect themselves from threats. A variety of stimuli have been employed to study defensive responses in flies including visual threat [73, 74], mechanical stimuli and odours. For example, Liu et al. showed that upon touching the mechano-sensory bristles on the wing margin, wild-type flies showed robust kicking behaviour [75]. Adults and larvae show avoidance behaviour towards the chemical iridomyrmecin secreted by a parasitic wasp *Leptopilina* [76].

The defensive responses to visual threats have been thoroughly investigated in flies. Mainly, two types of visual threat have been previously used: the looming stimulus and translational stimulus [73, 74]. Using a series of overhead shadow stimuli (translation threat), Gibson et al. showed that the wild-type flies increased their locomotion during the stimulation period (Fig 1.8) [74]. This increase in locomotion decayed after the stimulation period and depended on the number of

stimuli presentations [74]. Other defensive behaviours observed include hopping or jumping and freezing [74].

Other studies have employed the looming stimulus, a non-linearly expanding circle that mimics an approaching predator [73, 77]. Card and Dickson showed that in response to a looming object, flies initiated take-off jumps from platforms (Fig 1.8)[77, 78]. This takeoff consists of a sequence of behavioural modules including freezing, postural adjustment, elevation of the wings and jumping. Based on the time taken to escape, the escape behaviour was categorised into a short mode where flies prioritised speed and a long mode where flies spent time elevating their wings to ensure stability in flight [79]. Zacharias et al. showed that flies responded to looming stimulus differently when placed in an inescapable arena (Fig 1.8)[73]. In their assay, 70% of the flies mainly froze, while only 6.4% of the flies jumped. In addition, the flies that did not freeze reliably increased their baseline velocity or ran [73]. However, this freezing response was attenuated in the presence of other flies [80]. A single fly spent 76% of their time freezing while flies in groups of 6-10 spent <10% of their time freezing [80].

Barrios et al. showed that the flies experienced transient cardiac arrest in response to a loom [81]. The rates of cardiac reversal decreased among the flies that froze in response to the threat but increased among those who chose to flee [81]. Additionally, prolonged freezing behaviour led to a decrease in sugar levels in the hemolymph, making the flies more susceptible to starvation [81].

1.2.8 Neural control of defensive behaviours in *Drosophila*

Defensive responses involve reliable detection of approaching threats, processing and eliciting an appropriate action to evade the threat. In flies, the first level of threat detection happens at the optic lobes in the brain [82]. The optic lobes consist of four distinct regions namely lamina, medulla, lobula and lobula plate [82]. A special class of neurons emerging from the lobula or lobula plate called LC/LPLC neurons have been shown to be important in detecting and encoding visual objects [82]. A subset of these LC/LPLC neurons project onto optic glomeruli in the

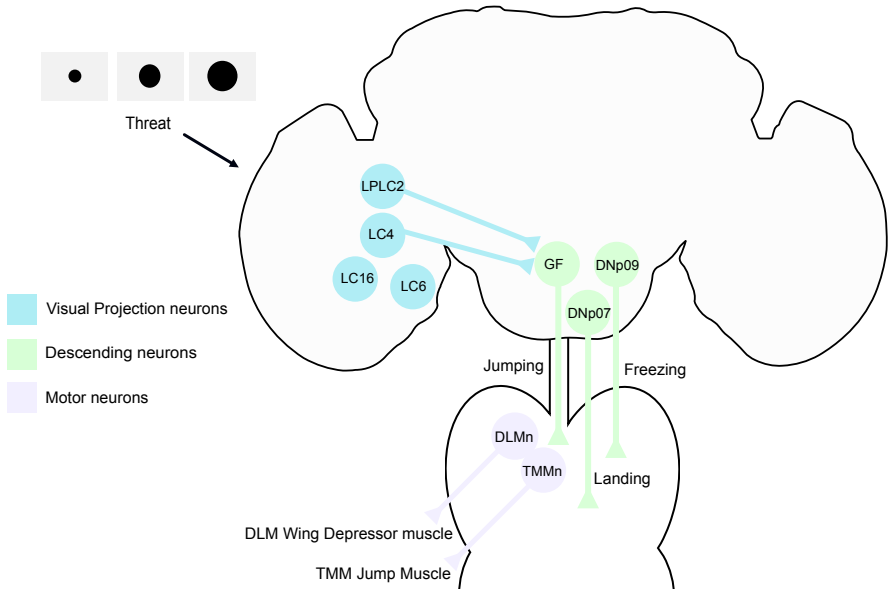


Figure 1.9: Schematic of neurons involved in detecting threat and controlling defensive behaviours in *Drosophila*. Visual projection neurons (LC4, LC6, LC16, LPLC1 and 2) in the optic lobe detect looming stimuli and convey the information to descending neurons. GF triggers takeoff while DNp09 elicits freezing behaviour.

central brain [82] and elicit the appropriate defensive behaviours. Using two-photon microscopy, Wu et al. showed that LC4, LC6 and LC16 neurons are selectively tuned to looming stimuli (Fig 1.9). Moreover, activation of LC4, LC6 and LC15 neurons elicits jumping whilst LC16 neuron activation causes backward walking (Fig 1.9)[82]. In addition, LC11 neurons have been shown to process motion cues from other flies and attenuate freezing in a social context [80]. Another group of visual projection neurons called Lobula plate/lobula columnar, type 2 (LPLC2) neurons, are important for threat response. While LPLC2 encodes the angular size of the threat, LC4 neurons encode speed information (Fig 1.9)[83]. Therefore, different visual projection neurons encode different aspects of the threat and trigger diverse defensive behaviours.

These defensive behaviours are further controlled by descending neurons, a group of neurons that convey information from the central brain to motor centres via the VNC [84]. Downstream of LPLC2 is the Giant fibre, a large descending neuron that connects to motor neurons TTMn and DLMn that further connect to the wing depressor and jump muscles (Fig 1.9)[79, 85–87]. Von Reyn et al. showed that activation of the Giant fibre was necessary and sufficient for inducing

short-mode takeoff but not the long-mode takeoff [79]. Two descending neurons downstream of LC4 neurons, DNp02 and DNp11 control the direction of takeoff. DNp11 neurons trigger forward jumps while DNp02 neurons trigger backward jumps [88]. These takeoffs are followed by a landing response and are under the control of descending neurons DNp07 and DNp10 (Fig 1.9)[89]. Additionally, Zacarias et al. found that activation of DNp09 evoked freezing behaviour (Fig 1.9)[73]. Despite these insights, the functions of the other $\sim$ 1000 descending neurons in *Drosophila* remain largely unknown.

1.2.9 Decision-making in *Drosophila*

Flies, like us, can make decisions. These decisions can range from simple instances, such as a fly's choice to approach or avoid an odour, to more intricate scenarios involving multiple sensory inputs like choosing a potential mate.

Decision-making processes have been meticulously explored in flies in the context of olfaction. In this regard, the approach/avoidance behaviour of flies in response to various odour cues has provided valuable insights. For instance, flies typically exhibit an attraction towards food-related odours such as vinegar. However, this attraction is attenuated when the same odour is presented in conjunction with geosmin, an aversive fungal metabolite [90]. Another example involves the avoidance behaviour of flies towards elevated concentrations of carbon dioxide (CO_2) above 0.02% [91]. Notably, this aversion response is diminished in starved flies when the CO_2 stimulus is presented alongside vinegar [91]. Similarly, flies can integrate gustatory cues with olfactory to make decisions too. For example, mated females show a preference for yeast over sucrose while male and virgin females show a preference for sucrose [59].

The examination of flies' choices towards visual cues remains comparatively less explored. When faced with a choice between light and dark, flies typically gravitate towards the light; however, this preference can shift towards the dark when their flying capability is compromised [92]. These studies highlight that flies integrate their internal state with external cues to produce the most

appropriate behaviour.

However, most of the studies have focused on the decision to initiate a single behaviour. The choice between two competing behaviours, often referred to as action-selection, has received comparatively little attention. Specifically, there is a notable gap in our understanding of how flies decide between two crucial yet mutually exclusive innate behaviours. There are a few studies that have tried to address this. For example, Chen et al. showed that when male flies are sleep-deprived, they prioritise sleep over courtship behaviours [93]. Conversely, they found that activation of the courtship-promoting P1 neurons decreased the amount of sleep in flies [93]. Similarly, focusing on P1 neurons, Hoopfer et al. explored the choice between courtship and aggression [38]. The authors found that lower frequency light stimulation of P1 neurons promoted aggressive behaviours while higher frequencies promoted both courtship and aggression.

Despite these insights, the choice between several crucial innate behaviours remains unexplored. For example, how do flies choose between feeding and escape? Or between feeding and courtship? What determinants govern their choice? To what extent do external and internal factors impact a fly's behavioural selection? Moreover, what are the neural mechanisms orchestrating the choice between these innate behaviours?

1.3 Scope of the thesis

The study of decision-making has a long and varied history. Despite decades of investigation, very little is known about the neuronal mechanisms underlying decisions. The neuro-ethology approach and the cognitive approach in decision neuroscience have proved complementary to better understanding how individuals make decisions in various contexts, from simple binary choices in sea slugs to high-stakes decisions in business. However, still several questions remain unanswered. How do animals resolve behavioural conflicts? Do animals take into account their internal state? What are the neural circuit mechanisms that drive behavioural choices? Do different species solve behavioural choices differently, depending on their ecological niche and

evolutionary history?

The fruit fly *Drosophila melanogaster* is a well-established model organism for investigating the mechanisms of decision-making. It has a simple, yet highly tractable, nervous system that gives rise to a wide range of complex innate behaviours including feeding, courtship, and escape. The neural circuits responsible for these behaviours have been the focus of extensive investigation, resulting in a highly detailed understanding of the underlying neuronal elements. However, how these circuit elements interact to produce behavioural choices remains largely unexplored. Moreover, how animals prioritise their behaviours in accordance with their internal state remains unknown.

These are the questions that I seek to address within the scope of my thesis. In the second Chapter of the thesis, I aimed to understand if flies prioritise survival over reproduction when confronted with an infection. Bacterial infections exert a huge metabolic cost on animals, therefore investing resources into other processes like reproduction might be detrimental. To test this, I implemented the micro-injection system in our lab in order to deliver consistent doses of bacteria into flies. After setting up the system, I performed behavioural assays looking at both male and female behaviour. My data showed that despite the vast metabolic costs of infection, male courtship behaviour and female sexual receptivity remain unaffected. I summarised my findings and published them in Proceedings of the Royal Society B: Biological Sciences [1].

Next, I focused on two more projects that involved the establishment of novel behavioural choice assays where flies were provided with a choice between two crucial but mutually exclusive behaviours. In Chapter 3, I focused on the choice between feeding and courtship in male flies. This assay was set up by a master's student in our lab, Sherry J. Cheriyamkunnel. I advanced the analysis by extracting data from ethograms and developing scripts for temporal behavioural analysis. I further performed experiments on P1 courtship command neurons to understand the neural mechanisms underlying the choice and was involved in writing the manuscript and addressing the reviewers' comments. This work went on to be published in Current Biology [2].

Finally, I devised an assay to identify the neural circuit involved in the choice between feeding and escape which I have covered in Chapter 4. With the help of Martha Moita's lab from Champalimaud Centre for the Unknown, I built the looming stimulus set-up in our lab that reliably triggers defensive behaviours in flies. Using intersectional genetic techniques, computational analysis of connectomics data, and optogenetics, I have outlined the neurons responsible for the behavioural choice connecting sensory input to motor output. These two projects allowed me to address the crucial question of how brains prioritise actions.

2 Reproduction-Immunity Trade-off

2.1 Abstract

Reproduction and immunity are energetically expensive traits that are critical for an animal's fitness. When confronted with an infection, an animal needs to allocate energetic resources towards fighting the infection at the cost of essential processes, such as reproduction. However, very little is known about the factors that dictate the trade-off between immunity and reproduction. Here, I use the fruit fly *Drosophila melanogaster* to understand the effects of bacterial infections on pre-copulatory reproductive behaviour. Using two different wild-type fly strains, I studied the impact of six other bacterial species that varied in their virulence in diverse behavioural setups. Surprisingly, I found that infected male flies displayed robust courtship levels and high mating success. Similarly, female flies infected with pathogens remained sexually receptive. Furthermore, activation of the immune system pathways by genetic means had a limited impact on both male courtship and female sexual receptivity. My results suggest that reproductive behaviours are sustained in flies with bacterial infection, despite its substantial metabolic cost. This work has been published in Rose et al. 2022 where I am the first author [1].

"Nothing in Biology Makes Sense Except in the Light of Evolution"

Theodosius Dobzhansky, The American Biology Teacher, March 1972

2.2 Introduction

2.2.1 Mate choice

In his book 'The Descent of Man, and Selection in Relation to Sex', Darwin eloquently laid down the theory of sexual selection [94].

"We are, however, here concerned only with that kind of selection, which I have called sexual selection. This depends on the advantage which certain individuals have over other individuals of the same sex and species, in exclusive relation to reproduction." - Charles Darwin, 1888

Darwin synthesised the concept of sexual selection as a distinct process to explain the evolution of conspicuous traits that could not be explained by natural selection [94]. However, sexual selection is not a distinct process, but rather a subset of natural selection, which enables selection for traits that increase an individual's reproductive success [95]. Darwin believed sexual selection can act through two mechanisms: male-male competition (where males compete for access over females) and female choice [94].

Mate choice is one of the most important decisions an animal will make in its lifetime with huge consequences on its fitness. This phenomenon is defined as "the process of non-random matings or allocation of reproductive investment with the opposite sex in a population as a result of the effects of traits expressed in one sex" [95, 96]. An animal can use a wide variety of traits to evaluate the features of a potential partner [97]. For example, individuals can directly benefit from choosing mates on the basis of resources [98], territorial defence [99], parental care [100]

and pathogen avoidance [101]. In this thesis, I will focus on understanding the effect of infection on mate decisions, from the perspective of an infected host (i.e. if infections can alter reproductive investment) and a healthy animal, which must choose to mate between an infected and healthy mate.

2.2.2 Effects of infection on reproductive investment in hosts

Animals can modulate their reproductive efforts to maximise their fitness. An animal's reproductive effort can be broken down into two components: current reproductive effort and future reproductive effort (or residual reproductive value) [102]. When animals are infected with a harmful pathogen, they are often faced with increased mortality and decreased fecundity, thus reducing their residual reproductive value (RRV). The **"terminal investment hypothesis"** predicts that an organism will increase reproductive effort as its chance for future reproduction decreases [103]. For example, older crickets (*Allonemobius socius*) infected with a bacterial component to simulate infection increase their calling rate [104]. *Drosophila* males infected with *Wolbachia* show an increase in mating rate [105]. Similar effects have been reported in other insects and birds (Table 2). The increase in investment is not limited to pre-copulatory traits. For example, infection with the entomopathogenic fungus *Metarhizium brunneum* increases the egg-laying rate in Queen ants [106].

However, this is not a general trend. Infected animals are riddled with energetic constraints as fighting the infection can be costly [115, 116]. As the energy resources are limited, there might be a trade-off between reproduction and immunity resulting in increased allocation towards immunity [117]. As a result, infected animals might experience malaise and fatigue. In these cases, one might expect the animals to court less. For example, guppies infected with flatworms and infected sticklebacks invest less time in courtship [110, 111]. Therefore, an animal's reproductive efforts post-infection can be influenced by energetic costs induced by the disease and its evolutionary past.

Host	Pathogen/Stimulant	Effect	References
Drosophila melanogaster	Wolbachia	Increase in mating rate in infected males	[105]
Allonemobius socius	Lipopolysaccharides (LPS)	Increase in calling rate in older infected males	[104]
Gryllodes sigillatus	Heat-killed *E. coli*	Older males increase their calling rate, the younger males decrease	[107]
Hetaerina Americana	Nylon implant	Infected older males were more aggressive and had higher mating success.	[108]
Chlamydotis undulata undulata	LPS	Infected males show reduced courtship display	[109]
Poecilia reticulata	*Gyrodactylus turnbulli*	Infected males spent less time courting	[110]
Gasterosteus aculeatus	*Schistocephalus solidus*	Infected males show decreased courtship levels, nesting activity	[111]
Gobiusculus flavescens	microsporidian	Infected males show decreased a courtship rate	[112]
Drosophila melanogaster	*P. entomophila*	Infected flies show reduced mating success	[113]
Tribolium castaneum	*Hymenolepis diminuta*	Infected males showed reduced courtship vigour	[114]

Table 2: Summary of the effect of infection on pre-copulatory reproductive investment in animals.

2.2.3 Infection and mate choice: can animals discriminate against infected individuals?

Mating with an infected individual is a costly affair. Animals risk transmission of the disease that could affect their survival and future reproduction [118, 119]. Infected mates can also be less fecund as there is a trade-off between the immune system and reproduction [120–122]. They can also transmit the pathogen to their progeny [123]. Therefore, mating with an infected individual could have severe consequences on their fitness. Can animals distinguish infected from healthy individuals?

Studies on birds and rodents have shown that individuals can indeed discriminate against infected mates [124]. Male birds have elaborate secondary sexual traits in the form of bright plumage like a peacock's train [125]. Infection can have a negative impact on these traits. For example, greenfinches with a coccidian infection display reduced plumage colouration as a result of a

decrease in carotenoid levels [126]. Female greenfinches can use these visual cues as an honest indicator of male quality [124].

Unlike birds, rodents use chemical cues like urine that contains a range of volatile and non-volatile compounds [127, 128]. A number of studies done in rodents show that infection can affect chemical cues [124]. These are indirect behavioural observations, where the female is simultaneously presented with the urine of healthy and infected males [124]. For example, when a female is presented with the urine of a healthy male and a male infected with the nematode, *Heligmosomoides polygyrus*, she has a preference for a healthy male [129]. However, some pathogens can trick their host by making the infected individuals more attractive [124]. For example, when a female rodent is presented with the urine of a healthy male and a male infected with *Toxoplasma*, she has a preference for an infected male [130]. This tactic is beneficial for the pathogen as it enables their transmission [124]. Similar trends have been observed in flies where bacterial infections can increase the production of aggregation pheromones in flies.

2.2.4 *Drosophila* immune system

The *Drosophila* immune system has been meticulously studied with a focus on the molecular players. *Drosophila* lacks an adaptive immune system and solely relies on innate immunity [131]. The epithelial tissues (cuticle, gut, reproductive tract) act as the first layer of protection [132]. Upon encountering pathogens, the host elicits a variety of processes in defence: encapsulation, phagocytosis, coagulation (clot formation to trap the microbes), and melanisation (deposition of melanin to encapsulate microbes) [133]. Additionally, the host induces the systemic immune response that is characterised by the production and secretion of Anti-Microbial Peptides (AMPs) [131, 133].

The systemic immune response is induced by two different cascades: Toll and Imd pathways. The two canonical pathways described below have been thoroughly reviewed in [131, 133]. The Toll pathway responds to pathogen-associated molecular patterns (PAMPs) from gram-positive bacteria (PGRP-SA, PGRP-SD, GNBP1) or fungi (GNBP3) [131, 133]. Unlike mammalian Toll

receptors, the fly Toll-like receptors (TLRs) don't bind to the PAMPs directly. The PAMPs bind to Spatzle which gets cleaved and thus activates TLRs [131, 133]. The TLRs have adaptors like Tube and MyD88 that help them to activate Nuclear factor-κB (NFKB) homologues Dif and Dorsal [131, 133]. Dif and Dorsal enter the nucleus and regulate the transcription of anti-microbial peptides(AMPs) including Drosomycin and Defensin [131, 133]. The Imd pathway works in a similar fashion but responds to Gram-negative bacteria [131, 133]. The PGRP-LC receptors bind to peptidoglycan (monomeric or polymeric) which leads to the recruitment of adaptors protein IMD [131, 133]. This, in turn, leads to the recruitment of caspases that cleave the phosphorylated version of Relish. Relish translocates to the nucleus and assists transcription of AMPs like Diptericin [131, 133].

2.2.5 Infection and mate choice in *Drosophila*

A few studies have shown that flies can discriminate against infected mates. For example, Wittman and Fedorka have shown that when presented with a choice of an infected (*P. aeurogi-nosa*) and a healthy decapitated female, males show a mating preference for uninfected females [134]. By using decapitated females, the authors eliminate the effect of female behaviour in mate choice, suggesting that males mainly use olfactory cues to discriminate infected from healthy flies. Whether this result is reproduced in mating assays with moving females remains to be tested. Similarly, Khan and Prasad showed that males display a mating bias towards uninfected females when they were given a choice between healthy and infected mates with *S.marcescens* [135]. In this study, the authors used a competitive mating assay and estimated mating bias as "the ratio of the number of infected females mated to the total number of females mated" post hoc. They found a small effect on mate selection (<15.2% fewer infected females were fertilised) but did not monitor if the males preferentially directed their courtship towards the uninfected females [135]. Keesey et al. show that infection with bacteria including *P.entomphila*, *S. marcescens* and *ECC15* leads to increased production of methylated pheromones which are important for mating [113]. However, infected flies showed a decrease in mating success when compared to controls

[113]. Infection with commensal bacteria doesn't have any consistent effect on mating success [136]. Similarly, Wolbachia seems to affect mate choice in a strain-specific way [137, 138].

Host species and strains	Pathogen/Stimulant	Effect on Mate choice	Ref
D.melanogaster(isofemale lines from the DGRP)	*Wolbachia*	No effect on female attractiveness or male mate choice	[138]
D.melanogaster(Strain R and W)	*Wolbachia*	Infected flies show positive assortative mating	[137]
D.melanogaster(OreR) and *D.simulans*	*Wolbachia*	No evidence of female or male pre-copulatory mate preferences	[105]
D.innubila(strain ST-1 and ST-4)	*Wolbachia*	No evidence for male mate choice	[139]
D.melanogaster	*Pseudomonas aeruginosa*	Males prefer unparasitized mates (decapitated)	[134]
D.melanogaster(CS)	*Pseudomonas entmophila*	Decrease in mating success in infected flies	[113]
D.melanogaster(LH)	*Serratia marcescens*	Males show a bias towards uninfected females	[135]
D.melanogaster	*Lactobacillus plantarum*	No assortative mating between flies subjected to different selection regimes	[136]
D.melanogaster	Sigma Virus	Females show no difference in copulation latency or remating rates when subjected to an infected fly.	[140]

Table 3: Summary of the effect of infection on mate choice in flies.

The above-mentioned studies have provided mixed evidence for mate preference either for or against infected individuals. Furthermore, considering the methodological and statistical limitations of these studies, it raises a significant question about whether flies are truly capable of distinguishing against infected partners. Additionally, how does the specific nature of the pathogen, such as whether it's natural or non-natural, or whether it's gram-positive or gram-negative, contribute to shaping these decisions?

2.3 Objectives

By taking advantage of a wide range of cutting-edge techniques, I will study the effects of bacterial infection on reproductive behaviours. In particular, I aim to answer the following questions.

1. Do bacterial infections alter courtship behaviour in flies? Are there any sex-specific differences?

2. Do flies discriminate against mates infected with bacteria?

3. Does the activation of the immune system by alternate means have an effect on courtship behaviour?

I used two different criteria to select the pathogens for my project: (A) virulence of the pathogen, and (B) whether they are natural or not. I hypothesised that depending on these features of pathogens, I might uncover different mechanisms by which pathogens influence mate choice. For instance, virulent pathogens that cause lethality in flies might impose higher fitness costs, therefore flies might modulate their reproductive behaviour in response. Given below is the list of pathogens used in the study:

- *Erwinia carotovora carotovora 15* or *ECC15* is a gram-negative phytopathogen that use flies as vectors including *Drosophila* sp.[141, 142]. Infection is not lethal but leads to a systemic immune response [143]. Interestingly, it leads to increased production of aggregation pheromones in flies [113].

- *E. coli* is a common gram-negative bacterium found in the gastrointestinal tracts of vertebrates including humans [144]. However, it is not natural to flies [145]. *E. coli* infection does not alter the lifespan of flies [146], and its effect on the pheromonal profile remains unknown.

- *Micrococcus luteus* is a gram-positive bacterium commonly found on the skin of mammals [147]. It is not pathogenic to flies [146].

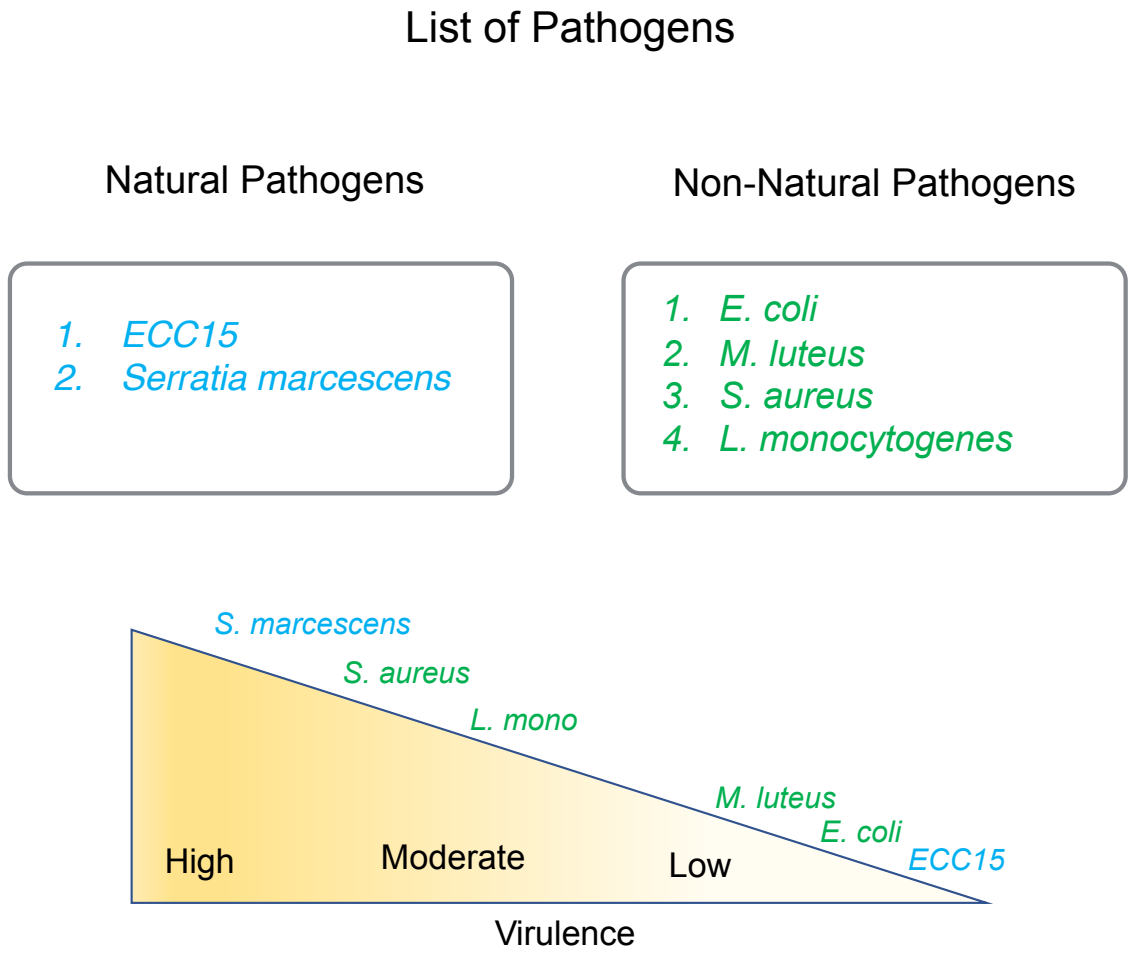


Figure 2.1: List of bacterial pathogens selected for this study, displayed according to their virulence and whether they are natural or not to *Drosophila*.

- *Serratia marcescens* is a gram-negative enterobacterium with a wide range of hosts [148]. It's a frequent cause of nosocomial infections in hospitals [148]. *S. marcescens* is natural and lethal to flies [146] and produces a variety of virulence factors including hemolysin, lipopolysaccharide [149]. Infection in flies leads to increased production of methylated pheromones that lead to aggregation behaviour [113].

- *Staphylococcus aureus* is a gram-positive pathogenic bacterium [150]. They are commonly found in nasal membranes and skin of poikilothermic animals and secrete numerous exotoxins[150, 151]. *S. aureus* is not naturally found in flies, but the members of the same genus including *S. succinus* have been reported to be found in wild-caught *Drosophila* strains [152]. When directly injected into the body cavity, *S. aureus* infection is lethal to

flies [153]. However, there are no reports of how *S. aureus* affects courtship behaviour in flies or the pheromonal profile.

- *Listeria monocytogenes* is a non-natural gram-positive bacterium that is usually transmitted via food [154]. *Listeria* infections are lethal to flies, killing the adults within six to eight days post-infection [154]

2.4 Methods

2.4.1 Drosophila stocks and husbandry

The wild-type *D. melanogaster* strains in this Chapter include Canton-S and Dahomey [1]. The list of transgenic flies used is given below (Table 4). Flies were raised on a standard cornmeal diet at 25°C (recipe given below in Table 5). The incubators maintained a humidity level of 50-60%, and a 12-hour light/dark cycle. Newly emerged virgin male and female flies were sorted within 8 hours of eclosion. They were then grouped by sex, with 15-20 flies per group, and allowed to age for 5-7 days for the behavioural experiments.

List of transgenic fly strains used in Chapter 2	Source
C564-GAL4	BDSC 6982
UAS-Toll10B	BDSC 58987
UAS-Imd	gift from Markus Knaden

Table 4: List of transgenic fly strains used in Chapter 2

Ingredient	Quantity
Water	14.0004 L
Agar	156 g
Cornmeal Flour	828 g
Yeast	444 g
Propionic acid	72 ml
Nipagin(100g/L ethanol solution)	360 ml

Table 5: Fly food recipe

2.4.2 Infection protocol

Six different bacterial pathogens, consisting of three virulent strains and three non-virulent strains were used in this chapter (elaborated in Table 7). These bacterial strains were cultured overnight under the conditions specified below.

[1] Methods section has been published in Rose et al. 2022 [1].

Ingredients for TSB Media	Quantity
Tryptone	17.0 g
Soytone	3.0 g
Glucose	2.5 g
Sodium Chloride	5 g
Dipotassium Phosphate	2.5 g
Ingredients BHI Media	**Quantity**
HM infusion powder	12.50 g
BHI powder	5.0 g
Proteose peptone	10.0 g
Dextrose	2.0 g
Sodium chloride	5.0 g
Disodium phosphate	2.50 g

Table 6: Recipe for Tryptic Soy Broth (TSB) and Brain Heart Infusion (BHI) media.

Subsequently, the cultures underwent centrifugation at 4500 x g for two minutes to form a pellet. This pellet was then diluted in filter-sterilised PBS (phosphate-buffered saline) to achieve a pre-determined concentration. For the experimental setup, 50 nl of the diluted bacterial solution were injected using a nano-injector (MPP1-3 Pressure Injector, Applied Scientific Instrumentation) into the abdomen of anaesthetised flies as previously described [155].

Strain	Nature	Media	Growth conditions
E. coli	Non-pathogenic	LB Media	37° C (shaking)
ECC15	Non-pathogenic	LB Media	30° C (shaking)
M.luteus	Non-pathogenic	LB Media	37° C (shaking)
S. marcescens	Pathogenic	LB Media	37° C (shaking)
S.aureus	Pathogenic	TSB Media	37° C (shaking)
L. monocytogenes	Pathogenic	BHI Media	37° C (shaking)

Table 7: List of bacterial strains used in Chapter 2

2.4.3 Survival assay

Both infected flies and their respective controls (PBS mock injections) were placed in vials, with each group comprising 10 individuals. These vials were maintained at a temperature of 29°C to promote bacterial growth. The count of surviving flies, infected with pathogenic strains, was conducted at consistent time intervals (either hourly or daily, depending on the bacterial strain),

continuing until all the infected flies had died. In contrast, flies injected with non-pathogenic strains were monitored regularly for a time period of 72 hours, as within this time frame, most of the bacteria were naturally eliminated from the flies' systems.

2.4.4 Behavioural assays

Behavioural experiments were performed between zeitgeber time (ZT01) and (ZT10) at a temperature of 25°C over a period of 2-5 days, to account for daily variation. Most of the experiments and data analysis were performed by two experimenters and were not performed blind. Flies that expired during the behavioural trials were excluded from the analysis. For courtship assays, circular chambers with a diameter of 20mm and a height of 5mm were employed. These chambers included built-in partitions that allowed for the separation of flies prior to the experiments.

In the single-pair mating assay, infected flies or the PBS controls were directly placed in the courtship chamber with fly food to prevent starvation after the injection protocols. The courtship chambers were double sealed to prevent the escape of flies and placed in 29°C incubators until experimentation. Uninfected flies of the opposite sex were introduced into the chamber using a fly aspirator right before the start of the behavioural experiment. Following this, the partitions were opened, and the behaviours of the flies were recorded for a duration of one hour using a Sony Handy Camera (model DCR-HC38).

For the competitive mating assays, a healthy fly was presented with a choice between an infected fly and PBS control of the opposite sex. Both infected and PBS flies were marked with acrylic paint of different colours 48 hours before the experimentation to distinguish them.

For single-pair mating assays, the following parameters were quantified:

1. **Courtship Latency:** The time taken by the male to court from the start of the experiment.
2. **Courtship Index:** The percentage of time the male spends courting from the beginning of courtship until 10 minutes or end of copulation.

3. **Mating Success:** % of fly pairs that have copulated within one hour.

4. **Copulation Latency:** The time taken to copulate from the start of courtship.

5. **Copulation Duration:** The duration of the copulation.

In competitive mating assays, where a choice was given to the flies between an infected and a healthy mate, the same parameters were recorded. Additionally, the first mate choice was noted, i.e., whether the focal fly mated with a healthy or an infected mate. The entire set of behavioural data was analysed using the Behavioral Observation Research Interactive Software (BORIS) software [37].

2.4.5 Locomotion assay

An individual male fly (aged 4-7 days) was infected, then gently aspirated into a circular chamber measuring 2 cm in diameter and 0.5 cm in height. Their behaviour was captured for 5 minutes at a high frame rate of 60 frames per second using the Blackfly S U3-13Y3M camera. To analyse the recorded locomotion data, a custom-written Bonsai script [156] was used to compute individual trajectories. A fly was considered to be walking if its instantaneous speed exceeded 4 mm/s [73]. Two essential parameters were computed from these trajectories. The walking speed parameter refers to the mean instantaneous speed recorded during the 5-minute interval following the 4mm/s thresholding, while the total distance parameter denotes the total Euclidean distance traversed by the fly within 5 minutes.

2.4.6 Target-based gene activation experiments

TARGET system was used to control the expression of Toll^{10B} only in the adult flies [157]. C564-GAL4>tub-GAL80^{ts}, UAS-Toll^{10B} and the respective genetic controls were raised at 18°C during development. Adults were collected within 8 hours after eclosion, then placed at 31°C to inactivate tub-Gal80^{ts} for 5 days before testing at 25°C.

2.4.7 Data visualisation

All data visualisation was performed using R (version 3.6.3). Courtship index and mating success are expressed in percentages while copulation latency is measured in seconds. Courtship index and copulation latency data are presented as boxplots with the lower and upper hinges representing the first and third quartiles. Whiskers of the boxplots are in the form of Tukey's fences (1.5*IQR). Mating success is presented in the form of bar plots.

2.4.8 Statistical analysis

All statistical analyses were performed using R (version 3.6). Sample sizes were not pre-determined. All data were tested for normality using the Shapiro-Wilk test. As most of the behavioural data do not follow a normal distribution, Kruskal-Wallis followed by the Dunn test with Bonferroni corrections was used for post-hoc comparisons. To explore the effects of infection timing and concentration, a mixed effects model was used. Fisher's exact test was used to analyse count data and the log-rank test for survival data.

To confirm the validity of the results, a parametric method was employed where courtship data was transformed into fractions (between 0-1) and fitted into a beta regression model (zero and one-augmented) using the R package "GAMLSS". Tukey's test was performed for pairwise comparisons of the model parameters between the infected group and controls using the Emmeans Package. Copulation latency data was formatted in the form of time-to-event and analysed using log-rank tests. The alpha value was set at <0.05. Statistical differences are indicated by different letters. Groups sharing the same letter exhibit non-significant differences in median values. Conversely, when two groups are labelled with different letters, their differences are statistically significant.

2.5 Results

2.5.1 Establishing a reliable infection protocol

In order to understand how infections affect reproductive decisions in flies, I had to establish a protocol for reliably injecting bacteria into flies [2]. Different methods have been used to induce a systemic infection in flies including feeding, pin-pricking and micro-injection [158]. Pin-pricking involves dipping an insect pin in a bacterial solution and pricking the flies in the abdomen [158]. While pin-pricking is a fast procedure and easy to execute, it fails to deliver consistent doses of bacteria [158]. On the other hand, micro-injection uses a micro-capillary under a pressurised system to deliver a precise volume of bacterial solution into flies, hence producing a robust and repeatable infection phenotype [158]. Therefore, I decided to use the micro-injection technique for Chapter 2 of my thesis.

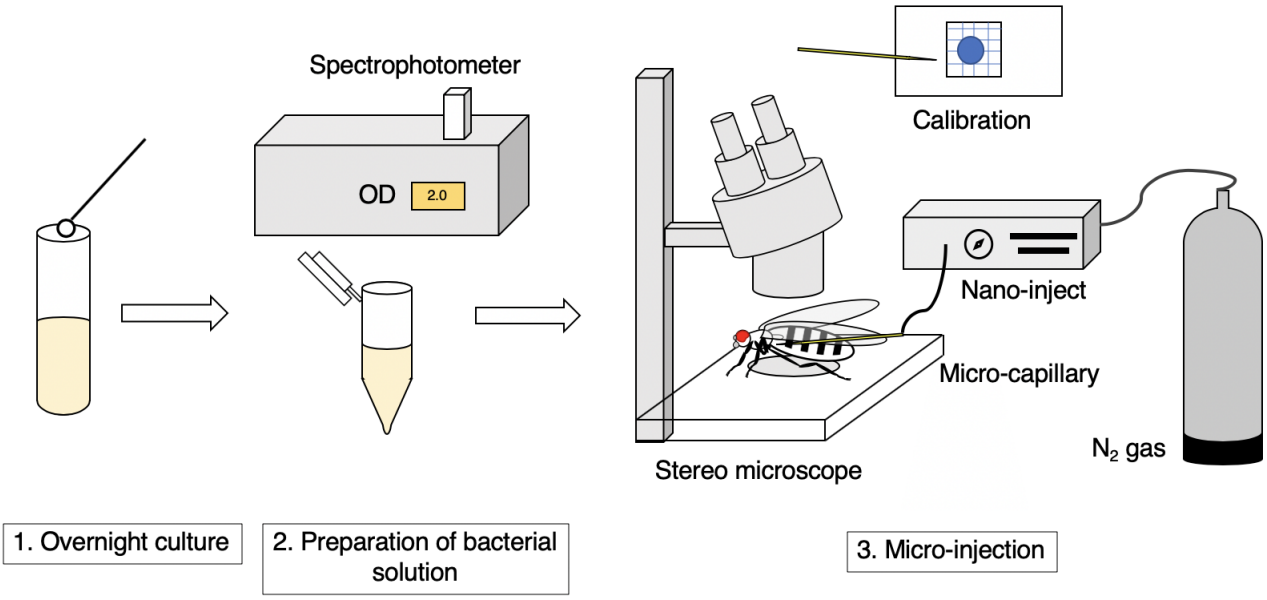


Figure 2.2: Protocol for micro-injection in flies. The infection protocol involves three main steps. (1) I set up an overnight culture by inoculating the bacteria in culture media. (2) After 12 hours of incubation, I measured the optical density using a spectrophotometer and diluted the bacteria in PBS solution to OD_{600}=0.1. (3) I used a micro-injection system to deliver a precise volume of bacterial solution into flies. The system consists of a pulled micro-capillary under the control of an air-pressure injecting device (Nitrogen gas) and a regulator. I first calibrated the pulled micro-capillary tubes to deliver a defined volume of liquid using a hemocytometer. Then, I anaesthetised flies on the CO_2 pad and inject 50 nl of bacterial solution(or PBS) into the abdomen.

[2]Results have been published in Rose et al. 2022 [1]

To get trained in the micro-injection technique, I spent one month in Dr. Marc Dionne's lab at Imperial College London, which has expertise in fly innate immunity. I first practised injecting blue dye into flies to ensure the solution was being introduced into the abdomen of the flies. I monitored the survival of the flies on a daily basis, to make sure that the injection technique itself was not injurious to flies. After perfecting the injection technique, I proceeded to infect CS wild-type flies with bacteria. To do this, I set up an overnight culture by inoculating the bacteria in culture media. After 12 hours of incubation, I measured the optical density using a spectrometer and diluted the bacteria in PBS solution to a defined concentration. I calibrated the pulled micro-capillary tubes to deliver a defined volume of liquid using a hemocytometer. Then, I anaesthetised the flies on the CO_2 pad and injected 50 nl of bacterial solution (or PBS) in the abdomen. I kept the injection time consistent throughout the project as circadian rhythms can influence infection phenotypes [158].

2.5.2 Bacterial infections impact survival in a strain-specific way

To confirm the reproducibility of the infection protocol, I first characterised the survival curves post-infection. To this end, I injected 4-5 days-old Canton-S males or virgin females with 50 nl of bacterial solution (OD_{600}=0.1) or sterile PBS solution. I transferred the infected flies in vials (groups of ten) and I counted the proportion of dead flies at regular intervals.

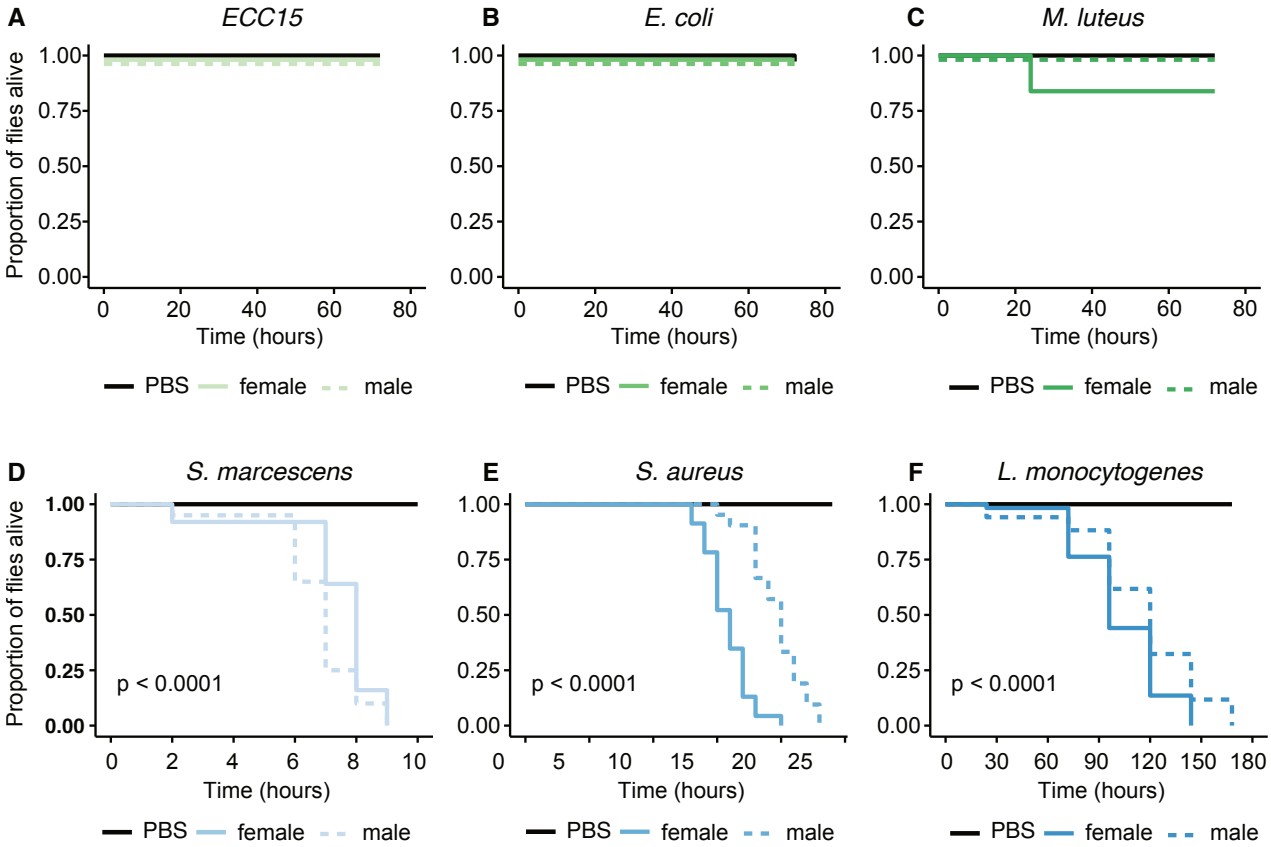


Figure 2.3: Survival curves depicting the lifespan of both Canton-S male and female flies following bacterial infections used in this Chapter. The flies were subjected to an injection of either a bacterial solution or PBS, and subsequently, the number of surviving flies was tallied at regular intervals. Survival curves of (A) *ECC15* (n= 12-25 (♀), 15-45 (♂)), (B)*E. coli* (n= 10-37 (♀), 11-41(♂)) and (C) *M. luteus* (n=12-31 (♀), 12-41 (♂) (D) *S. marcescens* (n= 10-25 (♀), 10-20 (♂)), (E) *S. aureus* (n = 13-23 (♀), 15-21 (♂)) and (F) *L. monocytogenes* (n=25-56 (♀), 20-34 (♂)). (n) indicates number of flies used. Statistical analyses using the log-rank test (A-F). The figure has been published in Rose et al. 2022 [1] and is used under the terms of the Creative Commons Attribution License.

I found that non-pathogenic bacteria including *E. coli*, *ECC15* and *M. luteus* have no effect on the survival of flies (Fig 2.3A-C). In contrast, pathogenic bacteria dramatically reduced the lifespan of wild-type flies (Fig 2.3D-E). Infection with *S. marcescens*, a natural virulent pathogen, killed

the male flies within 9 hours (Fig 2.3D, $\chi^2 = 26, p = 3e^{-7}$, Log-rank test). Similarly, *S. aureus*, a non-natural pathogen, caused 100% mortality within 24 hours (Fig 2.3E, $\chi^2 = 35.8, p = 2e^{-9}$, Log-rank test) and *L. monocytogenes* killed all flies within 7 days (Fig 2.3F, $\chi^2 = 29.3, p = 6e^{-8}$, Log-rank test). Virgin females showed a similar survival profile when exposed to the same pathogens (Fig 2.3D-F). The survival data I obtained is in accordance with previous studies, thereby validating my injection technique. [146, 153, 159].

2.5.3 Male courtship behaviour remains unaffected upon bacterial infections in wild-type CS flies

I first aimed to test the effects of non-pathogenic bacteria on courtship behaviour [3]. To do this, I chose three bacteria that activate the fly's innate immune system without causing mortality, namely *ECC15* which naturally uses flies as hosts, and *E. coli* and *M. luteus* [131, 146]. *ECC15* and *E. coli* are gram-negative bacteria that activate the Imd cascade, while the gram-positive bacterium *M. luteus* activates the Toll pathway [131, 133]. Following 5-6 hours after injection, I performed behavioural assays when the bacterial load was still high.

When presented with a healthy virgin female (Fig 2.4A), control males spent most of the time courting the female ($\sim$ 90%, Fig 2.4B). Intriguingly, I observed that *ECC15*-infected males showed high levels of courtship similar to that of controls (H (Kruskal Wallis chi-squared) = 3.184, p = 0.2035, Fig 2.4B). Similarly, *E. coli* and *M. luteus* infection had no significant effect on male courtship behaviour (H = 2.4156, p = 0.2989, Fig 2.4C; H = 1.1954, p = 0.5501, Fig 2.4D). Most of the uninfected control males mated within an hour (Fig 2.4B-D). Similarly, infected males showed a high level of mating to that of controls (p=1, Fisher's Exact Test, Fig 2.4B-D).

Bacterial infections may impose different fitness costs depending on the virulence of the strain. Therefore, I next aimed to test the effects of pathogenic bacterial strains on male courtship behaviour namely *S. marcescens*, *S. aureus* and *L. monocytogenes*. As these bacterial strains induced lethality, I chose to perform behavioural experiments at time points at which infection is advanced but not too detrimental for the flies. In particular, I performed my courtship assays at 5-6h, 8-9h, and 24-25h post-infection for *S. marcescens*, *S. aureus* and *L. monocytogenes* respectively. To confirm that the infections were indeed not too detrimental, I quantified the walking speed and total distance travelled in 5 minutes by the infected flies and found no significant differences between the infection group and controls (Fig 2.5B-G).

[3] Results have been published in Rose et al. 2022 [1]

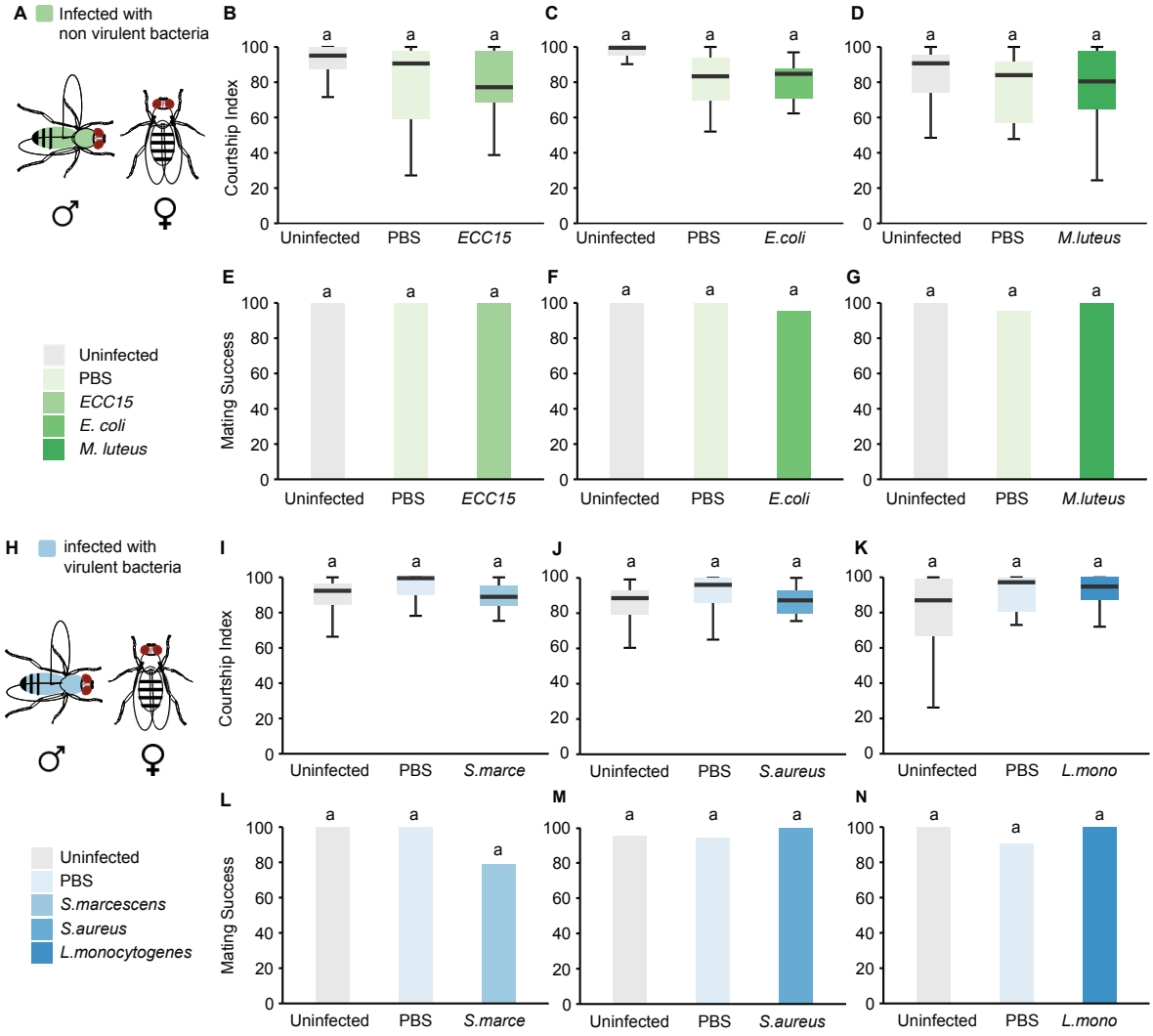


Figure 2.4: Male courtship behaviour remains unaffected upon bacterial infections in wild-type CS flies. Male Canton-S flies were subjected to injection with six distinct bacterial strains, non-pathogenic (A) and pathogenic (H). Subsequently, these flies were tested in a single-pair courtship assay, paired with uninfected virgin females. The courtship index and the mating success of males infected with the non-pathogenic strains *ECC15* (B and E, n=20-21), *E. coli* (C and F, n=19-23), and *M. luteus* (D and G, n=21-22) along with their corresponding control groups. The courtship index and mating success of males infected with the pathogenic strains *S. marcescens* (I and L, n=20-21), *S. aureus* (J and M, n=17-21), and *L. monocytogenes* (K and N, n=19-20) alongside their respective controls. (n) indicates number of flies used. Statistical analyses using the Dunn Test for comparisons in panels B-D, I-K, and the Fisher Test for panels E-G, L-N. Box plots labelled with different letters are statistically significantly different. The figure has been published in Rose et al. 2022 [1] and is used under the terms of the Creative Commons Attribution License.

Contrary to my hypothesis, none of these pathogenic bacterial infections had a significant influence on male courtship behaviour. There were no observable changes in the courtship index of

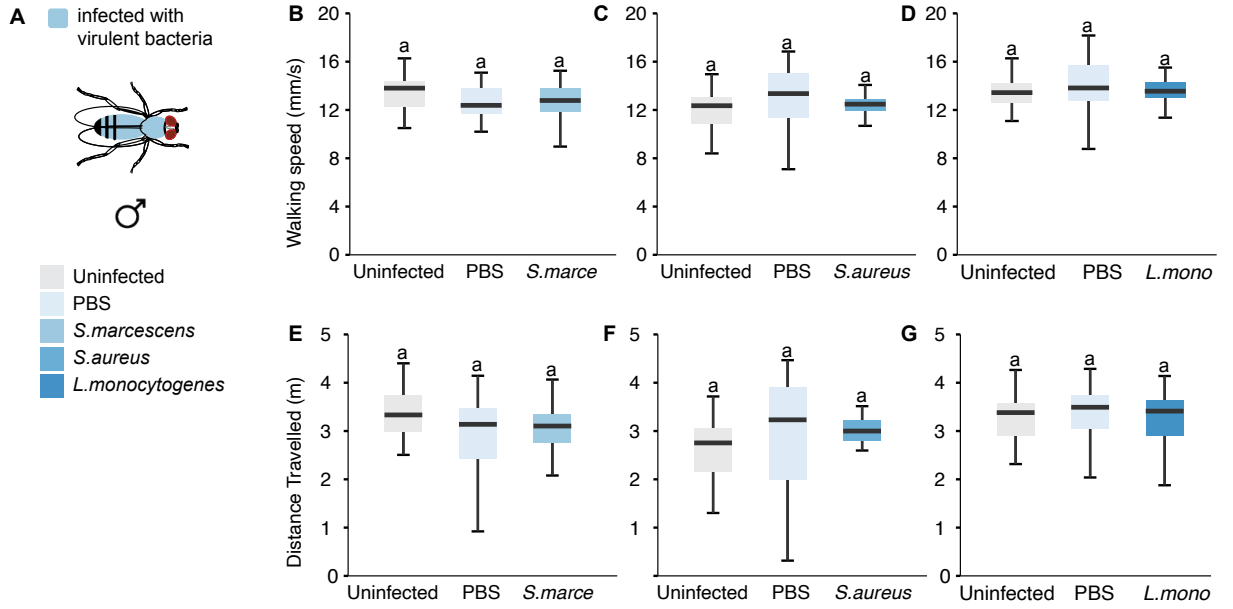


Figure 2.5: Lethal bacterial infections do not affect locomotion of wild-type CS male flies during the time of behavioural experiments. Male Canton-S flies were subjected to injection with one of the three pathogenic strains, followed by testing in a locomotion assay lasting for a duration of 5 minutes. Walking speed and total distance travelled by males infected with *S. marcescens* (B and E, 5 hours post-infection, n=24-36), *S. aureus* (C and F, 13 h post-infection, n=24-32) and *L. monocytogenes* (D and G, 24 hours post-infection, n=20-24) and their respective controls. (n) indicates number of flies used. Statistical analyses using the Kruskal-Wallis followed by the Dunn Test in B-G. Box plots labelled with different letters are statistically significantly different. The figure has been published in Rose et al. 2022 [1] and is used under the terms of the Creative Commons Attribution License.

infected males with either *S. marcescens* (H = 6.1299, p = 0.04666, Fig 2.4B), *S. aureus* (H = 3.0853, p = 0.2138, Fig 2.4C) or *L. monocytogenes* (H = 3.002, p = 0.2229, Fig 2.4D). Moreover, lethal infections did not decrease male mating success (p=1, Fisher's Exact Test, Fig 2.4E; p = 1, Fisher's Exact Test, Fig 2.4F; p = 1, Fisher's Exact Test, Fig 2.4G). Therefore, both non-pathogenic and pathogenic bacterial infections do not impact male courtship behaviour.

2.5.4 Timing of *ECC15* infection and bacterial load have a limited impact on male courtship behaviour in CS wild-type flies

Previous studies have shown that the immune system induction peaks 6 hours after non-pathogenic infections after which its activity decreases [133]. Therefore, I next enquired if infection timing and dosage have an effect on courtship behaviour. To this end, I quantified the behaviour of *ECC15*-infected flies at 3 different time points (3h, 5h, 8h post-infection) while simultaneously varying the bacterial dosage (OD_{600} = 0.5, 1, 2) (Fig 2.6). Interestingly, manipulating bacterial load and the timing of infection failed to reveal any impact on courtship levels or the success of copulation in males. (Fig 2.6, estimate for OD = -0.25068, p = 0.1391; estimate for infection time = -0.05746, p = 0.1323). Moreover, there was no interaction between these two factors (p = 0.0916).

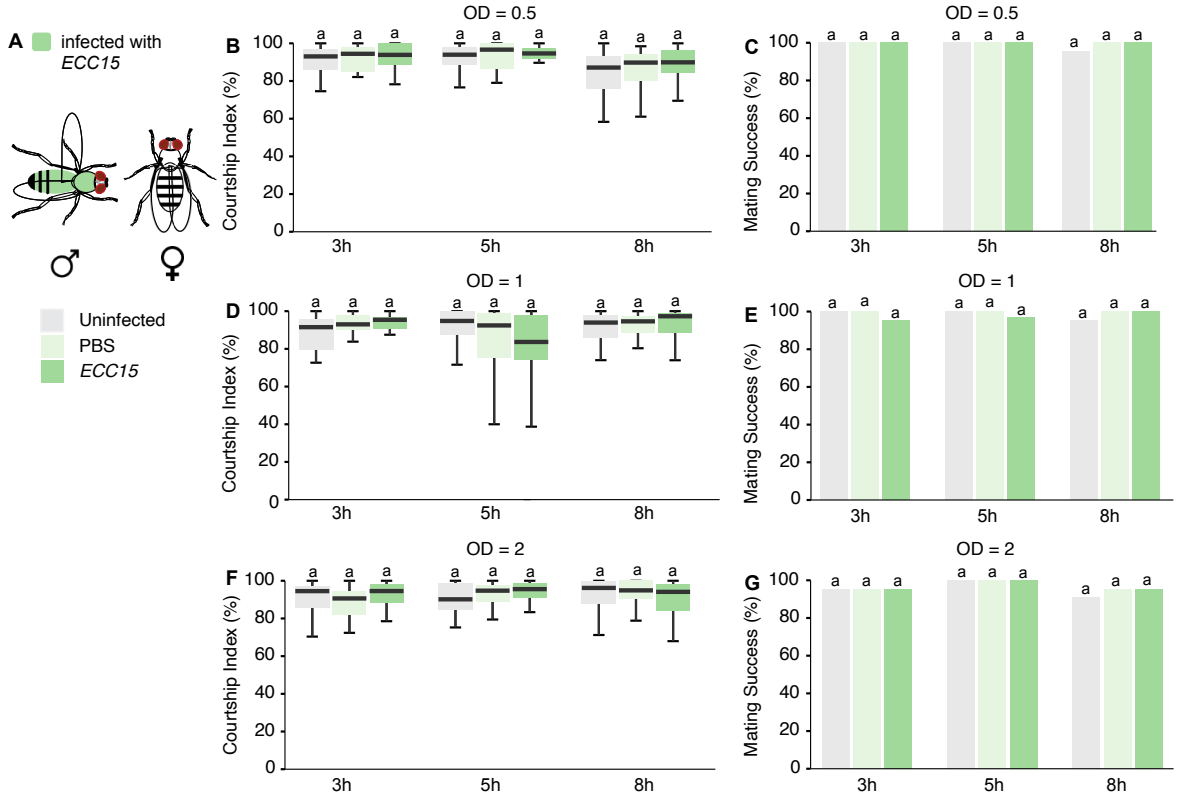


Figure 2.6: Timing of *ECC15* infection and bacterial load has a limited impact on male courtship behaviour in CS wild-type flies. Canton-S male flies received injections of three varying concentrations of *ECC15* solution(OD_{600}=0.5, 1.0, 2.0), after which they were subjected to a single-pair courtship assay alongside uninfected virgin females at 3, 5, and 8 hours following infection. Courtship index and mating success of males infected with *ECC15* (OD_{600}=0.5, A and B, n=21-22; OD_{600}=1.0, C and D, n=21-32; OD600 = 2.0, E and F, n=21-22). (n) indicates number of flies used. Statistical analyses using the Dunn Test in B, D, and F and Fisher Test in C, E and G. Box plots labelled with different letters are statistically significantly different. The figure has been published in Rose et al. 2022 [1] and is used under the terms of the Creative Commons Attribution License.

2.5.5 Males infected with *S. marcescens* decrease their courtship behaviour in time points closer to death

Next, I wanted to explore if different infection timings with a pathogenic strain might reveal a trade-off between immunity and reproductive behaviours. Therefore, I tested *S. marcescens*-infected males in the courtship assay at earlier and later time points (4 and 6 hours post-infection). While CS males displayed robust courtship behaviour at 4 and 5 hours post-infection, I observed a significant decrease in the courtship index of *S. marcescens*-infected males at 6 hours post-infection (H = 21.728, p = 1.913e-05, Fig 2.7B). Moreover, their mating success dropped drastically to 4% (Fig 2.7C). However, these flies were lethargic and displayed a drastic decrease in walking speed (H = 17.154, p = 0.0001884, Fig 2.7D-F), arguing that locomotor defects underlie a decrease in courtship behaviours.

My data suggests that *S. marcescens* infections trigger rapid mortality in flies. Next, I asked if lower doses of *S. marcescens* would have an effect of infection on male courtship behaviour. I thus tested two more infection doses of this bacteria (OD_{600}=0.01 and OD_{600}=0.001). Both doses increased the lifespan of flies only by a few hours suggesting the potency of infections (Fig 2.8A). However, there were still no noticeable effects on pre-copulatory behaviours (H = 0.61564, p = 0.735, Fig 2.8C-D).

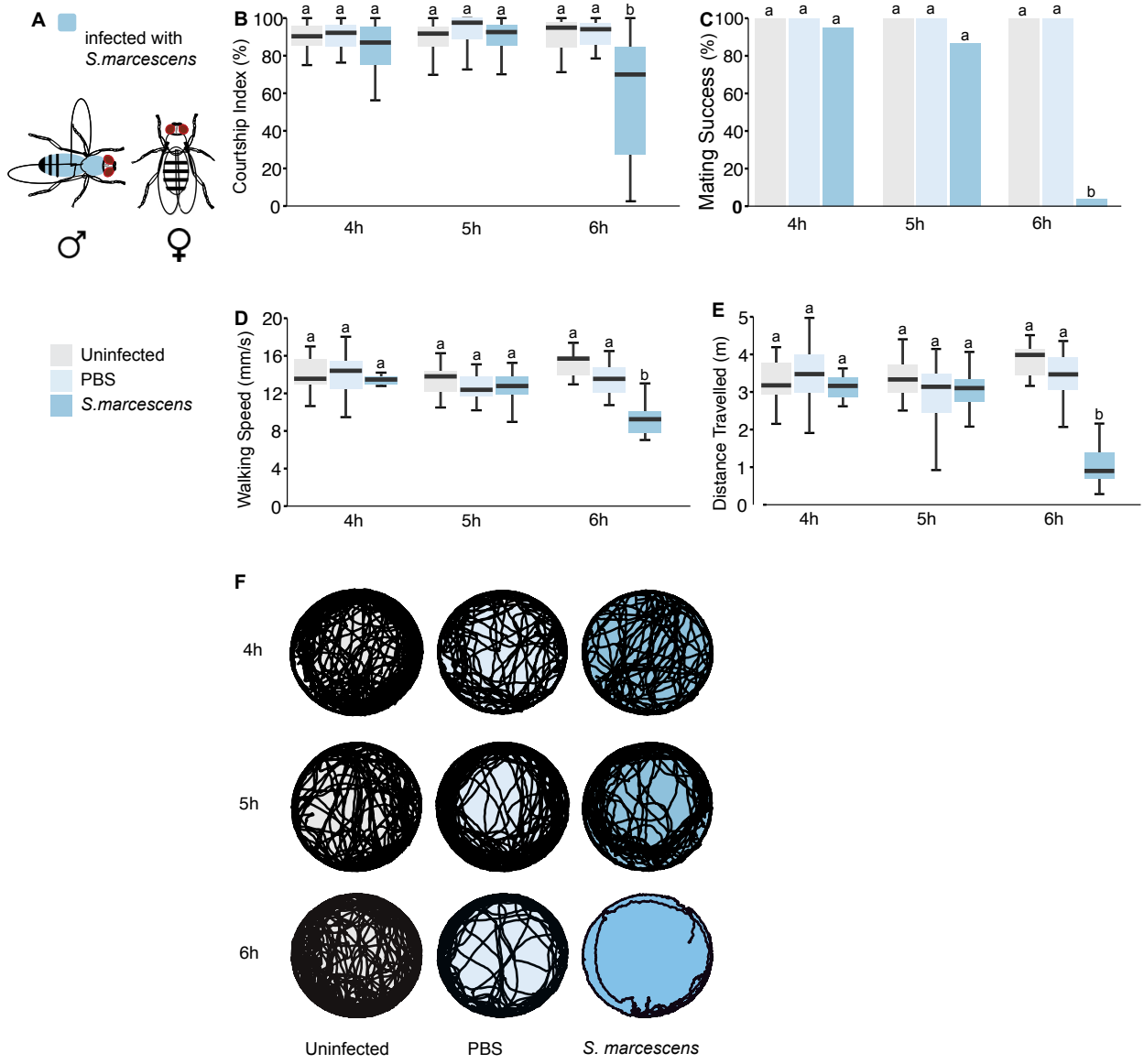


Figure 2.7: Males infected with *S. marcescens* exhibit locomotor defects at 6 h post-infection accompanied by a large decrease in their courtship index and mating success. Male Canton-S flies were injected with *S. marcescens* (OD_{600}=0.1) and tested in a single-pair courtship assay with an uninfected virgin female at three different time points (4, 5 and 6 h post-infection). (A-B) Courtship index and mating success of *S. marcescens*-infected males and their respective controls at different time points (n=20-30). (C-D) Walking speed and total distance travelled within 5 minutes of males infected with *S. marcescens* and their respective controls in a locomotion assay (n=12-36). (F) Trajectories of infected flies and their controls plotted for various time points after infection(4-6hrs). (n) indicates number of flies used. Statistical analyses using the Dunn Test in B, D, and E and Fisher Test in C. Box plots labelled with different letters are statistically significantly different. The figure has been published in Rose et al. 2022 [1] and is used under the terms of the Creative Commons Attribution License.

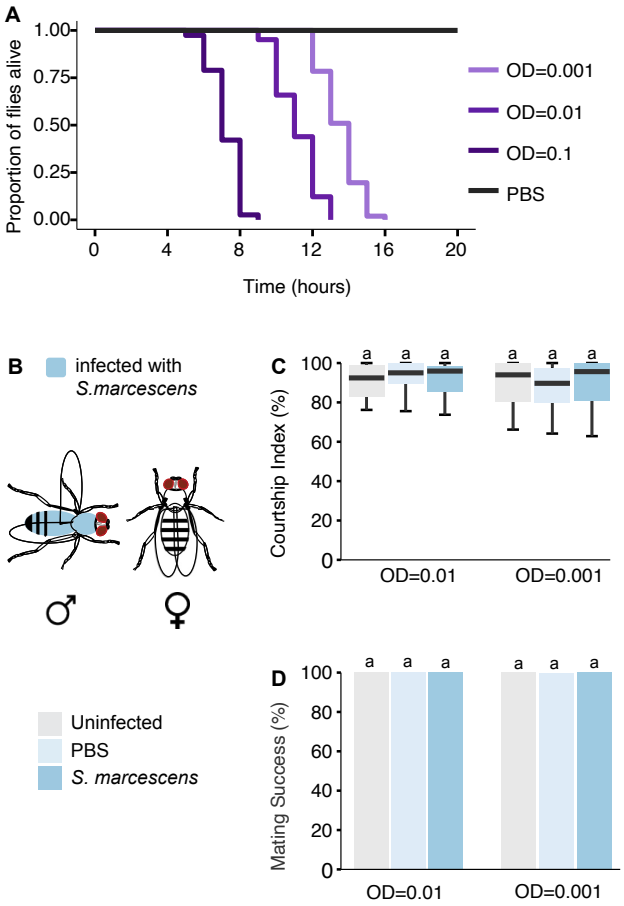


Figure 2.8: Reducing bacterial load has no impact on courtship behaviour in Canton-S males infected with *S. marcescens*. Canton-S male flies received injections of lower concentrations of *S. marcescens* (OD_{600}=0.01 or 0.001), and were subsequently evaluated in survival assays and single-pair courtship assays alongside uninfected virgin females at 5 hours post-infection. (A) Survival curve of Canton-S males flies injected with different doses of *S. marcescens* (n=20-51). (C-D) Courtship index and mating success of males infected with *S. marcescens* and their respective controls (n=21-22). (n) indicates number of flies used. Box plots labelled with different letters are statistically significantly different. Statistical analyses using the log-rank test in A, Dunn Test in C, and Fisher Test in D. The figure has been published in Rose et al. 2022 [1] and is used under the terms of the Creative Commons Attribution License.

2.5.6 Male courtship behaviour remains unaffected upon bacterial infections in wild-type Dahomey flies.

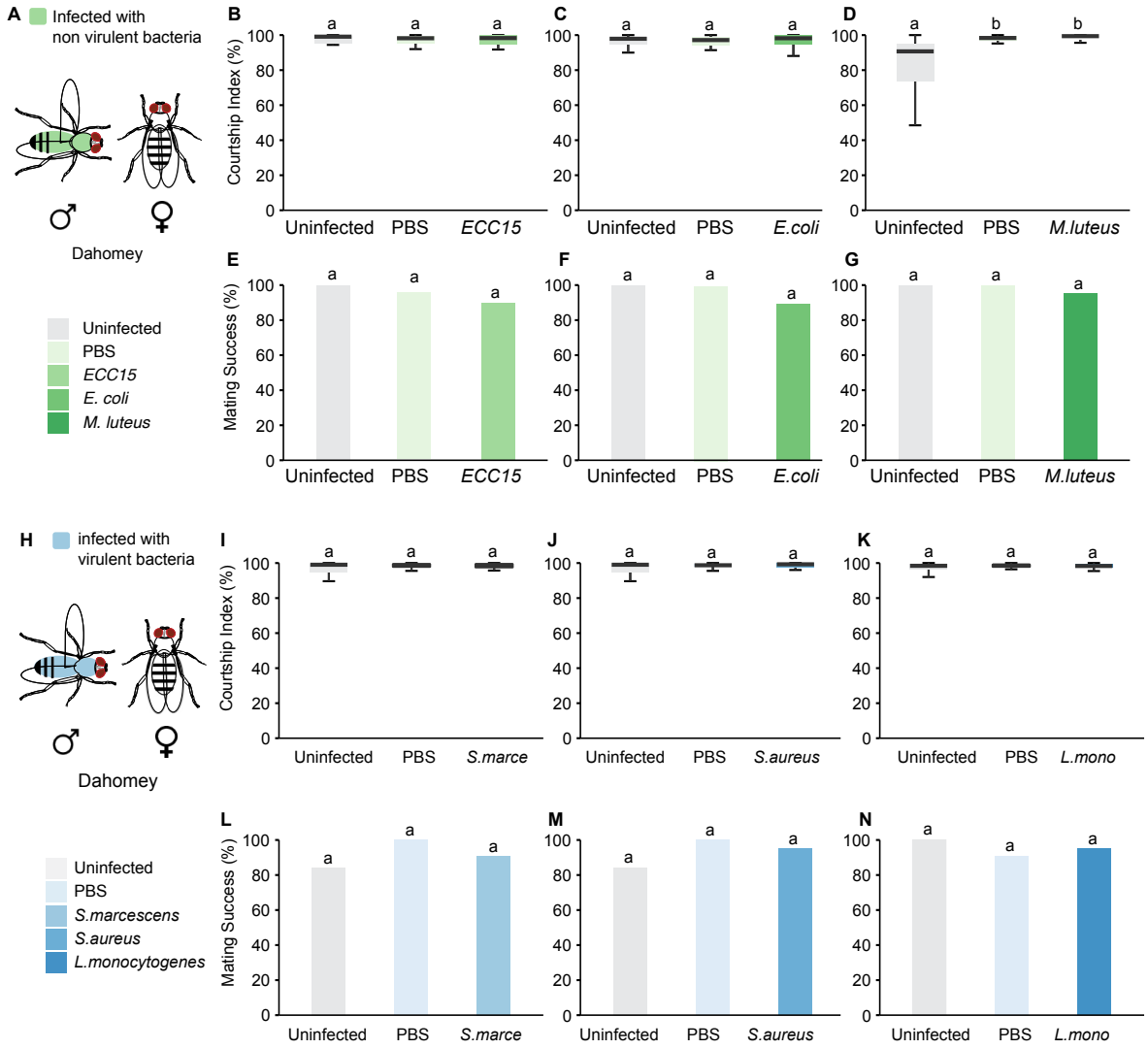


Figure 2.9: Male courtship behaviour remains unaffected upon bacterial infections in wild-type Dahomey flies. (A, H) Male Dahomey flies were subjected to injections with three distinct non-pathogenic and three pathogenic strains and were subsequently assessed using a single-pair courtship assay alongside uninfected virgin females. Courtship index and mating success of males infected with *ECC15* (B and E, n=19-21), *E. coli* (C and F, n=18-20) and *M. luteus* (D and G, n=20-22) and their respective controls. Courtship index and mating success of males infected with *S. marcescens* (I and L, n=20-22), *S. aureus* (J and M, n=19-21) and *L. monocytogenes* (K and N, n=21-22) and their respective controls. (n) indicates number of flies used. Statistical analyses using the Dunn Test in B-D & I-K and Fisher Test in E-G, L-N. Box plots labelled with different letters are statistically significantly different. The figure has been published in Rose et al. 2022 [1] and is used under the terms of the Creative Commons Attribution License.

Next, I asked if these findings were specific to the Canton-S strain or a more generalised phe-

nomenon. To tackle this, I performed behavioural assays with a second wild-type strain Dahomey, isolated from Benin that has spent relatively fewer years in laboratory conditions [160]. I found that, like Canton-S, Dahomey males did not modify their courtship behaviour in response to all the six bacterial infections (Fig 2.9B-G, I-N). These results indicate that courtship behaviours in males are very robust and preserved upon infections and this effect was not strain-specific and generalisable.

2.5.7 Sexual receptivity remains unaltered upon infection in wild-type female flies.

Host response to infections has been shown to be sexually dimorphic in many species, including flies [161, 162]. Furthermore, the costs associated with reproduction vary between the sexes. For example, males invest considerable energy in courting a potential mate while females do not play an active role during courtship. But, females may invest more energy in post-copulatory behaviours like egg-laying. Given that the costs of immunity and reproduction are different between males and females, I wondered if bacterial infections will have a greater impact on female behaviour. To test this, I injected wild-type CS virgin females with the same non-pathogenic bacterial strains (Fig 2.10H). I measured two behavioural parameters that are proxies for female receptivity: copulation latency and mating success (Fig 2.10H). Uninfected CS virgin females readily accept males and copulate within 2-5 minutes. I found that infected females and PBS controls exhibited a higher latency to copulation than uninfected controls (H = 18.8, df = 2, p = 8.274e-05, Fig 2.10B, H = 27.584, p = 1.024e-06, Fig 2.10C, H = 5.9608, p = 0.05077, Fig 2.10D). But, there were no significant differences between PBS controls and infected females, suggesting that the injection technique itself affects copulation latency, but not the bacterial infection (|ECC15-PBS|, p = 1, Fig 2.10B, |*E.coli*-PBS|, p = 1, Fig 2.10C, |*M.luteus*-PBS|, p = 1, Fig 2.10D). Likewise, the infections did not affect the mating success of females. (Fig 2.10E-G). Next, I tested the effects of pathogenic strains *S. aureus*, *S. marcescens* and *L. monocytogenes* in female behaviour. All three pathogens caused a dramatic reduction in female lifespan and there were no sex-specific differences in survival (Fig 2.3D-F). However, similar to males, infection status did not affect latency to copulation in females or mating success (Fig 2.10). Dahomey virgin females infected with non-pathogenic and pathogenic strains showed a similar trend (Fig 2.11). Therefore, female receptivity remains unaffected in flies with bacterial infections.

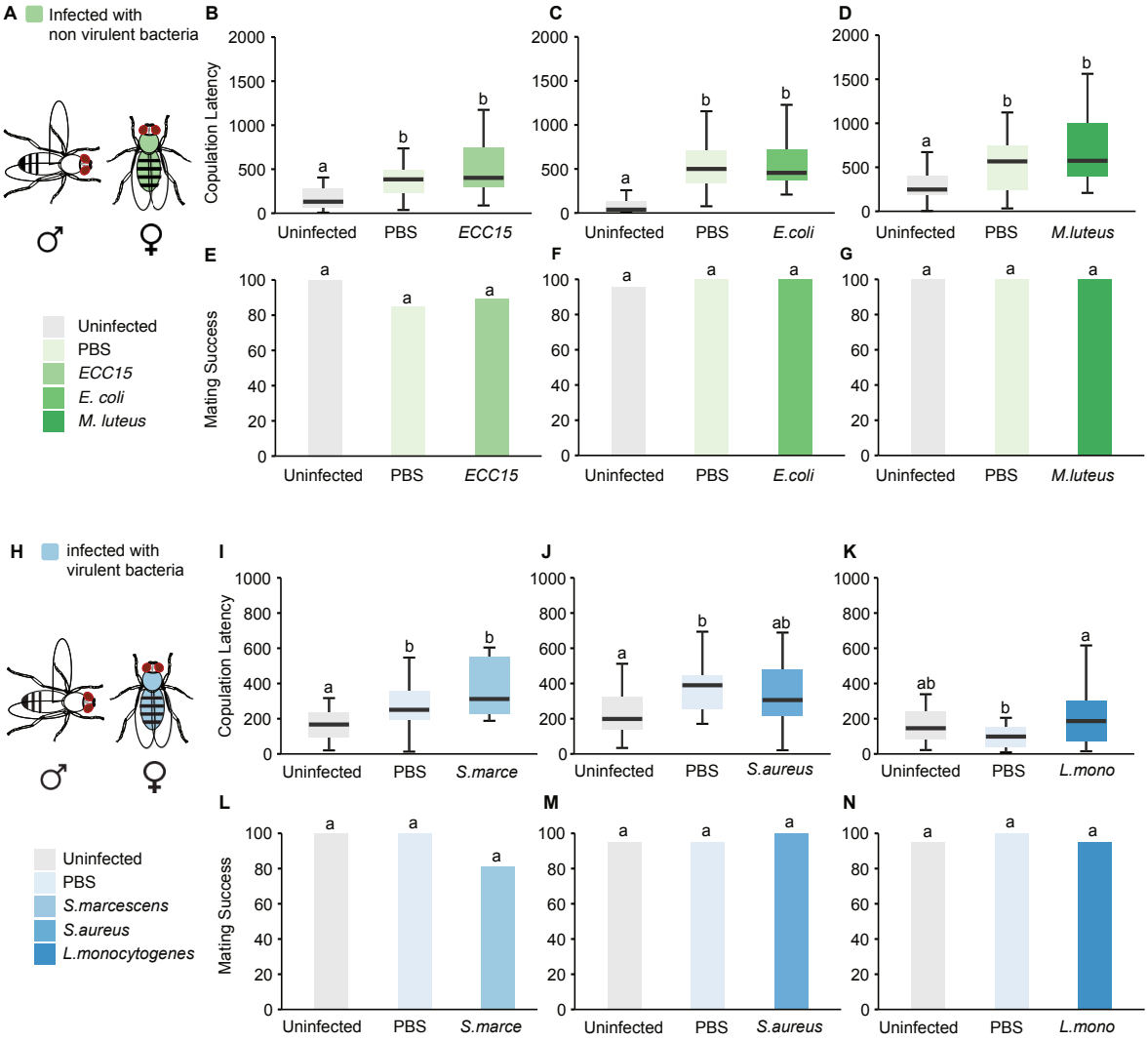


Figure 2.10: Bacterial infections have a limited impact on female sexual receptivity in wild-type Canton-S flies. (A, H) Virgin female Canton-S flies received injections of three distinct non-pathogenic and pathogenic strains and were subsequently examined in a single-pair courtship assay alongside uninfected males. Copulation latency and mating success of females infected with non-pathogenic species *ECC15*(B and E, n=20-24), *E. coli*(C and F, n=20-21), *M. luteus*(D and G, n=19-20) and their respective controls. Copulation latency and mating success of females infected with pathogenic species *S. marcescens*(I and L, n=16-19), *S. aureus*(J and M, n=19-20), *L. monocytogenes*(K and N, n=19-20) and their respective controls. (n) indicates number of flies used. Statistical analyses using the Dunn Test in B-D, I-K and Fisher Test in E- G, L-N. Box plots labelled with different letters are statistically significantly different. The figure has been published in Rose et al. 2022 [1] and is used under the terms of the Creative Commons Attribution License.

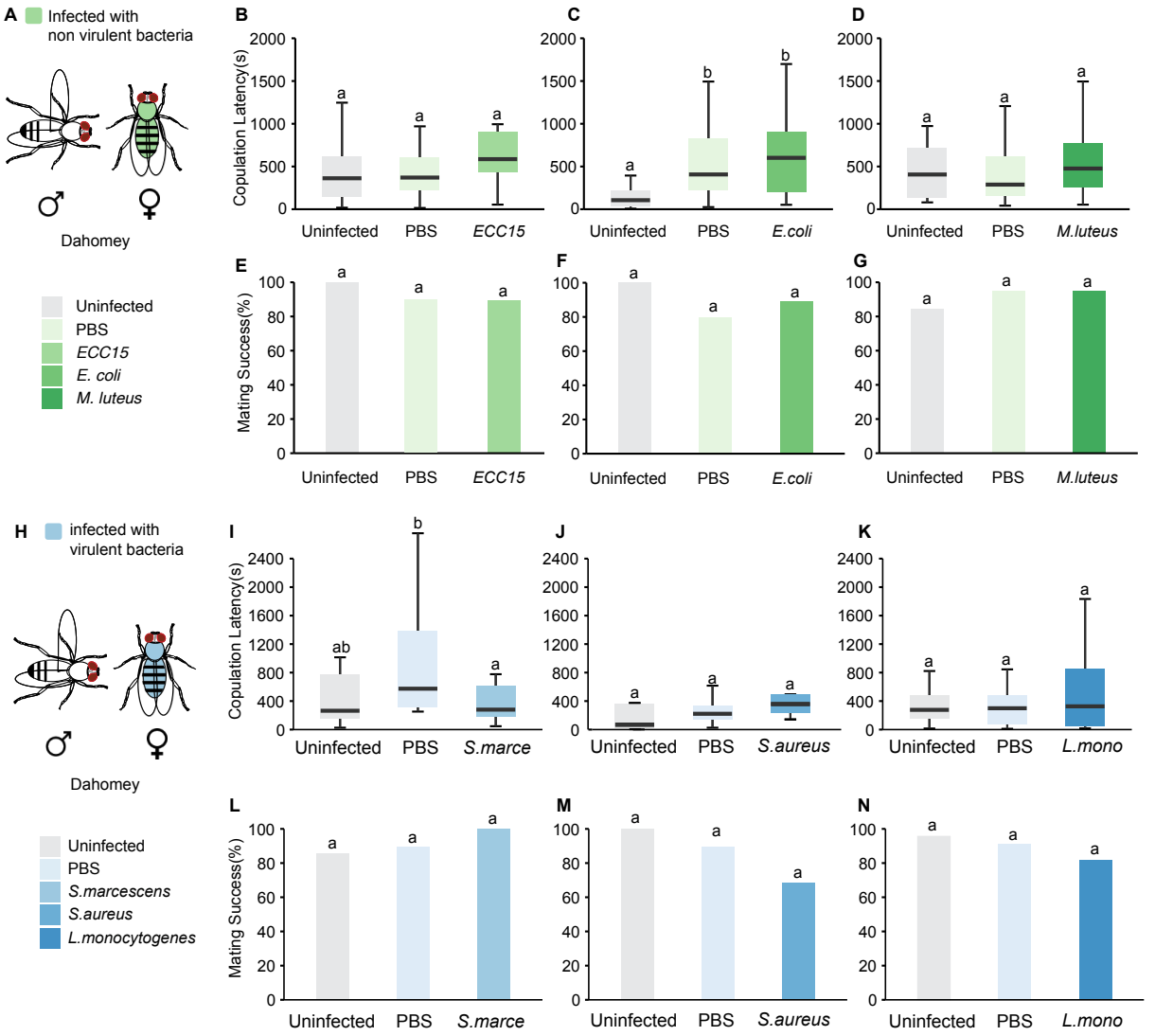


Figure 2.11: Bacterial infections have a limited impact on female sexual receptivity in wild-type Dahomey flies. (A, H) Virgin Dahomey females received injections of three distinct non-pathogenic and pathogenic strains and were subsequently examined in a single-pair courtship assay alongside uninfected males. Copulation latency and mating success of females infected with *ECC15* (D and E, n=18-20), *E. coli* (C and F, n=19-21) *M. luteus* (D and G, n=13-20) and their respective controls. Copulation latency and mating success of females infected with *S. marcescens* (I and L, n=19), *S. aureus* (J and M, n=22-23), *L. monocytogenes* (K and N, n=19-20) and their respective controls. (n) indicates number of flies used. Statistical analyses using Dunn Test in B-D, I-K and Fisher Test in E-G, L-N. Box plots labelled with different letters are statistically significantly different. The figure has been published in Rose et al. 2022 [1] and is used under the terms of the Creative Commons Attribution License.

2.5.8 Wild-type CS flies do not discriminate against mates infected with pathogenic bacteria.

Previous studies have shown that animals can recognise mates infected with pathogens [124, 135]. I thus asked if healthy flies are able to discriminate against infected mates in a competitive mating assay (Fig 2.12A). I performed a choice assay where I presented a CS virgin female with a choice of two mates: a healthy male and an infected male (Fig 2.12A). To my surprise, infected males with *S. aureus* courted to the same level as the uninfected male (p = 0.524, Wilcoxon signed rank test, Fig 2.12B). Moreover, infected males had a similar mating success to the healthy males, suggesting that females do not have a preference for healthy or infected males ($\chi^2 = 0.16981$, p = 0.6803, Fig 2.12C). I next performed the reverse experiment where I presented a healthy male with a choice of healthy or infected female (Fig 2.12D). When given a simultaneous choice, the focal males invested similar amount of time into courting the infected and the control females (p = 0.4817, Wilcoxon signed rank test, Fig 2.12E). They also did not bias their mating towards either healthy or *S. aureus*-infected females ($\chi^2 = 0.94737$, p = 0.3304, Fig 2.12F). I extended my mate choice experiments to a second pathogenic strain, *S. marcescens* and found no effects on courtship or first mating partner (Fig 2.12G-L). These results suggest that flies cannot discriminate against infected mates.

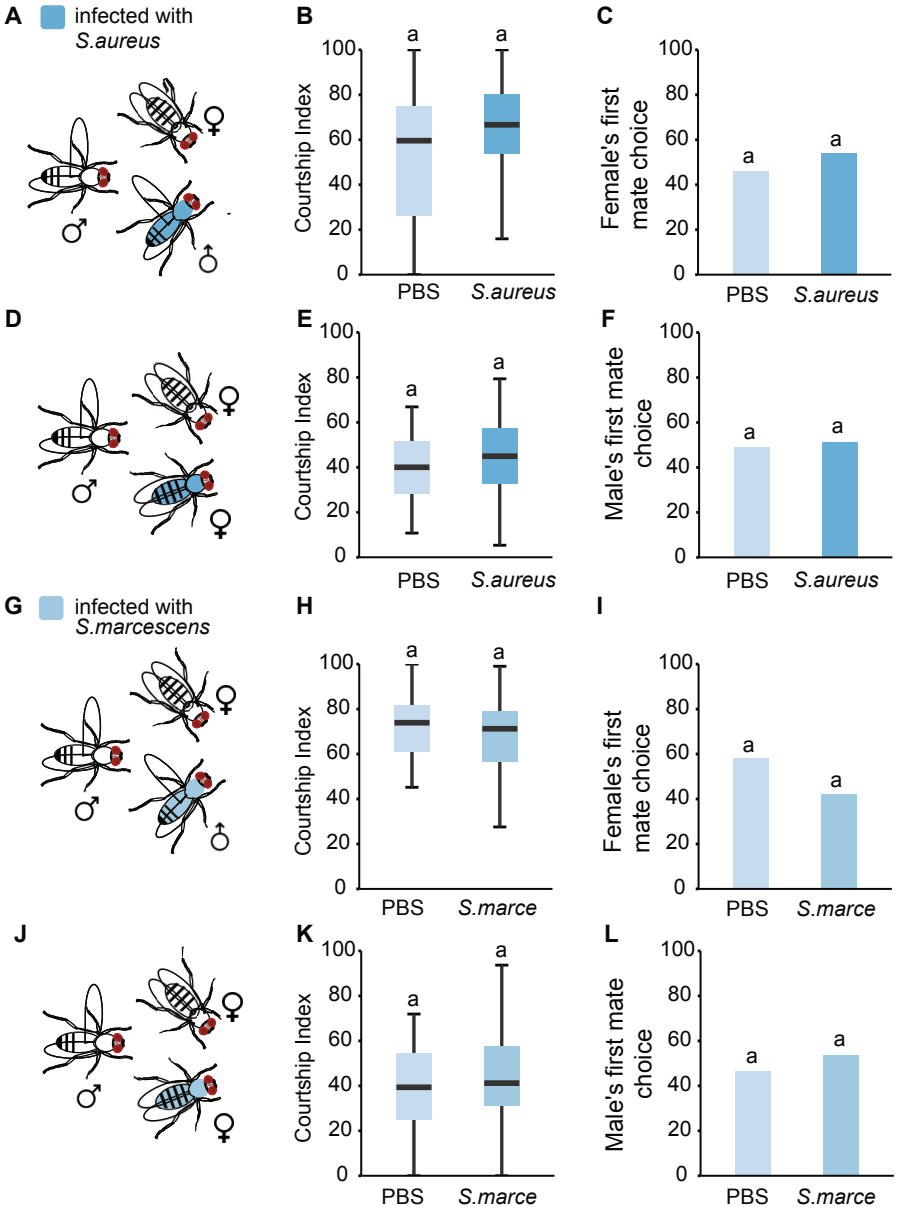


Figure 2.12: Canton-S flies do not discriminate against mates infected with bacteria. (A, G) In a choice experiment, a focal female fly was presented with the option of selecting between a male injected with PBS and a male infected with either *S. aureus*(A) or *S. marcescens*(G). (B, H) Courtship Index of the PBS or infected male towards the focal female (C, I) Female's first mate choice (n = 45-55). (D, J) In a similar choice setup, a focal male had the choice between a PBS-injected female and a female infected with *S. aureus*(G) or *S. marcescens*(J). (E, K) Courtship Index of the focal male (F, L) Male's first mate choice (n = 38-58). (n) indicates number of flies used. Statistical analyses using Wilcoxon test in B, E, H and K and Fisher Test in C, F, I and L. Box plots labelled with different letters are statistically significantly different. Figure has been published in Rose et al. 2022 [1] and is used under the terms of the Creative Commons Attribution License.

2.5.9 Artificial activation of the immune system has a limited impact on reproductive behaviours.

The *Drosophila* immune system detects pathogens mainly by two different pathways: Toll and Imd [131]. Next, I asked if activation of these pathways without bacterial infections itself would affect courtship behaviours. To this end, I chose to genetically activate the immune pathways in the fat body (the primary site of immune activation), using the GAL4/UAS system. I used the C564-GAL4 [163], which mainly targets the fat body to overexpress UAS-Imd [164] or UAS-Toll^{10B} [165]. I found that the activation of the Imd pathway did not significantly affect the courtship behaviour in *C564-GAL4>UAS-Imd* males (H = 2.6248, p = 0.2692, Fig 2.13A-B), (Mating success, p=1, Fisher's Exact Test, Fig 2.13C). Likewise, the activation of the Imd pathway did not have an impact on the copulation latency of *C564-GAL4>UAS-Imd* females (H = 4.5705, p = 0.1017, Fig. 2.13D-E) or their mating success (Fig. 2.13F).

Next, I explored the effects of Toll pathway activation on reproductive behaviours. I expressed the dominant-negative form of Toll protein Toll^{10B} only through adulthood, as the expression of Toll^{10B} disrupts growth and causes developmental lethality [166]. To do this, I used the fly line C564-GAL4 combined with the temperature-sensitive tub-GAL80^{ts} (GAL80^{ts}) [157], which inhibits GAL4. I raised the larvae in low temperatures (18°C) where the GAL4 is inactive. After eclosion, I placed the adults at 31°C, where GAL80 stops suppressing GAL4, thereby allowing the expression of Toll^{10B}. Consistent with the previous results, adult-specific activation of Toll^{10B} did not impact the reproductive behaviours of *C564-GAL4; tub-GAL80^{ts}>UAS-Toll^{10B}* males (H = 1.879, p = 0.3908, Fig 2.13G–I) or females (H = 2.3694, p = 0.3058, Fig 2.13J–L). These findings indicate that inducing artificial activation of the immune system does not influence pre-copulatory behaviours in *Drosophila*.

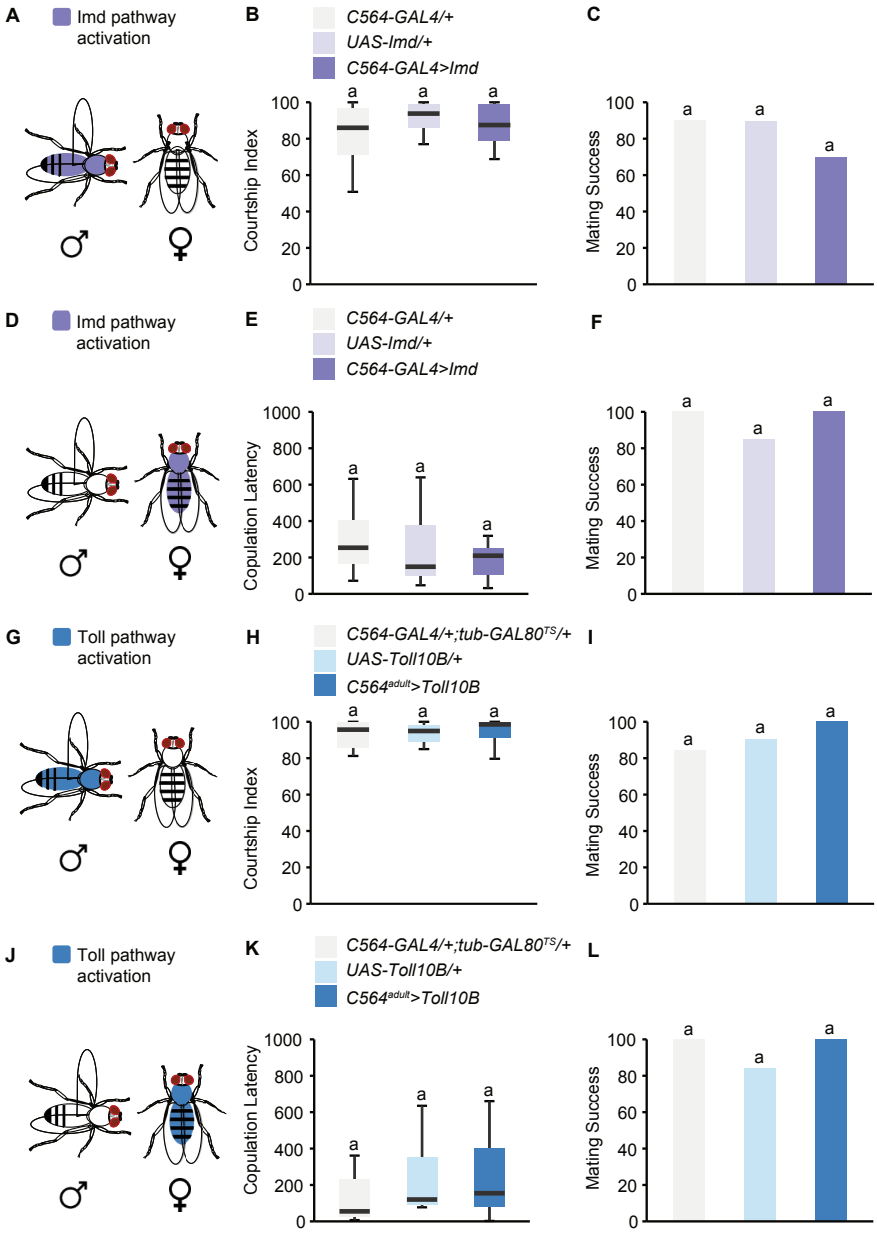


Figure 2.13: Artificial activation of the immune system activation has a limited impact on reproductive behaviours. Toll(G, J) and Imd(A, D) pathways were induced specifically in the fat body of male or virgin female flies and tested in a single-pair mating assay. Courtship index and mating success of *C564-GAL4>UAS-Imd* male flies (B-C, n=19-20) and C*564 GAL4; tub-GAL80TS>UAS-Toll10B* male flies (H-I, n=19-21). Copulation latency and mating success of *C564-GAL4>UAS-Imd* female flies (E-F, n=20-24) and *C564-GAL4; tub-GAL80TS>UAS-Toll^{10B}* female flies (K-L, n=19-20). (n) indicates number of flies used. Statistical analyses using the Dunn Test in B, E, H and K and Fisher test in C, F, I and L. Box plots labelled with different letters are statistically significantly different. Figure has been published in Rose et al. 2022 [1] and is used under the terms of the Creative Commons Attribution License.

2.6 Discussion

2.6.1 Immunity-reproduction trade-off

According to the "life history theory", there exists a trade-off between energetically expensive traits like reproduction and immunity [117]. What happens when an animal is infected? Does it prioritise reproduction over survival? To address this, I systematically investigated the effects of bacterial infections on reproductive behaviour in flies using diverse behavioural paradigms. Using the micro-injection technique, I assessed the effects of six bacterial species on courtship behaviour in males and sexual receptivity in females namely, pathogenic (*S. marcescens*, *S. aureus* and *L. monocytogenes*) and non-pathogenic bacterial strains (*ECC15*, *E. coli* and *M. luteus*). In addition, I studied other parameters that might dictate the trade-off between immunity and reproduction like infection timing, concentration of bacterial solution and genetic background of the host.

Contrary to my expectations, my results show that infection with both pathogenic and non-pathogenic bacterial strains does not lead to changes in courtship behaviour in males of both wild-type strains Canton-S and Dahomey. Similarly, female sexual receptivity remained unaffected upon all six bacterial infections. Genetic activation of the *Drosophila* immune pathways Toll and Imd did not have a significant influence on both male and female behaviours. Finally, altering the infection timing and bacterial concentration had negligible effects on reproduction. These results suggest that males sustain normal levels of courtship and females remain receptive upon immune system activation either by bacterial infection or genetic activation. Similarly, Keesey et al. observed there were no observable changes in courtship when male flies were infected with *Pseudomonas entomophila* [113]. Khan and Prasad showed that infection with *S. marcescens* did not alter the receptivity of wild-type females [135].

Studies in frogs and male crickets have also found that courtship abilities remain unaffected in the presence of infection [167, 168]. For example, Drayton et al. showed that field cricket *Teleogryllus commodus* injected with LPS did not take longer to court the females [167]. Tree frogs

infected with a deadly fungus *Batrachochytrium dendrobatidis* showed similar calling abilities to that of uninfected males. In contrast, other reports have revealed a trade-off between immunity and reproductive behaviour where infected animals show a decrease in reproductive activity post-infection. Male hobubara bustards injected with LPS showed a drastic reduction in courtship activity until they recovered from infection [109]. Differential energetic costs associated with courtship and immunity across different species could account for the behavioural differences in response to infections.

Interestingly, infections have been shown to negatively impact other behaviours in *Drosophila*. For example, flies infected with *L. monocytogenes* decrease their food intake [169]. Kuo et al. showed that infection with gram-negative bacteria increased sleep in flies [170]. Flies infected with the gram-positive bacteria *Micrococcus luteus* show a transient increase in activity until the bacteria are cleared [171]. Furthermore, infections significantly alter post-copulatory traits in *Drosophila*. Kurz et al. showed female flies infected with *E. coli* transiently decreased their egg-laying [122]. They showed that peptidoglycan (PGN) derived from bacteria activates octopaminergic neurons via the NF-KB signalling pathway, thereby arresting egg release from the ovaries. Similarly, activation of the immune system by PGN reduced sperm viability in *D. melanogaster* males [172].

In addition, infections are known to disrupt metabolism in flies. Activation of the toll pathway or infection with pathogens like *M.luteus* and *L. monocytogenes* has been shown to influence the insulin pathway and to limit nutrient storage [166, 173]. To maintain normal levels of courtship, flies might have to invest more energetic resources into reproduction which can be construed as a terminal investment [103]. However, while I observed the sustenance of courtship behaviours, my behavioural experiments did not reveal an increased expression of these reproductive traits. Therefore, pre-copulatory behaviours remain conserved, even flies are subjected to rapidly lethal bacterial infections that incur huge metabolic costs.

2.6.2 Infection and mate choice

Mate choice is one of the most important decisions an animal has to make. Animals have been shown to avoid mating with partners infected with pathogens to increase their fitness [124]. For example, female birds use plumage colouration as a reliable indicator of the health of their mate [124]. Studies done in rodents have shown females use olfactory cues to distinguish between infected and healthy mates.

Similarly, Khan et al. showed that male fruit flies bias their mating against mates infected with *S. marcescens* [135]. By contrast, my study shows that healthy and infected female flies with *S. marcescens* are equally chosen as partners in a choice context. There are a few differences in my approach: I used a different strain of *S. marcescens (DB11)* that was more virulent. In addition, I used a method based on micro-injections for delivering bacteria to flies which is more reliable and consistent in producing infection phenotypes. While these differences could account for the dissimilar results, additional evidence suggests that it is very unlikely that males show a bias against the infected individual. Keesey et al. showed that infection with *S. marcescens* leads to the production of methylated pheromones which would render the infected individuals more attractive [113]. This would translate to a mating bias towards infected mates. However, despite the increase in pheromone production, they too observed no differences in mate preference. Therefore, altogether my studies show that Canton S flies don't discriminate against mates infected with *S.marcescens* and *S. aureus*. Although I didn't expand my research to include the other four pathogens, I don't anticipate that they will produce positive results in favour of mate discrimination.

Despite the huge fitness costs of mating with an infected partner, why haven't flies evolved to discriminate against infected mates? Studies have even shown that despite having a shared evolutionary history between the host and pathogen, the infection may not lead to the evolution of mate choice. For example, *Drosophila innubila* is under high selection pressure from *Wolbachia* i.e. up to 45 % of the flies in the *D.innubila* population harbour *Wolbachia* [139]. The presence of *Wol-*

bachia bacteria seems to be detrimental to flies due to the embryonic male-killing effect [139]. Given the high prevalence of *Wolbachia* and high fitness costs, one would expect the evolution of male mate choice. However, Sullivan and Janenike found no evidence of male preference for uninfected females [139]. Similar effects are seen in the butterfly, *Acraea encedon* [174]. Authors argue that selection pressure imposed by the pathogen might either be fluctuating or recent [174], therefore there was not enough time for mate choice to evolve. Similarly, it is possible that the pathogens of my choice do not affect a large proportion of the *Drosophila* in the wild. Therefore, flies might have not evolved mate choice against infected individuals or have lost it in due course of laboratory breeding.

2.6.3 Limitations

> *"It is not his possession of knowledge, of irrefutable truth, that makes the man (or woman) of science, but his persistent and recklessly critical quest for truth."*
>
> Karl Popper, The Logic of Scientific Discovery, 1959

To study the effects of bacterial infection on reproductive behaviours in flies, I employed the micro-injection technique as a means of introducing the infection. While the micro-injection technique allows for precise control over the bacterial dosage, it may not accurately reflect the natural infection process that occurs in the wild. In natural settings, flies primarily acquire bacterial infections through ingestion from contaminated food sources or via damage to their cuticles. This natural mode of infection may result in a slower progression of the disease compared to the systemic infection induced by injection into the haemocoel. Additionally, the injection protocol required anaesthetising the flies using carbon dioxide (CO_2), which significantly alters fly behaviour. Previous studies have demonstrated that CO_2 exposure increases copulation latency

in females, and this effect can persist for up to two days [175]. Similarly, I observed that males exposed to CO_2 took longer to initiate courtship, and virgin females took longer to mate. While I made efforts to minimise CO_2 exposure during the injection process by reducing the pressure to 0.1 bar and limiting the duration of exposure, the impact of CO_2 on behaviour remained substantial. This confounding variable could make it challenging to detect subtle changes in mating success that may be influenced by the infection. Moreover, the chosen pathogens in my study caused rapid fly mortality (within a day) or triggered a transient activation of the immune system. Given this narrow infection window, it may be difficult to detect the effects of infection on courtship behaviour, as the influence of CO_2 exposure might overshadow any potential effects caused by the infection itself.

Secondly, I used lab-bred wild-type strains in my thesis i.e. Canton-S and Dahomey that have been bred in the laboratory for 100 years. It is worth noting that these flies may not encounter pathogens commonly found in natural environments and may have consequently lost certain behavioural responses to them over time. Therefore, it is possible that wild-caught *D. melanogaster* flies or other *Drosophila* species might exhibit different behavioural patterns in response to pathogens.

Furthermore, I performed the courtship assays in small circular chambers (20mm in diameter). This experimental setup does not fully replicate the natural reproductive biology of flies. In the wild, female flies have abundant space and exhibit behaviours such as decamping, that may not be adequately captured in the confined laboratory setting [35]. Finally, I manually annotated courtship behaviour using BORIS software throughout the project. However, this process could have been automated by using the Caltech fly-tracker, which tracks the fly's body and wings, and subsequently feeding the tracking data into machine learning software such as the Janelia Automatic Animal Behaviour Annotator (JAABA) to classify the behaviour automatically. This automated approach would have allowed for more efficient and objective behavioural analysis, saving time and minimising potential biases associated with manual annotations.

2.6.4 Summary

In this Chapter, I used bacterial infections to answer two main questions: (1) how infected flies change their reproductive behaviours, and (2) how healthy flies choose between infected and healthy mating partners. I developed a reliable infection protocol and tested the effects of six different bacterial infections on fly reproductive behaviour. The results show that infected flies maintain their usual level of male courtship and sexual receptivity. Moreover, flies do not appear to discriminate against infected mates.

3 Feeding-Courtship Behavioural Choice

3.1 Abstract

Hunger is one of the most powerful motivations for animal behaviour. But, how brains integrate hunger to make these crucial choices remains an enigma. To understand the mechanistic basis of behavioural choices, our laboratory developed a novel assay to investigate how fruit flies weigh the competing demands of feeding and courtship, which are both essential for their survival and reproduction. We found that sex-deprived fed flies prioritised courtship over feeding. Conversely, after 24 hours of starvation, the flies chose to feed before initiating courtship. We identified tyramine, the invertebrate analogue of norepinephrine, to be an important modulator of this behavioural choice. Tyramine steers the choice towards courtship in well-fed flies by activating P1 neurons, while simultaneously suppressing the feeding-promoting TyrR neurons. In contrast, the perception of food has the opposite effect, diminishing the activity of P1 neurons but activating the TyrR neurons. Our results highlight the role of antagonistic co-regulation of neurons for behavioural prioritisation in accordance with the internal state of the animal. This work has been published in Cheriyamkunnel, Rose et al. 2021 where I am the co-first author [2].

3.2 Introduction

Hunger is one of the most powerful motivations in the animal kingdom [176]. If an animal struggles to meet its energetic or nutritional requirements, it may experience a decline in health, immunity or even mortality in extreme cases. Therefore, food deprivation/starvation can have huge fitness consequences on the animal [176]. To combat that, animals have evolved a set of behavioural changes with their primary motivation shifting towards the acquisition of food. For example, hungry animals exhibit increased activity/foraging levels and heightened sensitivity to food-related chemical cues [176]. Hunger can also lead to changes in social behaviour as animals may choose to invest less in reproductive behaviours [176]. Similarly, hungry animals may become more competitive about their territory and show more aggressive behaviours towards their competitors [176]. In extreme cases, animals are willing to take greater risks in order to obtain food, which can negatively impact survival and reproductive success [176].

But, now what happens when an animal is deprived of both food and sex? Should it prioritise searching for resources to feed or look for a mate? Although food deprivation poses a threat to the animal's survival, mating is crucial for the propagation of genes and ensuring the continuation of a species. How do animals strike a balance between these two conflicting needs? Previous studies have looked into how starvation affects reproductive behaviours in animals. For example, food-deprived scorpionflies are less likely to copulate than their fed counterparts [176]. Similarly, Churchill et al. explored the role of starvation on reproductive behaviours either in male or female fruit flies. The authors found that female flies reduced their sexual receptivity upon starvation. In contrast, starved male flies exhibited high levels of courtship, regardless of the level of starvation, when presented with well-fed mating partners. [177]. Yet how starvation affects the choice between feeding and reproduction remains unexplored.

Drosophila is an excellent model system to tackle this question as both feeding and courtship behaviours have been meticulously characterised. Male fruit flies display a robust courtship ritual in order to woo their potential mate [45]. They use visual and chemical cues to assess the

suitability of their mate which are processed and integrated by neurons expressing the sex determination genes doublesex (*dsx*) and fruitless (*fru*) [45]. This information is sent to male-specific P1 neurons, that serve as a central node to initiate robust courtship behaviour in males [45].

Drosophila feeding behaviour is a series of dynamic behavioural sub-programs enabling these flies to efficiently forage and consume food until satiation. [57]. The neuromodulators (such as serotonin, leucokinin, DILPs, etc.) and neural circuitry responsible for these behavioural modules have been extensively studied [57]. Despite this progress, it remains unclear how the fly chooses between feeding and courting. Moreover, very little is known about the underlying neural mechanisms.

3.2.1 Objectives

Utilising the robust genetic and behavioural tools offered by the *Drosophila*, our lab aimed to explore the neural mechanisms responsible for the selection between feeding and courtship behaviours. In *Drosophila melanogaster*, feeding and courtship behaviours have been studied extensively, but only in isolation. How do these two crucial behaviours interact? What happens when a conflict arises between two behaviours? The three specific aims of the project are as follows:

1. To develop a behavioural assay to understand how flies choose between feeding and mating behaviours.

2. To understand how internal and external factors affect this behavioural choice.

3. To decipher the neural mechanism that governs the choice between feeding and mating.

3.3 Methods

3.3.1 Fly stocks and husbandry

The wild-type flies used in the Chapter belonged to the Canton-S strain. Transgenic lines used in this Chapter are given below (Table 8). All flies were raised on a standard cornmeal diet at 25°C (Table 5), 50-60% humidity with a 12 h light-dark cycle. Virgin male and female flies were collected <1 day after eclosion. Males were socially isolated and aged for 5-7 days before experimentation. Canton-S or Heat shock females were decapitated and used as courtship targets.

Number	Strain	Source
1	Canton-S	BL 64349
2	UAS TNT	BL 28837
3	UAS-dTrpA1	BL 26264
4	TyrR-GAL4	BL 67129
5	UAS-TAR1 RNAi	BL 28332
6	UAS-TAR2 RNAi	BL 25857
7	UAS-TAR3 RNAi	BL 27670
8	71G01-GAL4	BL 39599
9	P1-split GAL4	David Anderson Lab [38]
10	UAS-GCaMP6s	BL 42749
11	UAS-ASAP2	BL 65414
12	UAS MCD8:EGFP	BL 32185

Table 8: List of transgenic fly trains used in Chapter 3

3.3.2 Feeding-Courtship behavioural choice assay

Preparation of 100mM sucrose solution: A mixture of 0.17g of sucrose and 0.05g of agar was added to 4.5 ml of water. The solution was heated until the agar was melted and 0.5 ml of 4% (w/v) Brilliant Blue FCF dye was added. The final concentration of the solution was as follows: 100 mM sucrose, 1% (w/v) agar and 0.4% (w/v) Brilliant Blue FCF dye.

Starvation protocol: Fed males were transferred into vials with 1% agar with filter paper for 24 hours for starvation. The water in the agar ensures that the male flies remain hydrated and we only see the behavioural effects of starvation and not thirst.

Preparation of behavioural chamber: 20 µl of 100 mM sucrose solution was added in the centre of a 2 cm wide courtship chamber. Freshly decapitated CS/Heat shock virgin female was placed near the inner wall of the chamber so as not to touch the food. Individual male flies were released into the chamber and their behaviour was recorded for a time period of 15 minutes using a Sony DCR-HC38 camera.

Quantification of Behaviour: A male fly's first behavioural choice was classified as courting or feeding if the behaviour was consistently displayed for a duration of at least 5 seconds. Based on this binary categorisation, a group variable Courtship Preference Index (CPI) was calculated for each replicate of at least 10 flies:$(n^{\text{court first}} - n^{\text{feed first}})/n^{\text{total}}$. Flies that did not come into contact with the food patch were omitted from the analysis due to their lack of interaction with the sucrose-based food source, which is a non-volatile substance. Behavioural Observation Research Interactive Software (BORIS) was used to create ethograms of behaving flies. Based on the ethogram, custom R scripts were used to calculate the percentage of flies performing a behaviour.

3.3.3 FlyPAD assays for measuring food intake

Male flies (well-fed or 24h starved) were gently placed into flyPAD feeding arenas using an aspirator. These arenas were prepared with 5 µl of 100 mM sucrose containing 1% agarose, and the flies were given a 30-minute window to feed within these setups, as outlined by Itskov et al. in 2014 [58]. Throughout this feeding period, the fly behaviour was simultaneously captured using a Sony DCR-HC38 camera. Flies that did not make contact with the sucrose in the initial 20 minutes of observation were not considered in the subsequent analysis. The software developed by Itskov and colleagues was used to compute the total number of sips for all flies [58].

3.3.4 Thermogenetic activation of neurons

All thermogenetic neuronal activation experiments were performed at 28-30°C on a heat block. Both the experimental male flies and their corresponding genetic control counterparts were reared at 18°C until the time of experimentation. Prior to experimentation, the males were pre-heated

at 30°C for 10 minutes in an incubator after which they were subjected to the behavioural choice assay. As temperature control, fed flies of the identical genotype were subjected to testing at 22°C.

3.3.5 Two-photon live imaging

Virgin males were raised individually for 4-6 days post-eclosion, either with food (fed) or without (starved). After 24 hours, they were immobilised, and their head capsule was opened for imaging. Buffer composition for fed males is as follows: 103 mM NaCl, 3 mM KCl, 5mM N-Tris, 10 mM trehalose, 10 mM glucose, 7mM sucrose, 26 mM NaHCO3, 1mM NaH2PO4, 1.5 mM CaCl2, 4mM MgCl2, osmolarity 275 mOsm, pH 7.3. For starved males, a sugar free version of the buffer was used. Excitation was achieved using a Ti-Sapphire laser emitting 140 fs pulses at an 80 MHz repetition rate, centred on 910 nm. Images were subsequently acquired at a rate of 5.92 Hz.

For tyramine or octopamine experiments (1 mM or 5 mM), solutions were prepared and perfused through the fly's head capsule. Two-photon fluorescence images were manually segmented and analysed using custom Fiji and MATLAB scripts. For each stimulus response, the baseline fluorescence (F0) was established by averaging the fluorescence (F) during the initial 4 seconds of recording. Following this 4-second period, the mean fluorescence of the GCaMP signal was measured over a 90-second duration.

In sucrose feeding experiments, flies were prepared similarly, and a sucrose drop was placed near the fly. The baseline fluorescence was established, and the pre-feeding condition was recorded. Sucrose was then presented to the proboscis for 60 seconds, followed by its removal, and recording continued. Analysis included only flies visibly consuming sucrose.

3.3.6 Data visualisation

Courtship preference indices (CPI) are represented as dot plots with mean and standard error of the mean (SEM). Imaging data are represented in the form of boxplots with the lower and upper hinges representing the first and third quartiles.

3.3.7 Statistics

All statistical analyses was performed using R (version 3.6.3) or GraphPad (Prism). The normality of the data sets was assessed and P values were calculated accordingly. Non-parametric tests including Mann-Whitney or Kruskal-Wallis tests followed by Dunn's multiple comparison test were used for compare the behavioural data. Paired T-tests/Wilcoxon tests were used to compare the imaging data. The R package 'effsize' was used to the calculate the cliff's delta. Statistical differences indicated using asterisks: * $p< 0.05$, ** $p<0.01$ and *** $p<0.001$.

3.4 Results

3.4.1 Behavioural choice assay to study the conflict between feeding and courtship

In our laboratory, a team, led by a master's student named Sherry Cheriyamkunnel, designed a behavioural paradigm to delve into the neural mechanisms that govern behavioural choices, specifically focusing on the conflict between feeding and courtship. In this paradigm, virgin male flies were given a choice to feed from a nutritious food source (100 mM sucrose solution) or to court a decapitated virgin female, an attractive courtship target (Fig 3.1A). To quantify the behavioural choice, our team introduced a metric known as the Courtship Preference Index (CPI), which considered the behavioural priorities of the group of flies being tested:($n^{\text{court first}}$ – $n^{\text{feed first}}$)/n^{total}. A positive CPI value indicates that the group of flies showed a preference towards initiating courtship first while a negative value indicates a preference towards feeding behaviour.

Firstly, we sought to ask if the internal state of the fly affected the choice between feeding courtship. To this end, we tested males that were fed and food-deprived for 24h in our behavioural choice assay. Interestingly, 97% of sex and food-deprived males prioritised feeding over courtship (Courtship Preference Index [CPI] 0.8, Fig 3.1B). In contrast, 87% of fed males choose to initiate courtship first (CPI +0.7, Fig 3.1B, p= 0.0015, Wilcoxon test). To test if this behavioural choice depended on nutrient quality, we replaced the food source with another nutritive sugar (D-glucose). Here again, we observed similar results where food-deprived flies preferentially chose to feed from a nutritive food source rather than court a female (CPI 0.7 versus +0.7, Fig 3.1C, p = 0.0012, Wilcoxon test).

Next, we wanted to understand the temporal dynamics of the behaviour of male flies during the assay. Do male flies only perform one behaviour or do they distribute their time between the two behaviours? In particular, would starved flies switch to courtship after feeding? To this end, we created ethograms of the behaviour of individual male flies throughout the behavioural choice assay for a time period of 15 minutes. From the ethograms, we extracted the data of all flies

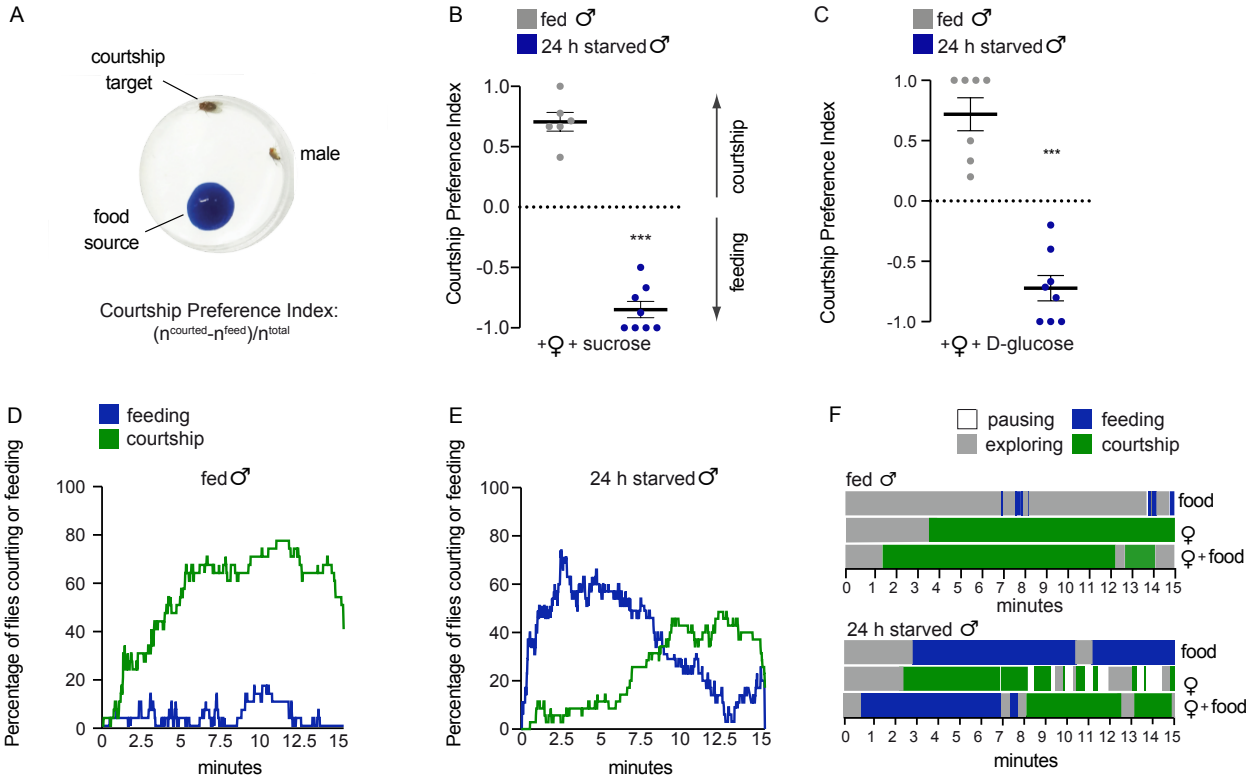


Figure 3.1: A new behavioural choice assay to study the conflict between feeding and courtship. (A) Both 24-hour starved and well-fed Canton-S male flies were presented with a choice between consuming a 100 mM sucrose solution and engaging in courtship behaviour with a decapitated virgin female. (B) The quantification of this behavioural choice was done using the parameter Courtship Preference Indices (CPI) calculated for both 24-hour starved and fed males during the choice between sucrose consumption and courtship (sample size: 52-56 flies). (C) CPI values were also obtained from flies offered a choice between D-Glucose, another nutritive sugar, and a decapitated virgin female (sample size: 29-35 flies). (D) Ethograms were constructed to visualise the behavioural dynamics during a 15-minute observation period, illustrating how flies alternated between feeding, courting, or engaging in both behaviours when presented with sucrose, a female, or both. (E and F) Temporal dynamics of behaviours were analysed, expressed as the percentage of flies displaying either courtship or feeding during 1-second time intervals (sample size: 30-35 flies). * p< 0.05, ** p<0.01 and *** p<0.001 calculated using Mann-Whitney U tests for data in B-C, absence of a * indicates no significant difference. Figure published in Cheriyamkunnel et al [2] and is used under the terms of the Creative Commons Attribution License.

using a custom R script and created charts representing the % of behaviour exhibited over time. We found that fed flies mainly displayed courtship throughout the observation time of 15 mins (Fig 3.1D-F). Conversely, starved males initially engaged in feeding for approximately four and a half minutes before shifting their focus to courting the decapitated female for an additional four minutes (Fig 3.1D-F). This behavioural switch to courtship was observed in 70% of starved

flies, suggesting that it is robust and generalisable across the population. Interestingly, the male flies devoted their time equally towards feeding and courtship behaviours (29% and 25%, respectively). Therefore, our results suggest that fed males prioritise courtship over feeding, however, this behavioural choice is plastic and is dependent on the hunger status of the fly (Fig 3.1).

3.4.2 Tyramine receptor neurons (TyrR) are important for the feeding-courtship behavioural choice

To probe into the neural mechanism of the behavioural choice, we performed a screen where we inhibited the activity of candidate neurons (i.e. neurons expressing neuropeptides and their receptors that control feeding/courtship behaviours including dNPF, SIFamide, Corazonin, OA, Dilp2 and 3, Hugin, Leucokinin, 5-HT, TyrR and AstA) using tetanus toxin which blocks synaptic neuro-transmission. Out of all the potential candidates, we found that inhibiting tyramine receptor neurons significantly altered the behavioural choice in starved males. While both genetic controls chose to initiate feeding first, a larger proportion of TyrR>TNT males prioritised courtship behaviour (CPI= 0.01 (TyrR>TNT), 0.9 (TyrR GAL4/+) and 0.6 (UAS TNT/+), Kruskal-Wallis chi-squared = 15.44, df = 2, p-value = 0.0004439, Fig 3.2A). Inactivating TyrR neurons in fed flies did not lead to any observable changes in behaviour (Kruskal-Wallis chi-squared = 3.5521, df = 2, p-value = 0.1693, Fig 3.2B).

If inhibition of TyrR neurons in starved males tipped the behavioural prioritisation towards courtship, we next wondered if activation of TyrR neurons would lead to the opposite effect i.e. tip the balance towards feeding. To activate the neurons, we sought to use a Ca^{2+} channel dTrpA1 that is directly activated by high temperatures (>28 °C), thereby allowing us to activate specific neurons temporally. We, therefore, activated TyrR neurons in fed males during the behavioural choice assay and quantified their behaviour. Consistent with the previous result, activation of TyrR neurons in fed males led to a decrease in courtship preference at 28 degrees when compared to genetic controls (CPI= 0.5 (TyrR>TrpA1), 0.4(TyrR GAL4/+) and 0.4(UAS TrpA1/+), at 28°C, H=9.591652, p=8.26E-03, Fig 3.2C). This effect was absent at the control temperature of 22°C where fed males behaved similarly to that of controls (CPI= +0.9(TyrR>TrpA1), 0.8(UAS TrpA1/+) and 0.6(TyrR GAL4/+) at 22°C, Figure Fig 3.2C). Furthermore, artificial activation of TyrR neurons did not significantly affect the behaviour of starved males (H=4.778, p=0.0917, Fig 3.2D).

Next, we explored the behaviour dynamics of manipulation of TyrR neurons during the be-

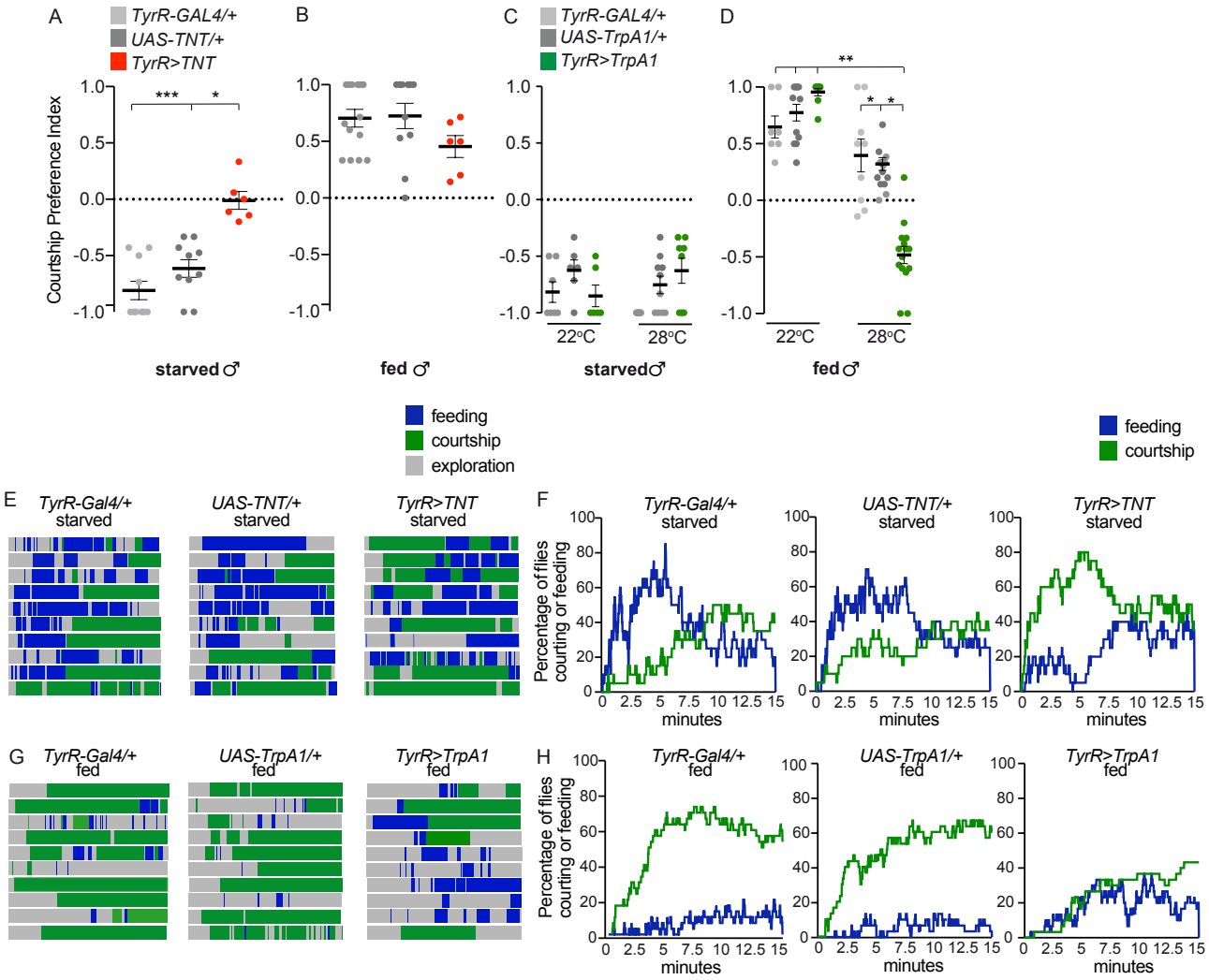


Figure 3.2: Tyramine receptor (TyrR) are important for the feeding-courtship behavioural choice. (A and B) Courtship preference indices of 24-h starved (A, sample size: 65–106 flies) or fed (B, sample size: 53–93 flies) TyrR>TNT males subjected to feeding-courtship behavioural choice. (C and D) Courtship preference indices of 24-h starved (C, sample size: 21–59 flies) or fed (D, sample size: 34–147 flies) TyrR>TrpA1 males, were allowed to choose between feeding and courting. (E-H) Representative ethograms and temporal dynamics of flies where TyrR neurons have been inhibited (E-F) and activated (G-H). * p< 0.05, ** p<0.01 and *** p<0.001 calculated using Kruskal Wallis followed by Dunn's post hoc multiple comparison test for data in A-D, absence of a * indicates no significant difference. Figure published in Cheriyamkunnel et al [2] and is used under the terms of the Creative Commons Attribution License.

haviour choice assay by looking at the ethogram data. Starved TyrR>TNT flies performed courtship behaviour for seven and a half minutes before switching to feeding while the starved control flies prioritised feeding over courtship for the first half of the behavioural choice assay (Fig 3.2E-F). This suggests that inhibition of TyrR neurons does not entirely eliminate feeding

behaviour per se but instead tilts the initial behavioural preference in favour of courtship. Conversely, we found that activating these TyrR neurons led to a significant decrease in courtship initiation during the choice scenario (Fig 3.2G-H).

3.4.3 Tyramine and sucrose ingestion differentially modulate the activity of TyrR^{PLP} neurons only in starved males

Our previous results suggest that TyrR neurons are crucial mediators of behavioural choice favouring feeding over courtship. In particular, activation of TyrR neurons tipped the balance towards feeding initiation. Our next objective was to pinpoint specific clusters within this neuronal population that are crucial for the behavioural choice. TyrR GAL4 labels approximately 50 cells within the central brain, which can be organised into four distinct clusters: the posterior lateral protocerebrum (PLP), the superior medial protocerebrum (SMP), the gnathal ganglia (GNG), and the inferior posterior slope (IPS). While Huang et al. showed that IPS cluster is important for courtship behaviour, work done in our lab found that manipulation of these neurons did not yield a phenotype in the behaviour choice assay (data not shown in the thesis, please refer to [2]).

To further narrow down the clusters, we performed two-photon calcium imaging in collaboration with Scott Wadell's lab at the University of Oxford. To measure the response to tyramine, we monitored the basal calcium levels using GCaMP6s, a genetically-encoded Ca^{2+}sensor, expressed under the control of the TyrR GAL4 driver [178]. Surprisingly, clusters TyrR^{SMP} or TyrR^{GNG} neurons did not show a change in activity levels in response to the tyramine application (data not shown). In contrast, the TyrR^{PLP} cluster showed a strong decrease in activity levels in starved flies (Fig 3.3A, C). This effect was absent in fed flies, possibly due to high levels of endogenous tyramine (Fig 3.3B, D). We confirmed these results using another genetic tool: the ASAP2 voltage indicator. Consistent with GCAMP imaging results, the ASAP2 signal diminished in response to tyramine in the Tyr^{PLP} cluster only in starved males suggesting that tyramine indeed inhibits Tyr^{PLP} neurons (Fig 3.3E-H). Additionally, we knocked down the TAR3 tyramine receptor using RNAi and found that a decrease in inhibitory effect of tyramine on TyrR^{PLP} neural activity (Fig

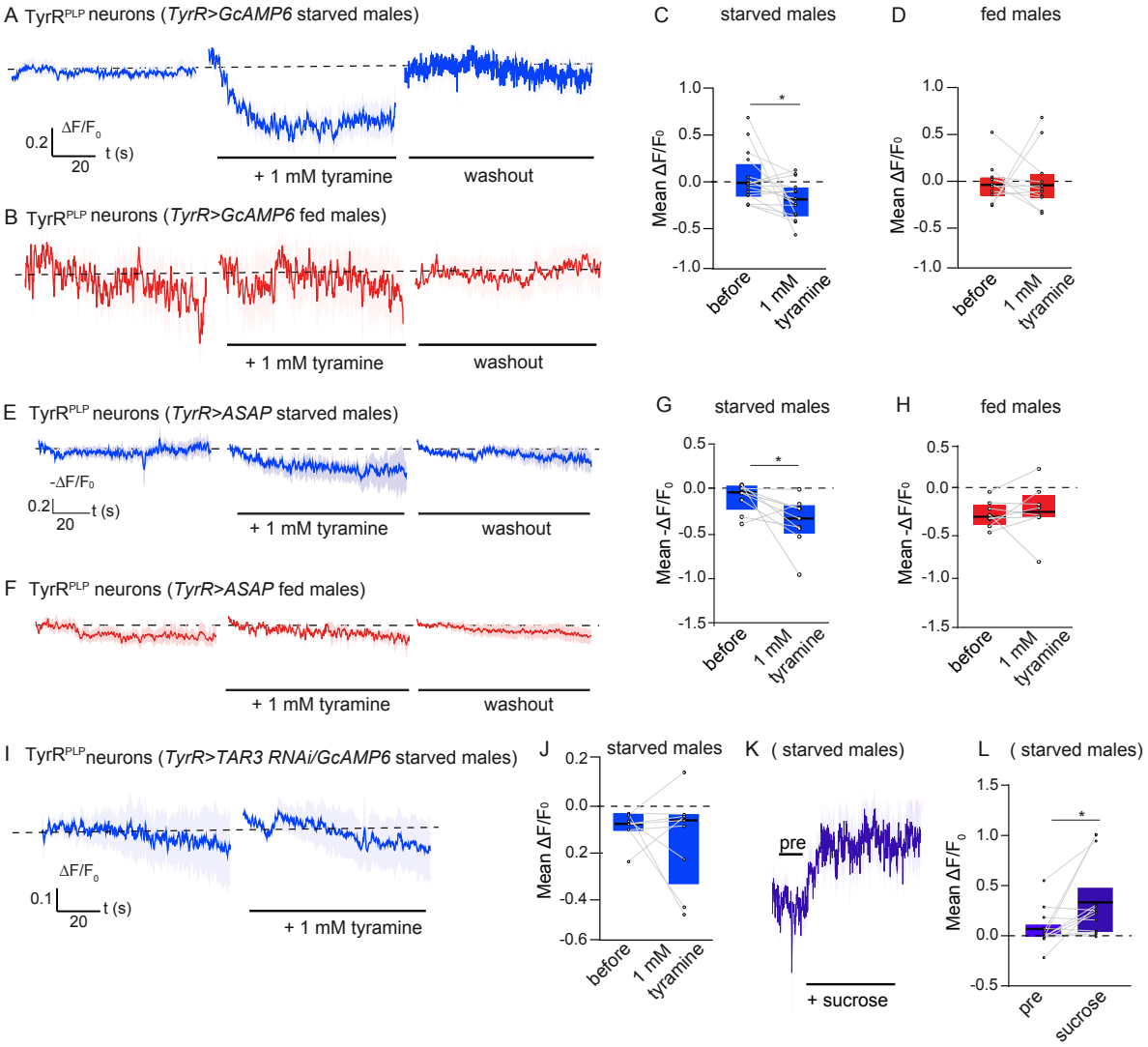


Figure 3.3: TyrR ^{PLP} neurons are activated upon sucrose perception but inhibited by tyramine in starved males. (A and B) A notable decrease in the Ca^{2+} levels of TyrR^{PLP} neurons is observed upon tyramine application only in starved (A) but not fed (B) males and their quantification (C and D, sample size: 11-16 flies). (E and F) Hyperpolarisation in TyrR^{PLP} neurons upon tyramine application in starved (E, blue) males and their quantification (G and H, sample size: 8-9 flies). (I and J) Down-regulation of tyramine receptor (TAR3I) levels in starved TyrR>TAR3 RNAi males did not change the mean Ca^{2+} levels in response to tyramine application when compared to baseline (sample size: 9 flies). (K) In starved males, an increase in Ca^{2+} in TyrR^{PLP} neurons is observed upon sucrose perception (sample size: 11 flies). * p< 0.05, ** p<0.01 and *** p<0.001 calculated using paired t test in (C) and (D); Wilcoxon matched-pairs signed rank test in G, H, J, and L, absence of a * indicates no significant difference. Figure published in Cheriyamkunnel et al [2] and is used under the terms of the Creative Commons Attribution License.

3.3I-J).

We next wondered if activity levels of TyrR could be modulated by the presence of food sources. To this end, we presented the male flies with 100 mM sucrose while simultaneously measuring the GCaMP6 fluorescence levels of $TyrR^{PLP}$ neurons. Interestingly, $TyrR^{PLP}$ neurons displayed an increase in GCaMP6 signal in response to sucrose ingestion, but not to water, arguing for the specificity of the effect (Fig 3.3K-L). These results suggest that $TyrR^{PLP}$ neurons are inhibited by tyramine but activated by food perception.

3.4.4 TyrR ^{PLP} neurons are crucial for the feeding-escape behavioural choice

To further narrow the $TyrR^{PLP}$ cluster, we used the AstC GAL4 driver that labels two bilaterally symmetric neurons in the PLP cluster along with other neurons. Co-labelling showed that $TyrR^{PLP}$ is indeed Ast C positive (Fig 3.3M-N). Furthermore, inhibiting AstC neurons using TNT tipped the balanced towards courtship over feeding in 24h starved males when compared to controls recapitulating the effects of TyrR>TNT(CPI= +0.1(AstC>TNT), 0.7(AstC GAL4/+) and 0.9(UAS TNT/+), Fig 3.3O). This further augments the crucial role of Tyr^{PLP} neurons in feeding-courtship behavioural choice. Our findings suggest that TyrR neurons might be inhibited in fed flies favouring courtship over feeding. However, in starved flies, this inhibition is decreased due to lower levels of tyramine, thereby tipping the balance towards feeding.

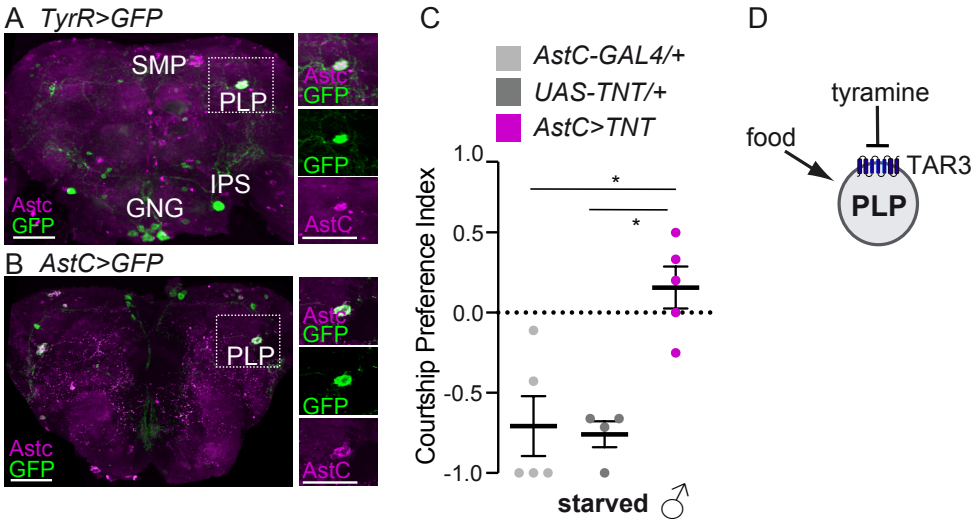


Figure 3.4: TyrR ^{PLP} neurons are crucial for the feeding-escape behavioural choice. (A) Expression pattern of TyrR-GAL4 in the male adult brain. Anti-GFP (green) and anti-AstC (magenta) staining are shown. (B) Expression pattern of AstC-GAL4 (II) in the male adult brain. Scale bar, 50 µm. (C) CPI of 24h starved AstC-GAL4(II)>TNT males and the controls presented with the feeding-courtship behavioural choice (sample size: 26–29 flies). (D) Working model. TyrR^{PLP} neurons' activity levels are antagonistically modulated by tyramine and sucrose perception. * p< 0.05, ** p<0.01 and *** p<0.001 calculated using Kruskal Wallis followed by Dunn's post hoc multiple comparison test for data in C, absence of a * indicates no significant difference. Figure published in Cheriyamkunnel et al [2] and is used under the terms of the Creative Commons Attribution License.

3.4.5 Hunger changes tyramine levels via Tdc2 and Tβh enzymes

We investigated the influence of starvation on tyramine levels in adult flies by assessing expression levels of enzymes involved in tyramine synthesis: Tβh and Tdc2. Tdc2 enzyme converts tyrosine into tyramine while Tβh converts tyramine into octopamine [70]. We observed a 40% reduction in Tdc2 mRNA levels (Fig 3.5A). Immunofluorescence analysis using an anti-TDC2 antibody showed a similar in TDC2 protein levels across all Tdc2 clusters in the fly brain (Fig 3.5C). In contrast, Tβh mRNA levels increased by approximately 60% after 24 hours of starvation (Fig 3.5B). To further test if the tyramine levels change upon starvation, we employed high-performance liquid chromatography (HPLC) in collaboration with Patrick J. Moynihan from University of Birmingham. Consistent with gene expression data, we observed a 30% reduction in brain tyramine levels upon starvation (Fig 3.5D).

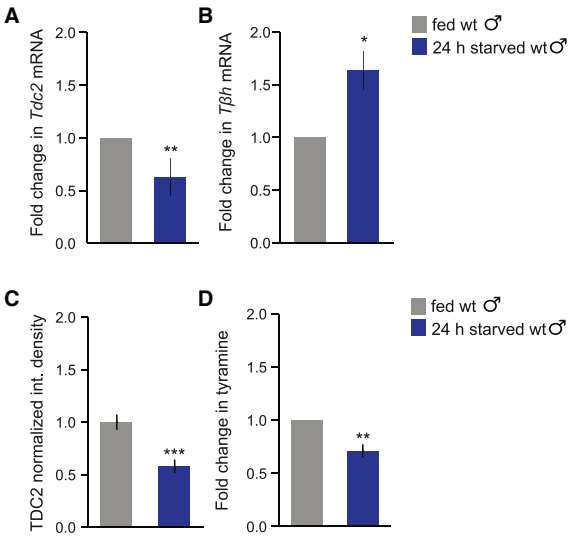


Figure 3.5: Hunger modulates tyramine biosynthesis in the brain via tdc2 and tbh enzymes. (A-B) Tdc2 and Tbh mRNA levels and (normalized to Rp15 mRNA) in 24-hour-starved male brains compared to non-starved males (n=18; 4 replicates).(C) GFP fluorescence under Tdc2-GAL4 control normalized to average fed fluorescence (n=18; 4 replicates).(M) Tyramine levels quantified by reverse-phase HPLC, expressed as starved/fed ratio (n=4, 40 heads per replicate). * p< 0.05, ** p<0.01 and *** p<0.001 calculated using Mann-Whitney U test for data in A-D, absence of a * indicates no significant difference. Figure published in Cheriyamkunnel et al [2] and is used under the terms of the Creative Commons Attribution License.

3.4.6 P1 courtship command neurons are important for feeding-courtship behavioural choice.

Having focused on the feeding-promoting neurons, we next enquired about which elements of the courtship circuit are involved in behavioural choice. We, therefore, focused our efforts on P1 neurons which are a central node in the male *Drosophila* brain that receives input from female cues and initiates male courtship [45]. To test if P1 neurons play a role in the behavioural choice, we activated P1 neurons in 24h starved flies using TrpA1 and tested them in our assay. Starved genetic controls, as expected, prioritised feeding over courtship. In contrast, P1>TrpA1 flies displayed an increased courtship preference at 28 degrees (CPI= 0 (P1>TrpA1), 0.6 (P1 Split GAL4/+), 0.6 (UAS TrpA1/+), Fig 3.6A-B). In line with this, starved P1>TrpA1 males also showed reduced food intake when compared to the controls.

We next tested if tyramine could potentially act on P1 neurons to modulate the feeding-escape behavioural choice (Fig 3.6A-B). We, therefore, knocked down the tyramine receptor TAR3 using RNAi in P1 neurons using 71G01 driver. We improved the efficiency of the RNAi tool by combining the GAL4 line with UAS Dicer. We found that P1>TAR3 RNAi flies showed a decrease in courtship preference when compared to the fed controls, suggesting that tyramine could potentially activate P1 neurons (CPI= 0.4(71G01-GAL4; dicer>TAR3 RNAi), 1(71G01 GAL4/+), 1((UAS TAR3 RNAi/+;dicer/+), Kruskal-Wallis chi-squared = 8.061, p-value = 0.01777, Fig 3.6D).

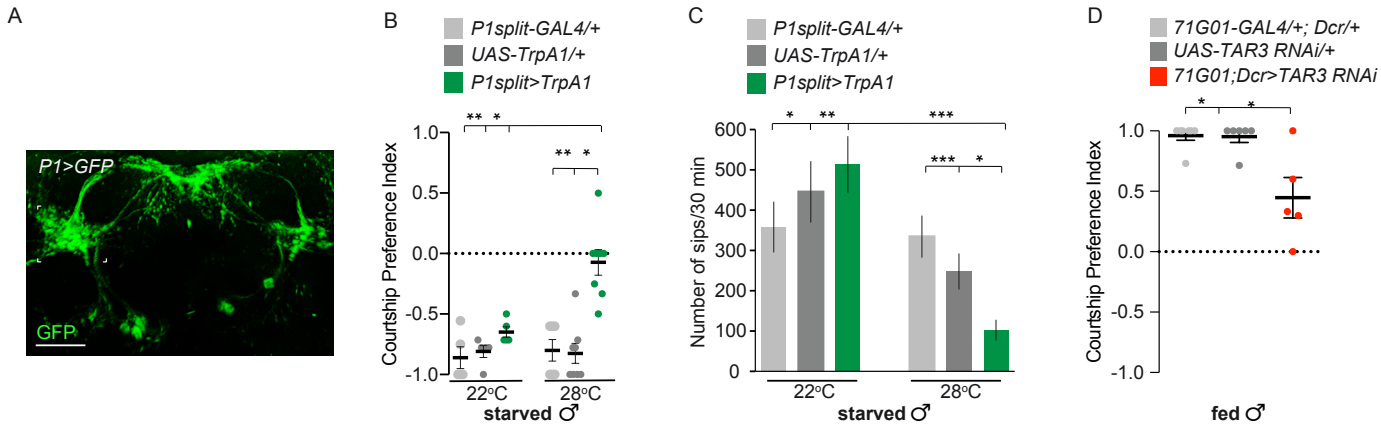


Figure 3.6: P1 courtship command neurons are important for feeding-courtship behavioural choice. (A) Expression pattern of P1 GAL4 in the adult brain. Green, anti-GFP. Scale bar, 50 µm. (B) Activation of P1 neurons increases courtship initiation in 24h starved males. CPI of 24h starved P1>TrpA1 and genetic controls at 28°C (sample size: 48–65 flies) and control temperature 22°C (sample size: 47–74 flies). (C) Activation of P1 neurons decreases food intake in P1>TrpA1 starved male flies measured using flyPAD at 28°C (sample size: 65–80 flies) and 22°C (sample size: 25–37 flies). (D) Down-regulation of TAR3 receptor using RNAi in P1 neurons alters behavioural choice. CPI of fed 71G01-GAL4; Dicer>UAS-TAR3 RNAi males and genetic controls subjected to feeding-escape behavioural choice (sample size: 18–40 flies). * p< 0.05, ** p<0.01 and *** p<0.001 calculated using Kruskal- Wallis test followed by Dunn's post hoc multiple comparison in B-D, absence of a * indicates no significant difference. Figure published in Cheriyamkunnel et al [2] and is used under the terms of the Creative Commons Attribution License.

3.4.7 Tyramine and sucrose ingestion antagonistically influence the activity of P1 neurons

Having tested the behavioural effects of P1, we next moved to live imaging experiments. We first tested if P1 neuronal activity is modulated by tyramine application using the 71G01-GAL4 driver. Interestingly, P1 neurons showed an increase in activity in response to tyramine application in both fed and starved males, which contrasts with the inhibitory effect of tyramine on $TyrR^{PLP}$ neurons (Fig3.7A-D). Given that the knockdown of TAR3 in P1 neurons altered behavioural choice in fed flies, we sought to determine whether TAR3 receptor in P1 plays responds to tyramine. Our imaging experiments revealed that 71G01>TAR3 RNAi flies exhibited a smaller, albeit significant, increase in P1 neuron activity upon tyramine application compared to controls (Cliff's delta= 0.396 (71G01>TAR3 RNAi), 0.969 (71G01 GAL4/+), Fig 3.7E-F), suggesting that TAR3 may be involved in this process. However, given that the down-regulation of TAR3 receptors in P1 neurons did not entirely eliminate the response to tyramine, we hypothesise that other tyramine receptors could be at play. Finally, we found ingestion of sucrose decreased the activity levels of P1 neurons in starved males (Fig 3.7G-H).

Altogether, tyramine and food perception have opposing roles on TyrR and P1 neurons. Tyramine inhibits TyrR neurons while sugar ingestion activates them. Conversely, tyramine activates P1 neurons while sugar perception inhibits them. Moreover, our HPLC data indicates that hunger levels modulate tyramine levels, with starved flies exhibiting lower tyramine levels when compared to well-fed flies. Therefore, these findings highlight how opposing regulatory mechanisms allow flies to efficiently combine their hunger status with external signals, helping them exhibit the most suitable behavioural response.

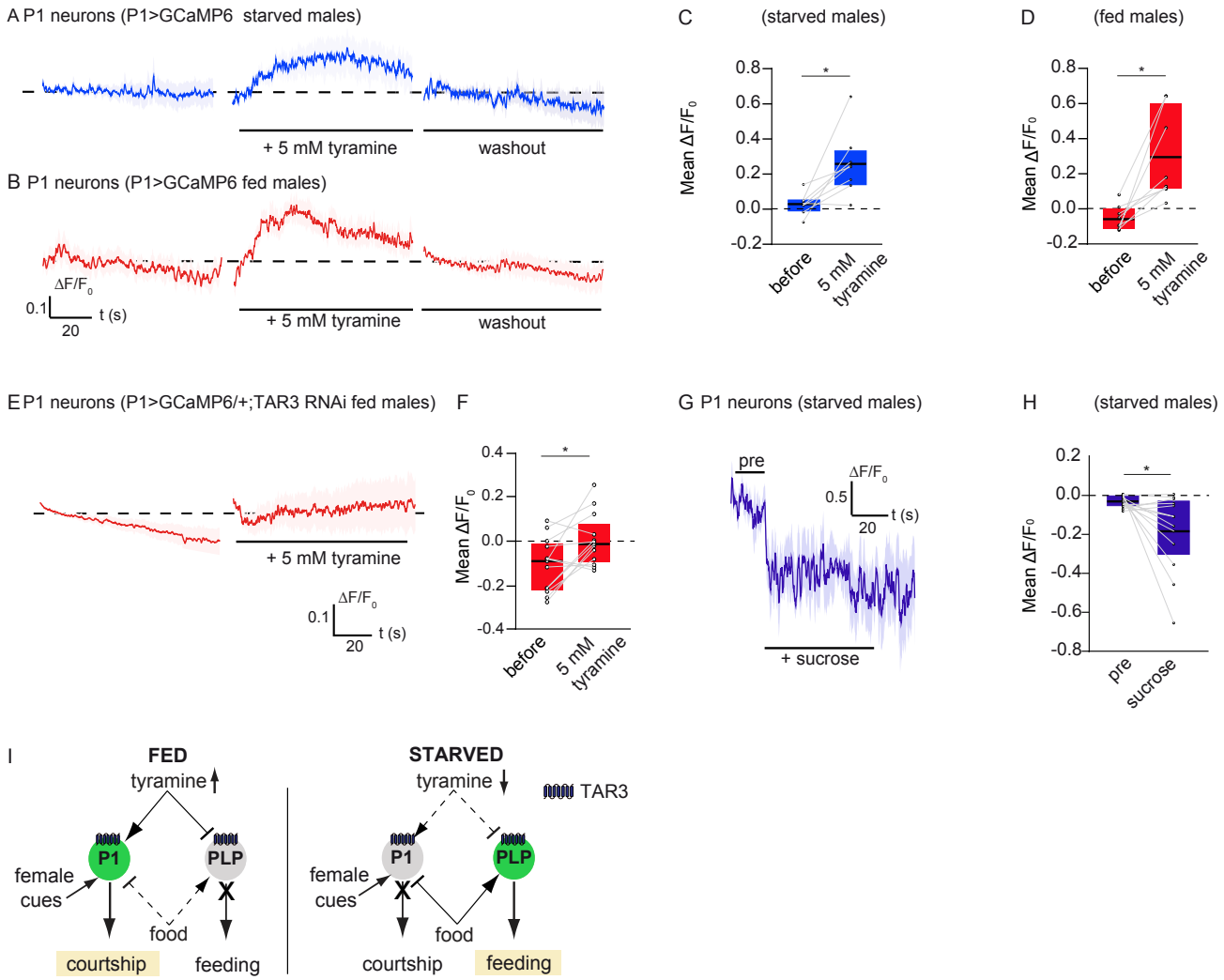


Figure 3.7: Tyramine and sucrose perception antagonistically influence the activity of P1 neurons. (A and B) Increase in the Ca^{2+} response of P1 neurons upon tyramine application in both starved (A) and fed (B) males and their quantification (C and D, sample size: 8 flies). (E) Fed males whose TAR3 levels are down-regulated TAR3 RNAi in P1 neurons show a smaller increase in Ca^{2+} response upon tyramine application (sample size: 13 flies) and their quantification (F). (G) In starved males, a Ca^{2+} decrease in P1 neurons is observed upon sucrose perception (sample size: 13 flies) and their quantification (H). * p< 0.05, ** p<0.01 and *** p<0.001 calculated using paired t test in C, D, and F; and Wilcoxon matched-pairs signed rank test in H, absence of a * indicates no significant difference. Figure published in Cheriyamkunnel et al [2] and is used under the terms of the Creative Commons Attribution License.

3.5 Discussion

3.5.1 Feeding-courtship behavioural choice

The hunger state of an animal plays a crucial role in shaping its behavioural prioritisation. However, the mechanisms through which the hunger state influences the neural processes underlying the selection between different behaviours have yet to be fully understood. To address this gap in knowledge, we developed a behavioural choice assay that focused on investigating the conflict between two fundamental drives: mating and feeding. To this end, we provided male *Drosophila* with a choice of a nutritious food source (sucrose) and a virgin female. We found that in sexually naive male flies that had been starved for 24 hours, the drive to feed overrode their courtship behaviour. However, fully sated males chose to initiate courtship first. These results align with Maslow's hierarchy of needs theory, that suggests that fulfilling basic physiological needs takes precedence over higher-level social needs like mating [179].

Maximising resource availability is crucial for efficient decision-making. While starved male flies do not decrease their courtship behaviour towards females in the absence of a food source, our results suggest that male flies first prioritise differently when presented with a nutritious food source. 24h starved flies fed for approximately four and a half minutes before transitioning to courtship, suggesting that flies may employ a behavioural strategy to effectively capitalise on the resources available in their environment. Similarly, studies by the Krashes lab have shown that hungry mice engage in a high degree of mating behaviours in the absence of food [179]. However, when simultaneously presented with a receptive female and food, male mice decrease the amount of time spent courting and feeding to utilise both resources equally [179].

In contrast, a recent paper by the Wang lab found that 24h starved flies only switched to courtship when fed with yeast but not sucrose [180]. Moreover, they claim that starved flies in the absence of food show decreased levels of courtship. This is in stark contrast with what we have found. Our results suggest that starved males court vigorously in the absence of food and transition to

courtship after feeding from sucrose when presented with a food source. However, there are a few differences in our assay. For example, we used a small chamber (20mm in diameter) with a decapitated virgin female. The Wang lab used a bigger arena and live 2-day-old virgin females. Traditionally, virgin females need more than 2 days to reach reproductive maturity and develop a stable pheromonal profile. Therefore, it is possible that the virgin females they used were not attractive courtship targets and were unreceptive to male courtship. Moreover, they elected to downsample their video frame rate to 1 Hz for analysis, a decision that introduces a potential compromise in the resolution and accuracy of their observations.

3.5.2 Neural mechanism underlying feeding-courtship behavioural choice

Our findings revealed that the tyramine pathway plays a crucial role in the choice between feeding and courtship. Tyramine acts as a satiety signal and its levels are modulated by the hunger status of the animal via expression levels of the enzymes Tdc2 and Tβh (Fig 3.5). While we have established the importance of the tyramine pathway in this context, the precise mechanism by which hunger signals are integrated within the neurons remains an intriguing question. How do the nutrient sensors in the brain detect and respond to the changes induced by starvation? Furthermore, how do these changes subsequently affect the transcriptional control of the Tdc2 and Tβh enzymes?

Our study highlights the antagonistic role of tyramine on feeding and courtship-promoting neurons. High levels of tyramine inhibit feeding-promoting TyrR neurons and activate courtship-promoting P1 neurons in fed flies helping them to prioritise courtship over feeding. Tyramine levels decrease upon starvation which reduces the inhibition of feeding-promoting TyrR neurons and further reduces P1 activity tipping the balance towards feeding. While previous research has demonstrated that P1 neurons promote courtship behaviour through interactions with the pIP10 descending neuron [181], the mechanisms by which $TyrR^{PLP}$ neurons promote feeding behaviour are yet to be investigated. It is unknown whether $TyrR^{PLP}$ neurons connect with the feeding circuitry in the subesophageal zone (SEZ) or if they communicate with other neurons expressing

neuro-peptides that regulate food intake (e.g. DILPs, NPF). Furthermore, the interplay between TyrR^{PLP} and P1 neurons could be examined further. It is uncertain whether TyrR^{PLP} neurons themselves connect to P1 neurons or if P1 neurons connect to TyrR^{PLP} neurons. The possibility of mutual inhibition between these neurons has not been explored. Investigating these connectivity patterns and potential inhibitory interactions between TyrR^{PLP} and P1 neurons will contribute to a better understanding of the choice between feeding and courtship behaviours.

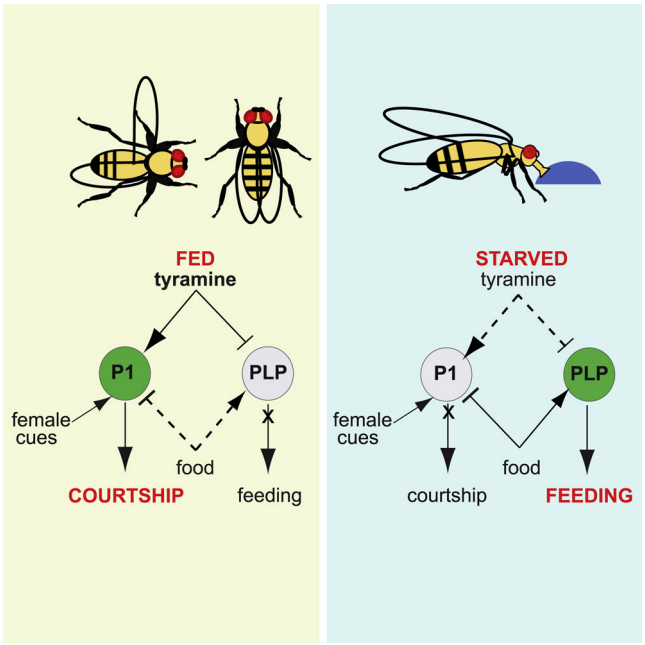


Figure 3.8: Feeding-courtship behavioural choice working model. Reduced levels of tyramine in starved males lead to increased TyrR neural activity (TyrR^{PLP}). These neurons are further activated upon ingestion of a food source, promoting feeding over courtship. P1 courtship-promoting neurons are antagonistically modulated by tyramine and sucrose, favouring courtship over feeding in fed flies. Figure published in Cheriyamkunnel et al [2] and is used under the terms of the Creative Commons Attribution License.

Tyramine plays a multi-functional role in the regulation of hunger-related processes in invertebrates (reviewed in [182]). Studies using *C. elegans* have demonstrated that tyramine decreases locomotion, enabling the worms to focus on feeding [182]. Similarly, tyramine suppresses locomotor behaviours such as crawling and flight in *Drosophila*. Furthermore, tyramine has been implicated in the modulation of feeding behaviour itself [182]. In honey bees, the injection of tyramine has been observed to increase the proboscis extension reflex, indicating a heightened response to sugar [182]. However, contrasting findings have been observed in the blowfly species

Phormia regina, where tyramine has been found to decrease the proboscis extension reflex [182]. While our findings indicate that tyramine functions as a satiety signal in flies, it is worth noting that the effects of tyramine on hunger might vary across species. Elucidating the precise mechanisms by which tyramine influences feeding behaviour still remains unknown.

Norepinephrine, the vertebrate analogue of tyramine, has also been shown to play a significant role in hunger-related behaviours. The release of norepinephrine has been shown to regulate appetite and drive food-seeking behaviours [183]. For instance, administration of norepinephrine directly into the hypothalamus of mice elicits robust feeding behaviour [183]. In addition, it acts on various brain regions involved in reward learning, attention and decision-making, including locus coeruleus (LC) [184]. Further investigations are required to gain a comprehensive understanding of how these biogenic amines contribute to the intricate process of behavioural choices in hunger-related contexts.

3.5.3 Limitations

To quantify the initial behavioural prioritisation of flies, a parameter CPI (courtship preference index) was devised in the lab. Firstly, a fly's choice was considered to be courting/feeding if the fly displayed that behaviour for at least 5s. Then, the initial choice of all the flies being tested at a given time (around 10 flies per genotype) was pooled to give rise to CPI calculated by the formula CPI= $(n^{court}\text{-}n^{feed})/(n^{court}+n^{feed})$. This parameter essentially transformed the dichotomous data into percentages to give continuous variables. However, this approach failed to capitulate the behavioural variability and reduced the statistical power of the tests due to the smaller sample size of replicates compared to the total number of flies tested in each experiment.

To address this, I also analysed the raw count data (i.e. the number of flies initiating courtship behaviour versus the number of flies feeding). I performed Fisher's exact test, followed by Bonferroni post-hoc corrections, to compare the count data among different groups. By employing this alternative method, I confirmed our initial findings and observed that the results did not qual-

itatively change. Both the original approach and the analysis using raw count data supported our findings, and the use of the latter method ensured the statistical robustness of the results.

Reflecting on the project, if I were to redo it, I would quantify the behaviour differently. I would devise a courtship preference index for each fly, calculated as (choice index = (time spent courting - time spent feeding in the first 5 minutes) / total time). This parameter would be more robust to the variability in the behavioural data. Behavioural expression is not always dichotomous on an individual level. For instance, if the fly feeds for 5 seconds but courts for the next five minutes, the original CPI parameter would consider its first choice to be feeding even though the fly performs courtship for the majority of the time. The choice index parameter would be able to capture the variability by measuring the time spent doing each behaviour. Moreover, it would provide more statistical power as it would consider continuous percentages for each fly rather than compressed count data.

To inhibit TyrR neurons, we expressed TNT through out the development which can result in morphological or developmental defects. To counter this, we expressed UAS TNT only in adulthood using GAL80^{ts} and observed similar results (see [2] for detailed results). Nevertheless, to validate the involvement of TyrR neurons, acute inhibition is essential exclusively during the behavioural experiments using Shi^{TS} or GtACR1. Consequently, interpreting the results requires a degree of caution due to the chronic nature of the genetic manipulations involved.

3.5.4 Summary

In summary, within this Chapter, we developed an innovative behavioural assay to explore the selection process between feeding and courtship behaviours in male flies. Our results reveal that well-fed flies tend to initiate courtship as their first choice, while flies deprived of food for 24 hours prioritise feeding. Additionally, we have identified two pivotal neuronal components, TyrR and P1 neurons, whose activity is influenced by tyramine levels and the presence of food.

4 Feeding-Escape Behavioural Choice

4.1 Abstract

The ability to prioritise one behaviour over others is a fundamental aspect of life. Behavioural choices involve weighing internal needs and environmental cues to choose the most appropriate behaviour for each situation. My work aims to shed light on the neural processes controlling this universally relevant brain computation.

To gain a mechanistic understanding of decision-making during a behavioural conflict, I devised a novel assay where *Drosophila* flies must decide whether to prioritise feeding or escaping from a visual threat, two crucial but mutually exclusive behaviours. My findings reveal that 24h starved flies cease feeding when exposed to a visual threat. However, when experiencing severe hunger, they ignore the threat and carry on feeding. Thus, the choice between feeding and escaping from threats is flexible and modulated by the nutritional state. Moreover, I uncover a sexual dimorphism in this behavioural choice, with females requiring longer periods of starvation to shift their behaviour from escape to feeding. By employing my innovative assay, along with neurogenetic approaches, behavioural techniques, and insights from the connectome, I identify the key neural circuitry that establishes priority during this sensory conflict, connecting visual input with motor output. My research provides insights into the neural circuit mechanisms that balance risks and rewards to prioritise actions during conflicting situations.

4.2 Introduction

"When in danger or in doubt, run in circles, scream and shout."

Herman Wouk, The Caine Mutiny, 1951

Animals must feed to acquire a sufficient source of both energy and essential nutrients, which are necessary for their growth, survival, and reproductive processes. Therefore, feeding is a crucial behaviour and food deprivation can have severe, even fatal, consequences for an animal. At the same time, defensive behaviours are necessary to avoid life-threatening dangers like escaping a predator. However, both feeding and defensive behaviours cannot be performed simultaneously. So, how do animals choose between these two behaviours?

When sea slugs *Pleurobranchea* are threatened with a noxious mechanical stimulus, they immediately stop feeding and show escape swimming behaviours [10]. This response is orchestrated by the A1 interneurons, which, upon stimulation by the noxious stimulus, trigger escape swimming while concurrently inducing proboscis retraction to cease feeding behaviour [10]. Conversely, in the medicinal leech *Hirudo*, feeding takes precedence over other behaviours like swimming and local bends [11]. Similarly, a study by Burnett and colleagues showed that hungry mice overcome their fear of a predatory odour to start feeding, while fed mice avoided the odour [185]. Therefore, the choice between feeding and escape behaviours can depend on the organism's niche and its hunger state. While this behavioural choice has been studied in multiple contexts, very little is known about the underlying neural mechanisms. More specifically, how the hunger state and threat visual cues are integrated into the brain to produce the most appropriate behaviour still remains unexplored.

Drosophila is a fantastic model system to tackle this question as both feeding and defensive behaviours have been meticulously characterised. As mentioned in the introduction Chapter 1, flies

exhibit robust defensive responses when confronted with visual threats that simulate approaching predators [73, 74, 78]. A commonly used visual threat is the looming stimulus that triggers defensive behaviours in several species including flies [186]. Research from the Card lab has revealed that flies exhibited take-off behaviour from a platform when exposed to the looming stimuli, and the choice of escape strategy hinges on the stimulus's speed [77, 78]. However, Zacarias et al. observed different defensive responses when flies were placed in an inescapable arena, where freezing and running behaviours were the predominant defensive responses [73]. The expression of these defensive behaviours relies on the accurate detection of threats by LC/LPLC visual projection neurons located in the optic lobes that are designed to extract specific features from the environment [82]. These neurons transmit the critical information to descending neurons that project onto the ventral nerve cord (VNC) and in turn, trigger a range of defensive behaviours [84]. Particularly noteworthy is the descending neuron DNp01, also known as Giant Fiber, which has been demonstrated to induce short-mode takeoff [79, 85–87]. Freezing behaviour is under the control of DNp09 neurons [73]. While several descending neurons have been characterised, how context and flexibility are conveyed to these neurons remains largely unknown.

Similarly, the feeding behaviour of *Drosophila* has undergone comprehensive investigation, using various methods to reliably measure food intake and explore different behavioural sub-programs within the feeding process [58, 59]. Previous studies have outlined the neuro-modulatory systems that regulate food-intake and neural circuit components that detect and process the gustatory cues [57]. Shiu et al. made notable strides using the hemi-brain connectome, discovering a network of 3-4 inter-neurons connecting sugar-sensing gustatory neurons to proboscis extension motor neurons [60]. Schwarz et al. further identified the specific motor neurons responsible for controlling the different steps of proboscis extension and retraction [61]. Despite these substantial advancements, it remains unclear how the fly chooses between feeding and escaping. Furthermore, the impact of the fly's hunger state on this critical behavioural choice remains unknown.

4.2.1 Objectives

Taking advantage of the powerful genetic and behavioural tools available in *Drosophila* and a novel behavioural assay that I have developed, I aimed to investigate the neural mechanisms underlying the choice between feeding and escape behaviours. In *Drosophila melanogaster*, feeding and defensive behaviours have been studied extensively, but only in isolation. How do these two crucial behaviours interact? What happens when a conflict arises between the two behaviours? This study had three specific objectives:

1. To understand how flies choose between feeding and escape behaviours.

2. To investigate the influence of both internal factors (such as hunger) and external factors on this behavioural choice.

3. To unravel the neural mechanisms that underlie the decision-making process between escape and feeding in *D. melanogaster*.

4.3 Methods

4.3.1 Fly stocks and husbandry

The wild-type and transgenic flies were cultured as described before (3.3.1). The list of transgenic lines used is given below (Table 9).

Number	Strain	Source
1	Canton-S	Kravitz Lab
2	LPLC1 Split GAL4	(R64G09 AD; R37H04 DBD), Moita Lab [82]
3	LPLC2 Split GAL4	(R19G02 AD; R75G12 DBD), Moita Lab [82]
4	LC6 Split GAL4	Bloomington (68247)
5	LC4 Split GAL4	(R47H03 AD, R72E01 DBD), Moita Lab [82]
6	LC16 Split GAL4	Bloomington (68331)
7	GF Split GAL4	Bloomington (79602)
8	DNP04 Split Gal4	Janelia (SS01080)
09	DNP06 Split Gal4	Janelia (SS01047)
10	UAS TNT	Bloomington (28837)
11	UAS CsChrimson	Bloomington (55134)
12	UAS MCD8:EGFP	Bloomington (32185)
13	nAChRα1 subunit RNAi	Bloomington (28688)
14	nAChRα3 subunit RNAi	Bloomington (27671)
15	nAChRα4 subunit RNAi	Bloomington (31985)
16	nAChRα5 subunit RNAi	Bloomington (25943)
17	nAChRα6 subunit RNAi	Bloomington (25835)
18	FDG splitGAL4	Janelia (SS31333)
19	Bract splitGAL4	Janelia (SS31320)
20	Roundup splitGAL4	Janelia (SS47736)
21	MN09 splitGAL4	(VT061715 AD, VT005008 DBD) Simpson lab [187]

Table 9: List of transgenic fly strains used in Chapter 4

4.3.2 Threat set-up

A modified version of the looming setup from Moita Lab as described in Zacarias et al. was used in this Chapter [73]. To create the setup, an infrared (IR) backlight was positioned beneath the experimental apparatus. On top of the IR backlight, an opaline plate was placed to diffuse the light uniformly. Additionally, a custom-built bridge was designed to securely hold the experi-

mental chambers (2 cm in diameter) in a consistent position on the opaline board across various experiments. For presenting the looming stimulus to the subjects, a monitor screen tilted at a 45-degree angle was placed at a distance of 21.5cm from the arena. The looming stimulus itself expanded non-linearly (following the equation size(time)=2tan^{-1}(length/velocity*time)) at a speed of 25 cm/s, creating a threatening visual experience for the flies [73]. To capture the fly behaviour during the experiments, a USB3 camera (Blackfly S) equipped with an infrared (IR) long-pass filter was used. This filter only allows IR light to pass through, ensuring that the camera avoids the changes in light intensity during threat stimulation. The threat delivery and video recording were done using Bonsai visual programming at 60 frames/s [156].

4.3.3 Starvation Protocol

Prior to starvation, the flies were flipped twice onto vials with fresh food as the food was prone to frequent bacterial contamination. Flies were wet starved in 1% agar vials with a filter paper for 12-96h. The agar vials were watered 1h before experiments to prevent dehydration.

4.3.4 Behavioural experiments

Behavioural experiments were performed between zeitgeber time (ZT01) and (ZT04) at a temperature of 25°C and humidity (40-60%) over at least 3 different days to account for daily variation. All experiments and data analysis were not performed blind. The flies were aged 4-8 days for behavioural experiments.

4.3.5 Solitary threat response

Individual male flies were introduced to a circular chamber (2 cm in diameter, 0.5 cm in height) and left to acclimatise for 180s. After 180s, 10 looming stimuli were introduced for 30s (10 cycles, frequency: 0.33 Hz) [73]. The trajectories of the flies and the change in pixels were computed using Bonsai software. A fly was considered to be freezing if the pixel change was less than 5 per frame or 300 per minute [73]. Jumps were automatically detected from changes in

instantaneous speed (velocity >75 mm/s) with a gap of at least 2 frames between each consecutive jump [73]. The flies were classified as runners if their freezing index was less than 30%. Walking speed was calculated by thresholding instantaneous velocity > 4mm/s. Change in walking speed was calculated by subtracting the mean walking speed (for 30s) before the stimulus from the mean walking speed during the looming stimulus.

4.3.6 Behavioural choice assay (feeding vs. escape)

Preparation of 100mM sucrose solution: 100mM was prepared as described before (3.3.1).

The assay: Individual 4-8 day old flies were introduced to a circular chamber (2 cm in diameter, 0.5 cm in height) with food 1% (w/v) agar and 0.4% (w/v) blue dye in 100 mM sucrose solution. After a 5s interval following feeding initiation, a total of 10 looming stimuli were administered continuously for 30s (10 cycles with a frequency of 0.33 Hz). The video was recorded for 2 minutes or until the fly returned back to the food patch after the delivery of the looming stimulus [73].

Female mating: Ten 4-6 day old virgin females were introduced to 30 virgin males in a vial. The males were discarded after 48 hours of social interaction. This gave ample time to make sure that all female flies were mated.

Quantification of behavioural parameters:

Feeding index: was calculated as the percentage of time in which the male spent feeding (extending its proboscis) on or near the food patch for 30s.

Escape index: was calculated as the percentage of time in which the male spent moving outside the food patch.

Freezing index: was calculated as the percentage of time in which the male spent freezing (or showing complete immobility)

Defensive index: was calculated as the percentage of time in which the male exhibited any of

two defensive behaviours: freezing and running/escaping.

Number of Jumps: was calculated as the total number of jumps during the stimulation period

The behaviour of the flies was manually annotated during the observatory period using Behavioural Observation Research Interactive Software (BORIS) [37]. For the feeding-escape project, 4 state events (feeding, freezing, escape and latency to return to food after threat) and one point event (jumping) were created.

Tracking and initial characterisation of locomotor parameters during feeding escape behavioural choice: The trajectories of all the flies were tracked using Ethovision software during the 30s of threat delivery. The food zone was defined as a circle that covered all the food patches in an experiment as there was variation in the position and the size of the food patch. The outer zone is the area of the arena excluding the food zone. Occupancy of both regions was quantified as the percentage of time spent in each zone. The mean speed was computed without applying any threshold, as flies in the control groups consistently displayed low speeds (< 4mm/s) while feeding.

4.3.7 Optogenetics

Preparing fly food for optogenetics: 20 µl of 50 mM *all*-trans retinal factor was added to the fly food so that all surface area was covered. The vial was wrapped with aluminium foil to prevent light exposure. The food was let dry for a day before use. For long-term use, the food was refrigerated.

Raising flies for optogenetics: The flies were collected after eclosion and placed on normal fly food for 2 days. They were flipped onto food with *all*-trans retinal factor on day 3 and again on the day before testing. During starvation, the flies were supplemented with 20 µl of 50 mM *all*-trans retinal in the starvation vials.

Behavioural Assay: Individual male flies were introduced to a transparent circular chamber (2

cm in diameter, 0.5 cm in height) with food 1% (w/v) agar and 0.4% (w/v) blue dye in 100 mM sucrose solution. Upon 5s after feeding initiation, 30s of 60 Hz continuous red light was used to light the arena from underneath. The assay was modified in 4.12 where the males were presented with a female or dusted with Brilliant Yellow powder. For solitary male experiments, male flies were introduced to a circular chamber and left to acclimatise for 180 seconds before light stimulation.

4.3.8 Flypad Assay

To measure food intake during threat delivery, single 24h starved flies were aspirated into flyPAD feeding arenas with 10 µl of 100mM sucrose [58] and their feeding behaviour was recorded for 30s of stimulation time. Both threat delivery and capacitance recording were done using the bonsai software [156]. Food intake was measured as the total number of sips taken by individual flies within one hour and was calculated in MATLAB using custom-written software as described in [58].

4.3.9 Connectomic analysis

To quantify the connections of the LPLC2 neurons and their downstream targets, the FlyWire and Neurprint database was used [19]. The synaptic connection matrix was downloaded and the number of synaptic connections was summed for neurons on the left hemisphere. The FlyWire IDs used for the analysis are tabulated in 6.

4.3.10 Geosmin Avoidance Assay

A modified version of the olfactory trap assay set up by Stensmyr et al. was used [90]. A group of fed male flies (30-50) were placed in circular plastic containers (diameter = 20cm) with two olfactory traps. Olfactory traps are smaller sample containers with a pipette tip to allow the entry of flies, but not the exit. The control trap contained 10 µl of balsamic vinegar while the experimental trap contained 10 µl of balsamic vinegar supplemented with 10 µl of 10^{-4}% w/v geosmin

solution in mineral oil. The trap was left to sit for 24h and the total number of flies in and outside traps was then counted after 24h to obtain preference indices using the formula PI= (number of flies in experimental trap-number of flies in control trap)/total number of flies.

4.3.11 Immuno-histochemistry

To check the expression pattern of the GAL4 lines, the flies were crossed with UAS mC8:eGFP, a membrane-targeted version of the green fluorescent protein [188]. Male flies were aged for 4-7 days before which their brains were dissected in PBS (Phosphate Buffered Saline). Following dissection, the brains were fixed using a 4% paraformaldehyde solution at room temperature for 20 minutes. The fixed brains underwent a series of four washes in 0.5% PBST (1X Phosphate-Buffered Saline, 0.5% Tween) lasting 30 minutes each. Subsequently, the brains were blocked with 5% normal goat serum for one hour. The brains were then incubated with primary antibodies (anti-GFP chicken 1:1000, (Abcam, #13970), anti-nc82 mouse 1:500 (DSHB, #528108)) for three days at 4°C. The brains were washed again 4 times in PBST, after which they were incubated with secondary antibodies (Alexa Fluor 488 goat anti-chicken IgG 1:1000 (ThermoFisher Scientific , #A28175), Alexa Fluor 543 goat anti-mouse IgG 1:1000 (ThermoFisher Scientific , #A21050)) overnight. The brains were mounted in Vectashield on a glass slide following 4 PBST washes again.

4.3.12 Data visualisation

All data visualisation was performed using R (version 3.6.3). Data is presented as boxplots with the lower and upper hinges representing the first and third quartiles. Whiskers of the boxplots are in the form of Tukey's fences (1.5*IQR).

To create the occupancy heat maps, the trajectory data of the flies were used and plotted using R. The centres of the behaviour arenas from different days were aligned to create the heatmaps using `stat_density_2d` function in ggplot2 using parameters h = 2 and n (number of bins) = 100. The colour scales were modified to visualise low-density regions clearly. To create the velocity

plots, the average velocity at each pixel was calculated and scatter plots for velocity corresponding to each coordinate within the range (0-30 mm/s) were plotted using `geom_scatter`.

4.3.13 Statistics

All statistical analysis was performed using R (version 4.1). Sample sizes were not predetermined. Median values of behavioural indices along with the interquartile range have been reported. To assess data normality, the Shapiro-Wilk normality test was used. As a majority of behavioural indices did not adhere to a normal distribution, non-parametric tests, specifically the Mann-Whitney test or Kruskal-Wallis tests, followed by Dunn's multiple comparison tests with Bonferroni correction, were used to assess differences between groups. The alpha value was set at 0.05. Logistic regression was performed to compare the effects of starvation on different sexes. Loess Regression was used to smooth the velocity data in Fig 4.11. R code used for analysis is described in 6. Statistical differences are indicated by distinct letters. Groups sharing the same letter exhibit non-significant differences in median values. Conversely, when two groups are labelled with different letters, their differences are statistically significant.

4.4 Results

4.4.1 Establishing a reliable visual threat set-up in the lab

To investigate the neural mechanism underlying a behavioural conflict, I aimed to develop a new behavioural paradigm in which wild-type *Drosophila* males are subjected to a choice between feeding and defensive behaviours. To this end, I employed the looming stimulus, a circle expanding non-linearly over time that mimics a predator approaching the fly (Fig 4.1A, for more details, see 'Methods')[73, 78].

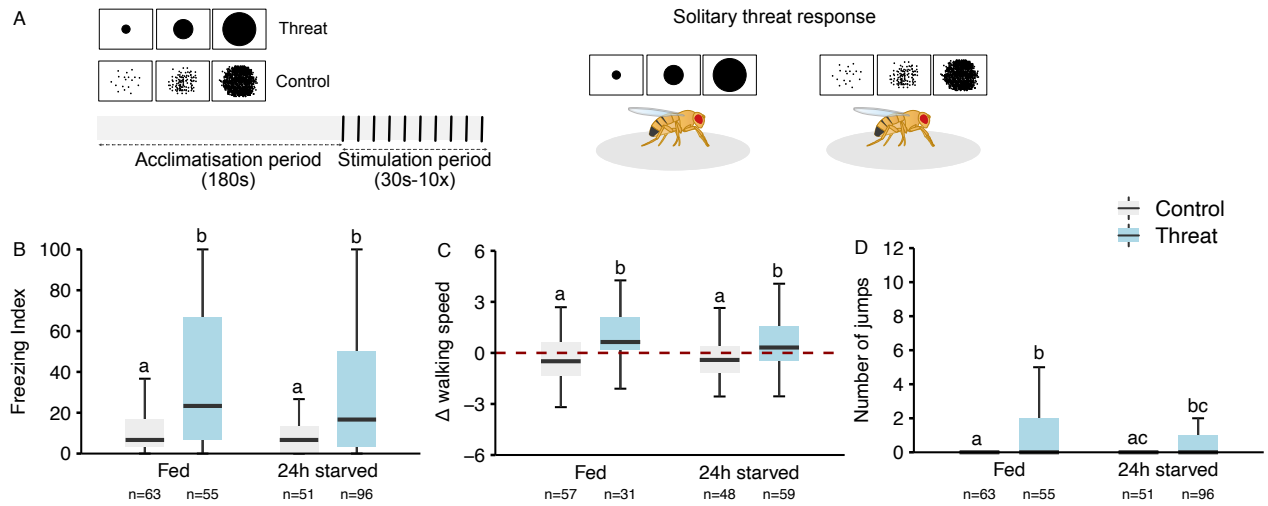


Figure 4.1: Robust defensive behaviours observed in wild-type Canton-S male flies subjected to a looming stimulus. (A) Individual Canton-S males were transferred into enclosed circular chambers where they acclimatised for 180s after which they were subjected to 10 looming stimuli within 30s. (B) Freezing index or percentage of time spent freezing during the stimulation period of 30s. (C) Change in walking speed caused by looming stimulation (baseline walking speed 30s before the loom subtracted from walking speed during the stimulation period). (D) Number of jumps detected during the stimulation period. Statistical analyses using Kruskal-Wallis followed by post-hoc Dunn Test in B, C and D. Sample size (number of flies) indicated below the boxplots. Box plots labelled with different letters are statistically significantly different.

Firstly, I tested if the looming stimulus reliably elicited defensive behaviours as shown in Zacarias et al. 2017 [73]. I adapted the threat protocol to be more acute and delivered 10 looms within a time period of 30 seconds (frequency = 0.33 Hz) in order to create a more threatening stimulus during the behavioural conflict (Fig 4.1A). As a control, I used a visual stimulus wherein random pixels appeared on the screen, mimicking the changes in brightness but without any actual

expansion (Fig 4.1A). I found that fed males exposed to the looming stimulus increased their time spent freezing when compared to males exposed to the control stimulus (Median freezing index = 6.66%(controls), 23.33%(loom), p = 8.00e^{-04}, Fig 4.1B). In particular, 43.63% of fed flies showed a freezing index >30%. 24h starved males showed a similar increase in the freezing index in response to the looming stimulus (Median freezing index = 6.66%(control), 16.67%(loom), p = 8.21e^{-04}, Fig 4.1B). Zacarias et al. found that flies increased their walking speed and this effect was more pronounced in flies that did not freeze (also called runners) [73, 81]. Using their criteria, I found that 56.36% of fed flies spent <30% of their time freezing in the presence of the looming stimulus and thus classified them as runners. These runners reliably increased their walking speed (Δ walking speed= -0.62 mm/s(control), 0.64 mm/s(loom), p = 3.6e^{-04}, Fig 4.1C). Finally, fed flies also displayed an increased number of jumps (median jumps + IQR = 0+/-0(control), 0+/-2(loom), p = 2.32e^{-05}, Fig 4.1D). In conclusion, wild-type Canton-S males exhibited freezing, running, and jumping in response to the looming threat. These findings align with previous studies [73, 81], demonstrating that my experimental setup replicates the conditions that trigger robust defensive responses in flies.

4.4.2 24h starved flies prioritise escape over feeding when presented with a visual threat

Having established a looming stimulus as a reliable visual threat for solitary flies, I subsequently designed a behavioural choice assay where flies would be subjected to a choice between feeding and defensive behaviours. To this end, I used 24h wet-starved males who were highly motivated to eat. I presented the starved males with a nutritious food source of 100 mM sucrose mixed with blue dye in order to visualise the food patch more effectively. After 5s of feeding initiation, I subjected the males to the looming or the control stimulus for 30s (Fig 4.2A). I found that 24h starved males exposed to control stimulus fed for 100% of the observation time (median feeding index (FI) = 100%, Fig 4.2B). In contrast, upon encountering the looming stimulus, starved males ceased feeding, left the food patch (FI = 22.3%, p = $1.634e^{-11}$, Fig 4.2B) and showed an increased expression of defensive behaviours (median defensive index (DI) = 73.51%, p = $1.609e^{-11}$, Fig 4.2C). After careful examination, I observed that the flies primarily exhibited escape behaviour rather than freezing in response to the looming threat (median escape index = 68.76%, Fig 4.2D). Specifically, only 12.24% of flies showed robust freezing behaviour resulting in a low freezing index (median freezing index = 0%, Fig 4.2E). Finally, flies showed an increase in the number of jumps (median number of jumps = 0(control), 1(loom), p = $3.65e^{-07}$, Fig 4.2F). These findings suggest that flies starved for 24h prioritise escape responses over feeding.

To better understand the behaviour of the flies, I tracked the locomotor parameters during the behavioural choice. I divided the chamber into two distinct zones: the food zone and the outer zone, after which I measured the time spent by the flies in each zone (Fig 4.2G-I). Flies exposed to the control stimulus primarily remained within the food zone, while flies exposed to the looming stimulus displayed reduced occupancy in the food zone and increased occupancy in the outer zone (Occupancy in the food zone= 100%(control), 36.14%(threat), p = $1.39e^{-07}$, Fig 4.2H-I). This shift is indicative of escape behaviour. I additionally calculated the average speed of the flies during the stimulation period without applying the 4mm/s threshold criteria. As expected, 24h starved flies engaged in feeding behaviour and maintained a low speed in response to the control

stimulus (speed = 1.92mm/s, Fig 4.2L-M). However, when confronted with the looming threat, the flies significantly increased their speed, providing further evidence of their escape response (speed= 10.613mm/s, p = $1.05e^{-08}$, Fig 4.2L-M).

Next, I used the Gaussian kernel density estimation function to create the 2D occupancy plots based on the cumulative time spent by the flies at each region (Fig 4.2H-I). To enhance the presentation of the occupancy plots, I further classified the behaviour into three categories based on the flies' feeding index: strong (FI = 0-33.33%), moderate (FI = 33.33-66.66%), and weak (FI = 66.66-99.99%) and plotted the occupancy of the corresponding groups. Flies presented with the control stimulus showed only weak responses with their occupancy in the centre near the food zone. When exposed to the looming stimulus, flies showed varying degrees of response. 59.2% of the flies exhibited a strong response where they drastically decreased their feeding index (<33.33%). Their occupancy plots showed a uniform density along the chamber walls indicating that they show a strong escape response (Fig 4.2H). 24.48% and 16.32% of flies showed a moderate and weak response to the threat with occupancy plots concentrated near the centre, close to the food zone (Fig 4.2I).

To further confirm that flies did indeed stop feeding upon threat detection, I coupled an automated device for measuring food intake, flyPAD (fly Proboscis and Activity Detector) [58], with the looming stimulus (Fig 4.2N). I measured the number of sips which is a proxy for food intake. As expected, flies exposed to the looming stimulus showed a significant decrease in the number of sips when compared to controls (Sips = 37(control), 6(loom), p = $6.047e^{-08}$, Fig 4.2O). Altogether, these results show that 24h starved males prioritise their immediate survival over feeding when exposed to a visual threat.

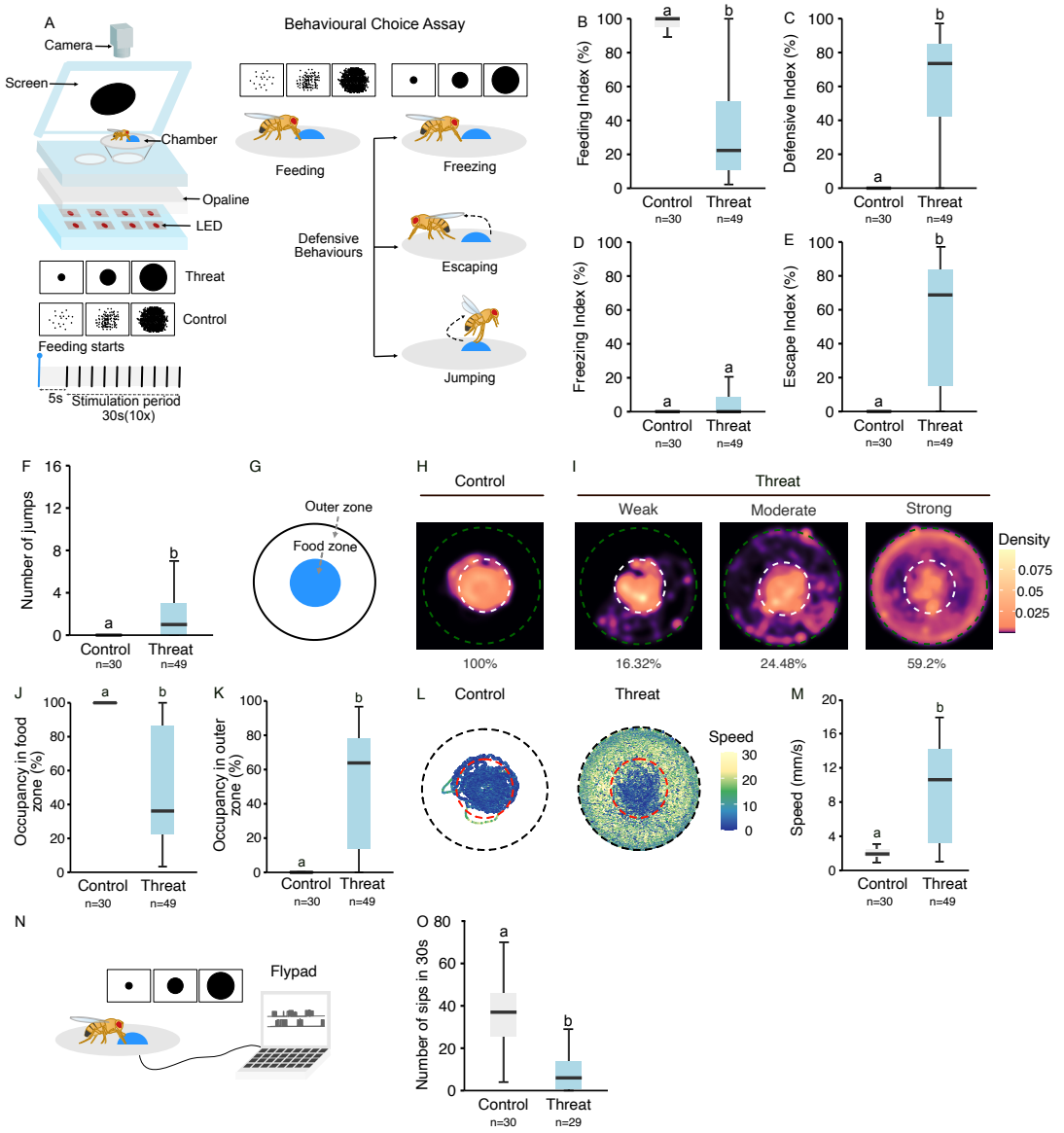


Figure 4.2: 24h starved flies prioritise escape over feeding when presented with a visual threat. (A) Schematic of the behavioural assay to study the choice between feeding and defensive behaviours. Male Canton-S flies were allowed to feed from a food source containing 100 mM sucrose. After 5s of feeding initiation, starved flies were exposed to 10 looming or control stimuli on the monitor screen for a time period of 30 seconds. (B) Feeding Index or % time spent feeding on the food patch during the stimulation period. (C) Defensive Index or % of time spent exhibiting freezing or escape behaviour. (D) Freezing Index or % of time spent exhibiting freezing. (E) Escape Index or % of time spent exhibiting escaping. (F) Number of jumps. (G-H) Occupancy plots showing commutative time spent in different areas within the chamber for flies exposed to the loom (H) or control stimulus (G). Occupancy plots for the threat group are categorised into three categories based on their feeding index. (J-K) Quantification of the occupancy time in the food zone (J) and outer zone (K). (L) Scatter plots showing average velocity at each pixel. (M) Quantification of average velocity through the stimulation time. (N) The looming stimulus was coupled with flyPAD (fly Proboscis and Activity Detector) and food intake was measured during the stimulation period. (O) Number of sips in 30 seconds. Statistical analyses using the Mann-Whitney U-test in B-F, J-K, M and O. Sample size (number of flies) indicated below the boxplots. Box plots labelled with different letters are statistically significantly different.

My results have established that 24h starved males prioritised defence over feeding when exposed to a looming stimulus. Previous studies have shown sex-specific differences in feeding behaviour in *Drosophila* [59]. Specifically, male flies consume more sugar when compared to females [59]. Additionally, the mating status of female flies plays a pivotal role in feeding, as mated females tend to increase their food intake and demonstrate a heightened preference for yeast to meet their increased reproductive needs [34]. Given the sex-specific disparities and the effects of the mating status on feeding behaviour, I asked whether virgin and mated female flies would show different behavioural prioritisation in the conflict between feeding and escape. Therefore, I tested both females in my behavioural assay and exposed them to the looming stimulus whilst feeding. In line with the observations in males, 24h starved females prioritised defensive behaviours over feeding when exposed to the loom (Fig 4.3A-C). Both virgin and mated females decreased their feeding index and increased their defensive index in the presence of the threat when compared to the control stimulus (FI = 11.78% (mated females), 7.91%(virgin females), Fig 4.3B). Strikingly, there were no noticeable differences in the feeding index between 24h starved mated and virgin females, indicating that a female's mating status doesn't play a role in determining behavioural choices (p = 1, mated vs virgin females, Fig 4.3B-C).

Similar to male counterparts, 24h starved females predominantly engaged in escape behaviours rather than freezing in response to the looming stimulus (Median escape index = 70.49% (virgin), 71.50% (mated); Median freezing index = 0% (virgin), 1.46%(mated), Fig 4.3D-E). Additionally, 24h starved females demonstrated an increased frequency of jumping in response to threats (Median number of jumps+IQR = 0+/- 4(virgin), 0.5+/-2.75(mated), Fig 4.3F). In line with the manual behavioural quantification, 24h starved female flies showed reduced occupancy in the food zone in the presence of the looming stimulus (occupancy = 30.372%(virgin), 38.728%(mated), Fig 4.3G-K).

After the stimulation period, both males and females returned to the food patch and resumed feeding. Here, I noticed key differences in the time to initiate feeding after the threat delivery (also

called feeding latency). While male flies took 10.76s to resume feeding, both virgin and mated females took significantly longer time to return to the food and initiate feeding after threat delivery (latency to feed = 22.16s(mated females), 26.56s(virgin females), Fig 4.3M). In conclusion, this set of experiments revealed that 24h starved male and female flies both prioritise escape over feeding.

When comparing the manual annotation with the automated behavioural parameters, I observed a discrepancy between zone occupancy and behavioural indices (% of time spent feeding or escaping) for both male and female flies. For example, among 24h starved males exposed to the looming stimulus, the feeding index stood at 22.3%, while the occupancy in the food zone reached 36.14%. For 24h starved virgin females exposed to the threat, this discrepancy was even more pronounced, with a feeding index of 7.91% contrasting with an occupancy rate of 30.37% in the food zone. As occupancy analysis in the food zone could potentially lead to an overestimation of feeding behaviour (e.g., flies may freeze, groom or even escape in the food zone, which could be misinterpreted as feeding), I decided to shift my focus towards manual, detailed behavioural quantification for the remainder of my thesis.

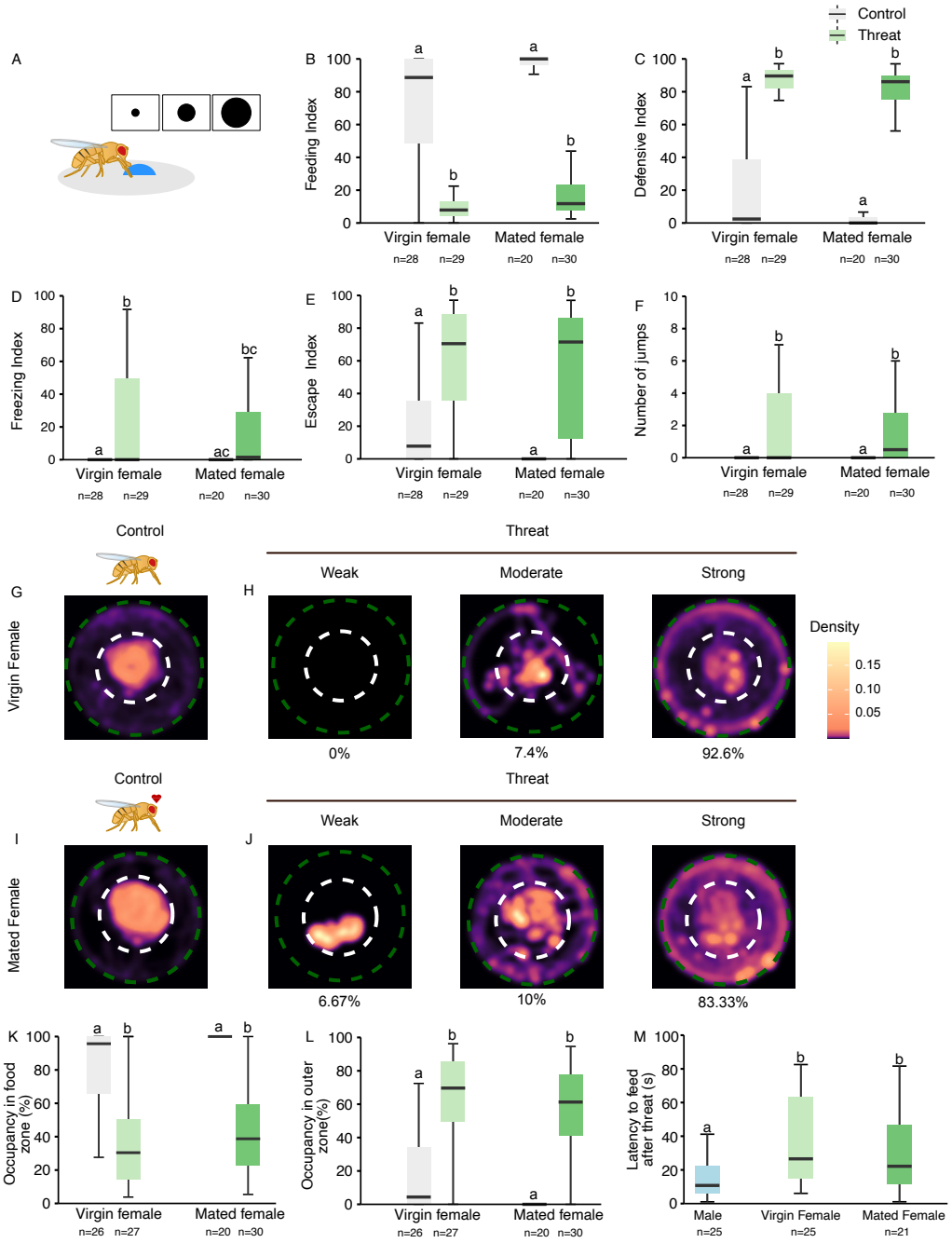


Figure 4.3: 24h starved wild-type female flies prioritise escape over feeding in response to the looming stimulus. (A) Individual 24h starved Canton-S virgin and mated female flies are subjected to a threatening looming stimulus whilst feeding from 100 mM sucrose solution. (B) Feeding Index. (C) Defensive Index. (D) Freezing Index. (E) Escape Index. (F) Number of Jumps. (G-J) Occupancy plots showing cumulative time spent in different areas within the chamber for flies exposed to the threat (H, J) or control stimulus (G, I). Occupancy plots for the threat group are categorised into three categories based on their feeding index. (K-L) Quantification of the occupancy time in the food zone (K) and outer zone (L). Starved wild-type females take longer to return to the food patch after the threat. (M) Latency to feed (in seconds) after the looming stimulation period in males and females. Statistical analyses using the Kruskal-Wallis test followed by the Dunn Test in B-F and K-M. Sample size (number of flies) indicated below the boxplots. Box plots labelled with different letters are statistically significantly different.

4.4.3 Other *Drosophila* species show a similar behavioural prioritisation

My studies so far indicate that 24h starved *Drosophila melanogaster* males prioritise escape over feeding, and this behavioural prioritisation is also observed in females, irrespective of their mating status. Behavioural choices might reflect the ecology of the species. Therefore, to explore the species-specific nature of behavioural prioritisation, I performed the behavioural choice assays on three additional *Drosophila* species, including *D. melanogaster*'s closest relative, *D. simulans*, and two other distant species: *D. erecta* and *D. melanica* (Fig 4.4A). Both *D. simulans* and *D. melanogaster* are generalist species that feed from a variety of sources while *D. erecta* specialises in feeding solely on *Pandanus* sp. Additionally, *D. melanica* inhabits regions of the south-eastern USA and Mexico while *D. erecta* is found in western Africa. I hypothesised that differences in ecological niches and feeding behaviour could reveal differences in behavioural choices in response to the loom.

Surprisingly, all of the species aborted feeding in response to the threat, although the effect was slightly smaller in *D. simulans* when compared to *D. melanogaster* (FI = 56.58% (*D. simulans*), Fig 4.4B). However, the type of defensive responses to the looming stimulus varied among the species. *D. melanogaster* and *D. simulans* primarily displayed escape behaviour (Escape Index = 82.27% (*D. melanogaster*), 20.1% (*D. simulans*), Fig 4.4E) while *D. melanica* and *D. erecta* mainly exhibited freezing behaviour in response to the looming threat (Freezing Index = 95.09% (*D. melanica*), 69.35% (*D. erecta*), Fig 4.4D). Furthermore, only *D. melanogaster* and *D. melanica* showed an increase in jumps in response to the loom (Fig 4.4F).

While it is plausible that these differences in defensive behaviours could be species-specific, it is also worth considering that the other species may not thrive as well on the same food that is optimal for *D. melanogaster* and this in turn could affect their responsiveness to the threat. To test this hypothesis, I monitored their locomotion in the absence of both food and threat for a 5-minute interval. In line with my expectations, *D. erecta* and *D. melanica* flies exhibited reduced mobility and sluggishness. In particular, the average walking speed of these two species was

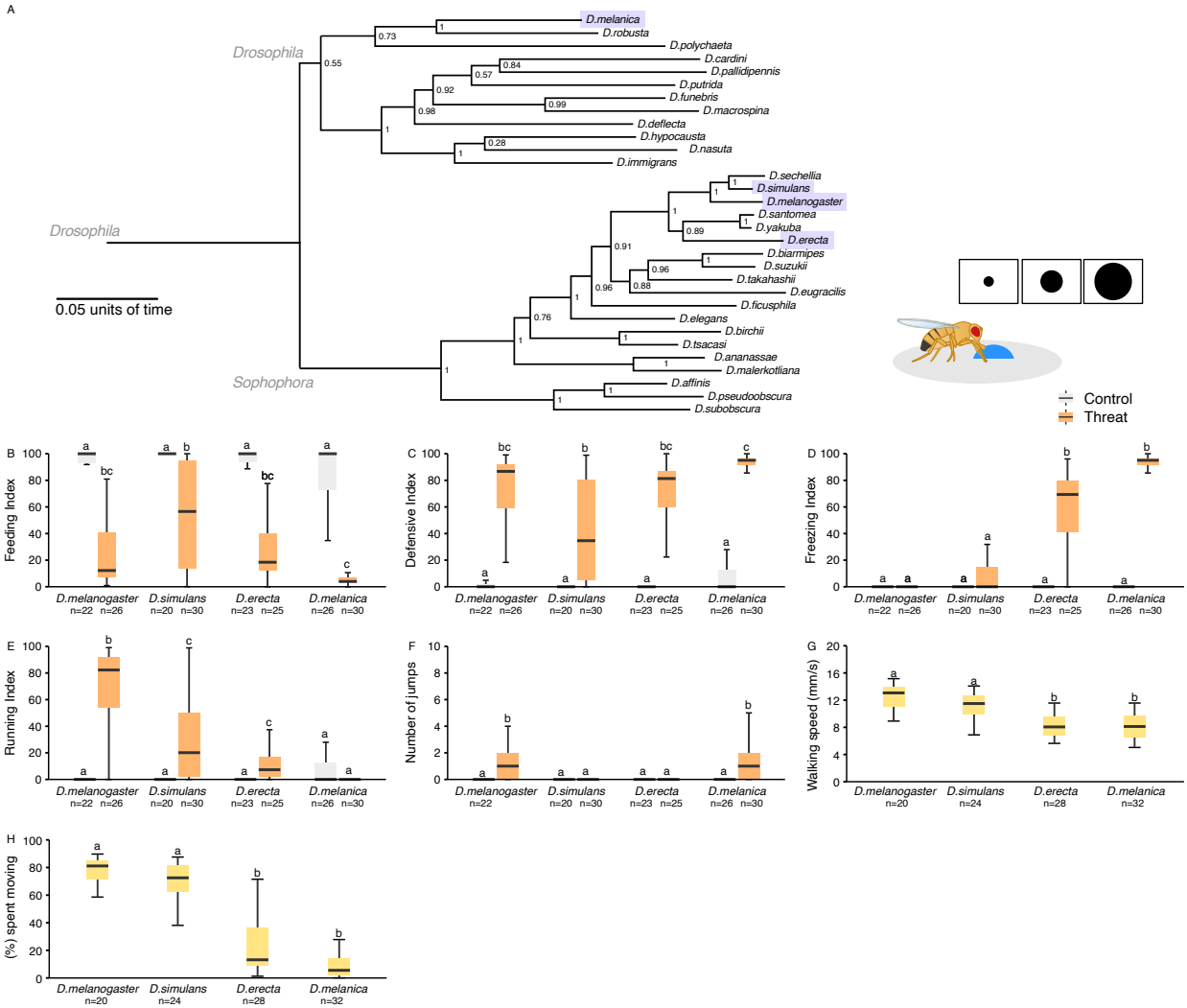


Figure 4.4: Other *Drosophilid* species species prioritise survival over feeding. (A) Phylogenetic tree showing relationships between *Drosophilid* species of sub-genus *Drosophila* and *Sophophora* (Adapted from Keesey et al. 2019 and is used under the terms of the Creative Commons Attribution License.) [189]. Four different *Drosophilid* species (*D.melanogaster*, *D. simulans*, *D. erecta* and *D. melanica*) are subjected to a threatening looming stimulus whilst feeding from 100mM sucrose. (B) Feeding Index. (C) Defensive Index. (D) Freezing Index. (E) Escape Index. (F) Number of Jumps. (G-H) Locomotor abilities of different species in the absence of food and threat. (G) Walking speed (mm/s). (H) The percentage of time spent moving is calculated as the percentage of time the fly's instantaneous velocity is above 4 mm/s. Statistical analyses using the Kruskal-Wallis test followed by the Dunn Test in B-H. The sample size (number of flies) is indicated below the boxplots. Box plots labelled with different letters are statistically significantly different.

lower than *D. melanogaster* (walking speed = 8.06mm/s (*D. erecta*), 8.12mm/s (*D. melanica*), 13.06mm/s (*D. melanogaster*) and 11.49mm/s (*D. simulans*), Fig 4.4G). Moreover, they spent less time moving around the chamber (13.17%(*D. erecta*), 5.53% (*D. melanica*), 81.11% (*D. melanogaster*) and 72.50% (*D. simulans*), Fig 4.4H). Therefore, reduced mobility could have

contributed to the observed differences in the freezing and jumping responses in solitary flies exposed to the threat.

4.4.4 Hunger shifts behavioural prioritisation in a sex-specific manner

Having explored the behavioural choice in both sexes and different Drosophilid species, I next aimed to understand how the internal state of the animal can influence this behavioural choice. Previous reports have shown that the severity of the hunger state tips the behavioural prioritisation towards feeding-related behaviours over other behaviours in a choice context [8]. Thus, my next goal was to examine whether the hunger state of the flies could modulate the choice between feeding and escape.

Previous studies have shown that flies can live without food for 3-5 days [190, 191], but this can vary according to the genetic background of the strain and the sex of the fly. Therefore, I first assessed how starvation affected the survival of the Canton-S strain used in this Chapter. I found that male flies died within 72 hours of starvation, while the females survived for an additional 2 days (Fig 4.5B). This observation aligns with previous literature indicating that females tend to exhibit greater resilience to prolonged hunger compared to males [191]. Based on the survival data, I progressively starved the male flies for 12-48h (12h, 24h, 36h and 48 h), and tested them in the behavioural choice assay. Male flies starved for 12 and 24h drastically decreased their feeding in response to the loom (FI= 9.53% (12h starved), 22.3% (24h starved), Fig4.5C). In contrast, flies starved for 36h and 48h showed a significantly higher feeding index (FI= 43.39% (36h starved), 64.83% (48h starved), Fig 4.5C). As expected, starvation had the opposite effect on defensive behaviours, with 48h starved flies showing the lowest defensive index (DI=34.9% (48h starved), Fig 4.5D). Therefore, male flies adapt their behavioural prioritisation depending on their hunger status.

I next inquired whether females would exhibit similar behavioural responses when subjected to longer periods of starvation. As female flies are more resilient to starvation, I starved them between 12-96 hours and tested them in my behavioural assay. The feeding indices consistently remained at low levels for both virgin and mated females until 48h of starvation (Fig 4.5E-H). However, a notable shift occurred as the females endured more extended periods of starvation.

Specifically, the feeding index of females exhibited an upward trend after 60 hours of starvation, peaking at 96 hours (FI = 63.93%(virgin female), 78.96%(mated female), Fig 4.5E-H). This suggests that, under extended periods of hunger, females seem to prioritise feeding over survival instincts, like escaping potential threats, like males do.

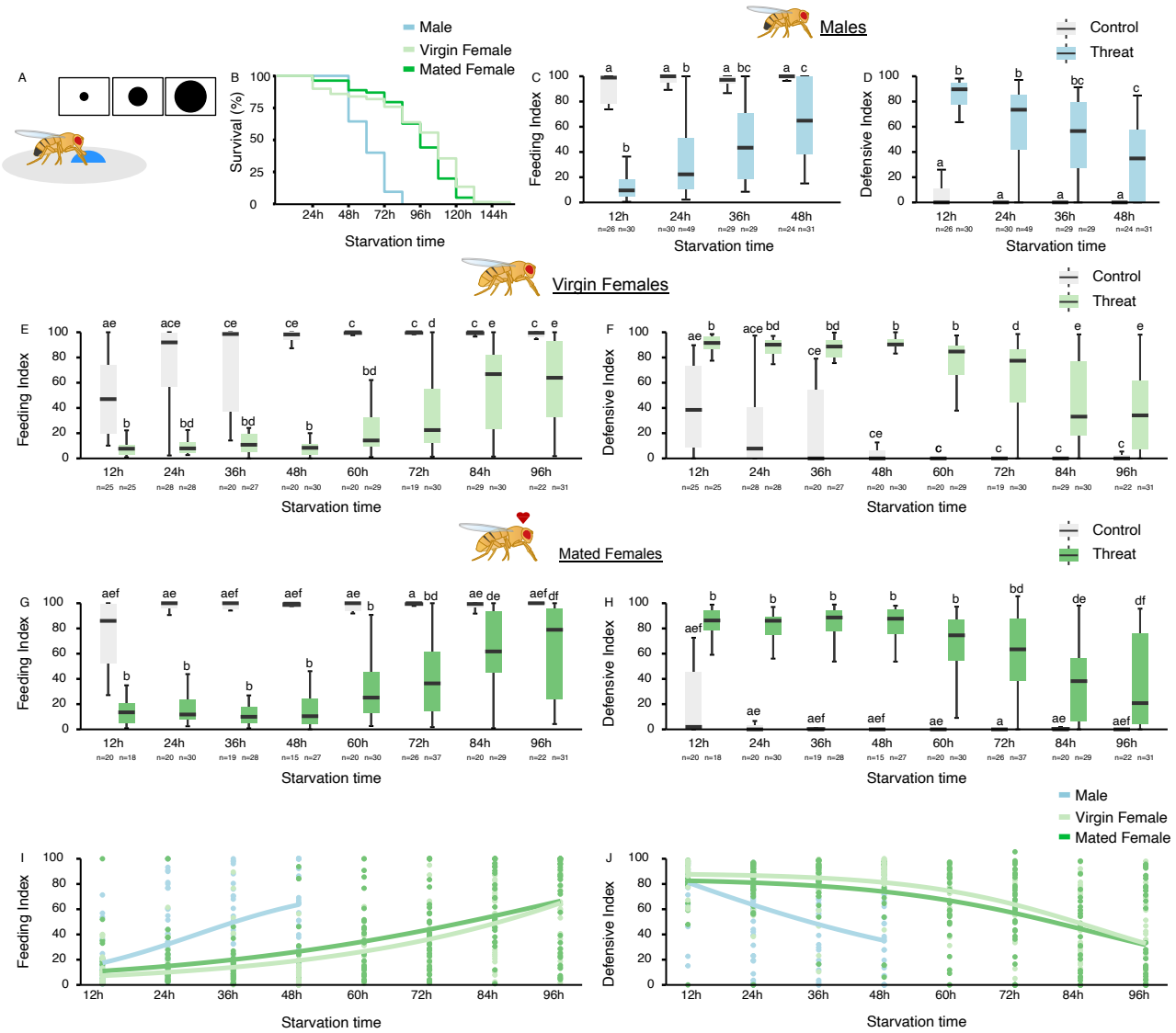


Figure 4.5: Hunger shifts behavioural prioritisation in a sex-specific manner. Male, virgin and mated female flies were starved progressively starved 12-96hrs and subjected to a threatening looming stimulus whilst feeding from 100mM sucrose solution. (C, E and G) Feeding Index or % time spent feeding on the food patch during the stimulation period. (D, F and H) Defensive Index or % of time spent exhibiting freezing or escape behaviour. Best fit of logistic regression of feeding index in (I) and defensive index in (J). Statistical analyses using pairwise comparisons after a logistic regression in (C-H). The sample size (number of flies) is indicated below the boxplots. Box plots labelled with different letters are statistically significantly different.

To quantify the increase in feeding index over time in males and females, I performed a logistic

regression analysis on the pooled data using the formula $p(t) = 1/[1 + exp(-(b0 + b1 * t + b2 * Condition))]$. The model revealed a positive log odds ratio over time for all three groups. These results suggest that as the duration of starvation increased, both male and female flies exhibited a greater likelihood of persisting in feeding behaviour when confronted with a visual threat. Next, I sought to examine the rate of increase in the feeding index between males and females. To this end, I compared the log odds ratio over time for the three groups (T= 0.058961 (Male), 0.035715 (Virgin Female), 0.030942 (Mated female), Fig 4.5G). Remarkably, a unit change in time resulted in a 6% increase in feeding index in males while virgin and mated females showed a 3.6% and 3.1% increase suggesting the males increase their feeding behaviour quicker in response to starvation (p=0.0199, |Male vs. Virgin female|; p= 0.0046 |Male vs. Mated female|). However, there were no differences between mated and virgin females (p=0.3752 |Mated female vs. Virgin female|). Taken together, these findings strongly indicate sexually dimorphic responses to starvation with males displaying a notably rapid increase in feeding behaviour when compared to virgin and mated females.

4.4.5 Deciphering the neural mechanism underlying the choice between feeding and defensive behaviours

My findings demonstrate that flies that have been starved for 24 hours prioritise escaping from a threat over feeding, and this holds true for both females and various *Drosophilid* species. Focusing on males, I next aimed to decipher the neural mechanism underlying this crucial behavioural choice. As a starting point, I aimed to understand how the visual threat was detected by the fly brain. I, therefore, focused on a class of visual projection neurons called Lobula Columnar (LC)/Lobula plate–lobula columnar (LPLC) neurons that act as feature detectors [82]. These neurons receive information from visual processing centres in the optic lobe and project it onto the central brain, therefore acting as good candidates to convey the threat signal to central circuit nodes involved in controlling behaviours [82].

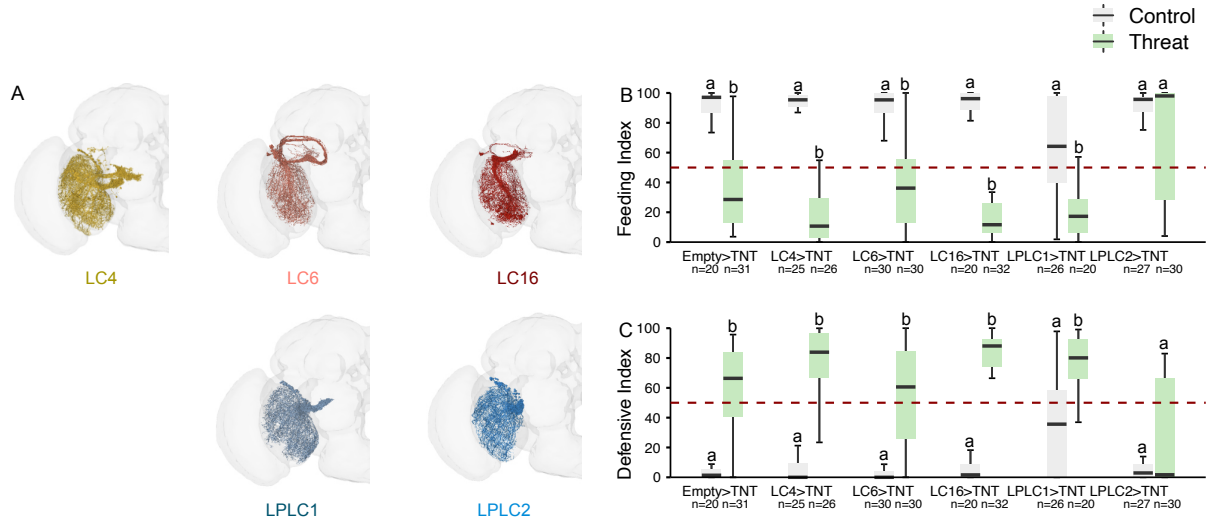


Figure 4.6: A behavioural screen to identify visual projection neurons involved in the choice between feeding and defensive behaviours. (A) FlyWire reconstruction of visual projections neurons in the left hemisphere of the fly brain [19]. (B-C) LC/LPLC neurons were inhibited in 24 h starved males using tetanus toxin and tested in the behavioural choice assay. (B) Feeding Index or % time spent feeding on the food patch during the stimulation period. (C) Defensive Index or % of time spent exhibiting freezing or escape behaviour. Each experimental group was compared to the control(Empty>TNT) using the Wilcoxon test. The sample size (number of flies) is indicated below the boxplots. Box plots labelled with different letters are statistically significantly different.

I performed a candidate-based screen using split GAL4 lines targeting LC/LPLC neurons (LC4, LC6, LC16, LPLC1 and LPLC2) that have been shown to be sensitive to the looming stimulus

(Fig 4.6A). Using tetanus toxin light chain (TNT), I inhibited the above-mentioned candidate neurons in 24h starved flies and tested them in my behavioural choice assay. To analyse the results of the screen, I compared the behaviour of experimental lines against Empty>TNT.

4.4.6 LPLC2 neurons are necessary for males to prioritise escape over feeding

My results showed that inhibition of either LC4, LC6, LC16 and LPLC1 neurons did not lead to observable changes in behaviour (Fig 4.6A-C). In contrast, inhibition of LPLC2 neurons significantly altered the behavioural prioritisation in male flies. 24h starved LPLC2>TNT males continued to feed throughout the stimulation period resulting in a high feeding index and a low defensive index (FI = 98.01%, DI = 1.70%, Fig 4.6B-C). This effect was absent in both of the genetic controls that decreased their feeding index in response to the looming stimulus (FI = 8.43%(LPLC2 GAL4/+), 12.7% (UAS TNT/+), Fig 4.7D-E). Hence, the inactivation of LPLC2 neurons specifically alters the behavioural prioritisation and tips the balance towards feeding over defensive response.

If indeed inhibiting LPLC2 neurons attenuates escape responses in the behavioural choice assay, activating them artificially, in the absence of threat, should induce escape behaviour. To activate LPLC2 neurons, I used CsChrimson, a red light-sensitive cation channel [24]. To test if LPLC2 activation can stop feeding and trigger defensive behaviours, I starved the flies for 24h and allowed them to feed on the food patch (Fig 4.7F). After 5s of feeding, I shone 60 Hz of continuous red light to activate LPLC2 neurons for 30s. Both genetic and light controls continued to feed and did not show any defensive responses in the presence or absence of light (Fig 4.7I-J). In contrast, LPLC2>CsChrimson flies stopped feeding (FI = 0%, Fig 4.7F) and displayed defensive behaviours (DI= 97.29%, Fig 4.7G-J). In particular, LPLC2>CsChrimson flies increased the percentage of time spent escaping, but not freezing (Escape Index = 97.29%). This effect was absent in LPLC2>CsChrimson flies in the absence of light, arguing for a specific effect of activation of LPLC2 and not responses to only light (LPLC2>CsChrimson(no light) FI = 98.11%, DI = 0%, Fig 4.7G-J). These results indicate that LPLC2 activation recapitulates the wild-type response to

threat during the behavioural choice assay where flies stop feeding and show escape responses. However, I noticed a notably larger effect size on the feeding index when LPLC2 neurons were activated (Cliff's Delta = 0.89(Threat), 0.99(LPLC2 activation)).

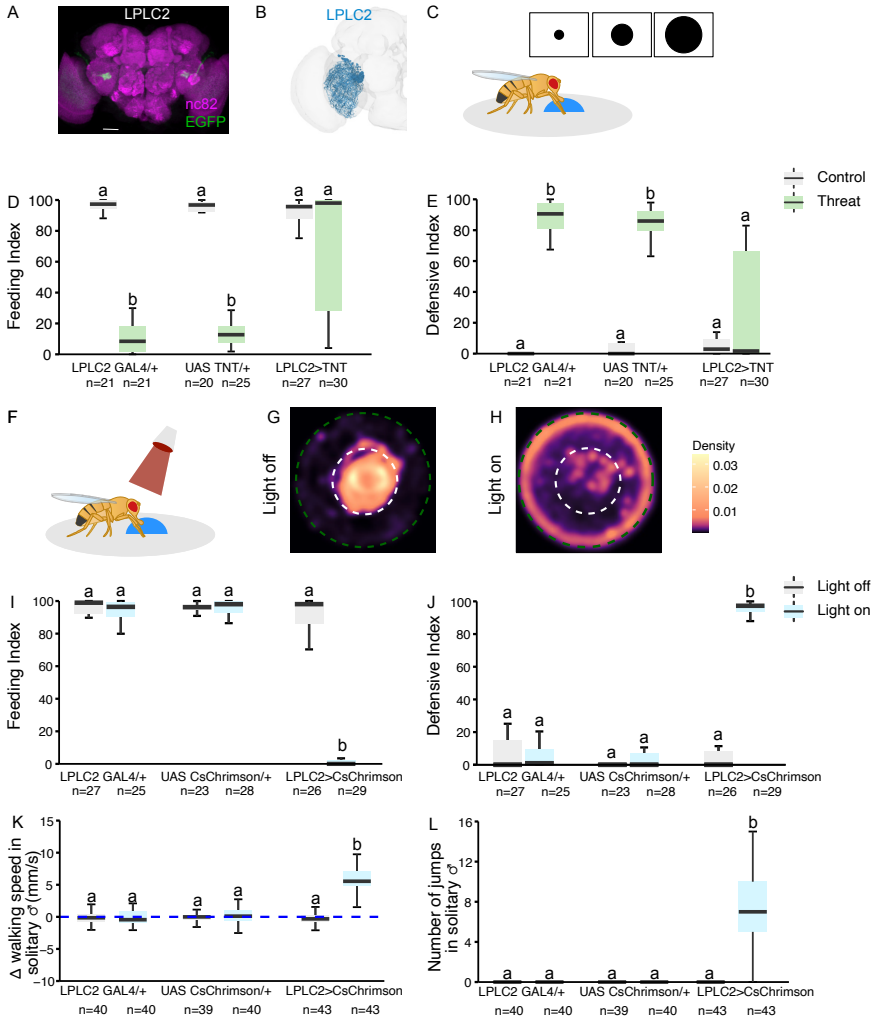


Figure 4.7: LPLC2 neurons are necessary for males to prioritise escape over feeding. (A) Expression pattern of LPLC2 Split GAL4 in the adult brain. GFP staining (green); neuropil counter-stained with anti-nC82 (white). Scale bar, 50 µm. (B) FlyWire reconstruction of LPLC2 neurons [19]. (C-E) LPLC2 neurons were inhibited in 24h starved males and tested in the behavioural choice assay. (D) Feeding Index (E) Defensive Index. (F) After 5 s of feeding initiation, LPLC2 neurons were activated in 24h starved flies subjected to continuous 60 Hz red light. (G-H) Occupancy plots of LPLC2>CsChrimson flies in the presence or absence of light. (I) Feeding Index. (J) Defensive Index. (K-L) LPLC2 neurons were activated in the absence of a food source in solitary males and their defensive behaviours were automatically classified. (K) Change in walking speed after light stimulation (mm/s). (L) The number of jumps after light stimulation. Statistical analyses using Kruskal Wallis followed by Dunn Test in D-E and I-L. The sample size (number of flies) is indicated below the boxplots. Box plots labelled with different letters are statistically significantly different.

To confirm that LPLC2 activation does indeed trigger escape behaviour, I quantified the effects

of optogenetic LPLC2 neural activation on walking speed in the absence of a food patch. I let solitary-fed males acclimatise to the chamber for 180s and then shone 60Hz red light for 30 seconds and quantified their defensive behaviours. Activation of LPLC2 neurons led to a significant increase in walking speed only in the presence of light (Δ walking speed LPLC2>CsChrimson = 8.10mm/s(light on), -0.31mm/s(light off), Fig 4.7K). It further led to an increased expression of jumps (number of jumps in LPLC2>CsChrimson flies = 10 (light on), 0 (light off), Fig 4.7L). Therefore, LPLC2 neurons induce running and jumping in response to the threat, prompting flies to prioritise escape over feeding.

4.4.7 Downstream targets of LPLC2

Having identified LPLC2 neurons as a key player in detecting the threat signal, I next asked what neurons downstream to LPLC2 are critical for behavioural choice. Previous studies from the Card lab and others have shown that LPLC2 neurons project to Giant Fibre (GF), a descending neuron that sends axons from the central brain to the VNC to trigger quick escape or takeoff [79, 83]. Simultaneously, I used the *Drosophila* connectome, the first complete map of the entire central brain of the fruit fly [19, 28], to identify the downstream targets. In addition to the Giant Fibre, I found two previously characterised descending neurons, DNp04 and DNp06, to be downstream of LPLC2 neurons. FlyWire data suggests that DNp06 neurons receive 334 connections from LPLC2 [19]. In addition, they receive 461 connections from other loom-sensitive visual projection neurons LPLC1 [19]. Similarly, DNp04 neurons receive 511 connections from LPLC2 neurons [19]. Given the high connection strength between GF, DNp04, DNp06 and LPLC2, I next asked if either of these descending neurons were important for feeding-defensive behavioural choice.

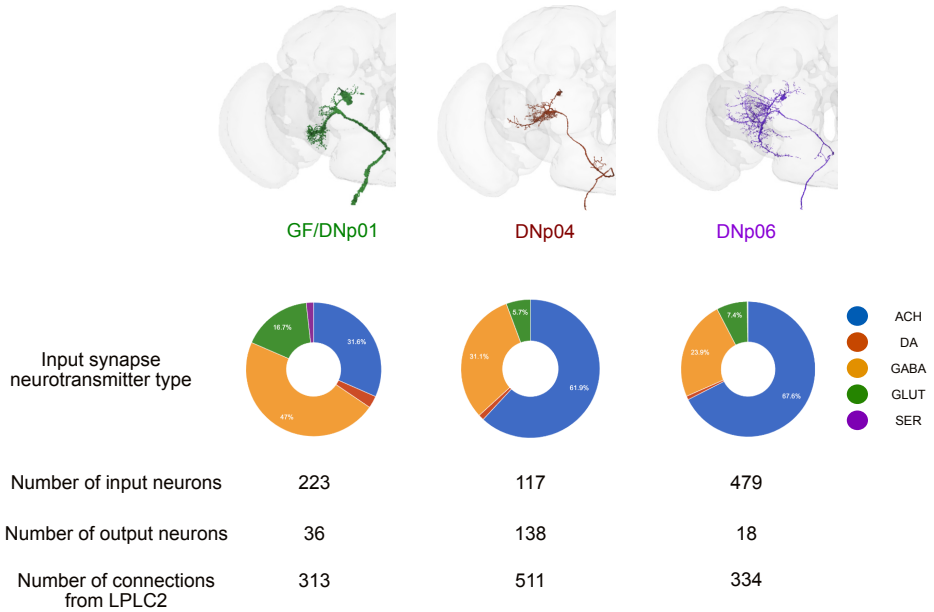


Figure 4.8: Descending neurons downstream of LPLC2: DNp01(GF), DNp04 and DNp06. DNp06 neuron receives 334 connections from LPLC2 while DNp04 and GF neuron receive 511 and 313 respectively. Neuronal skeletons obtained from FlyWire, connection strength only quantified from the neurons on the left hemisphere [19].

4.4.8 Giant Fibre neurons are dispensable for the choice between feeding and defensive behaviours

Considering the strength of behavioural evidence in the literature and connectomic data, I first asked if the choice between feeding and escape was mediated via the LPLC2-GF pathway. To test this, I inhibited the Giant Fibre neurons using TNT and tested the males in the behavioural choice assay. Surprisingly, GF>TNT flies responded to the threat and decreased their feeding index in the presence of the looming stimulus, and their behaviour was indistinguishable from the genetic controls (FI = 11.65%(GF>TNT), 10.88%(GF GAL4/+), 6.88% (UAS TNT/+), Fig 4.9D). Therefore, GF neurons are not necessary for the choice between feeding and escape.

To confirm that GF neurons indeed do not trigger escape responses during the behavioural choice assay, I activated GF neurons in 24h starved males feeding from a food source. Upon light onset, GF>CsChrimson flies reliably jumped but quickly returned to the food source and resumed feeding. This led to a modest decrease in feeding index and did not recapitulate the effects of LPLC2 activation (FI of GF>CsChrimson = 94.34%(light on), 98.14%(light off), Fig 4.9I-J). These results suggest that while GF neurons induce jumping, it does not trigger a sustained escape behaviour in the presence of food. This is in accordance with previous studies that have shown that GF neurons play an important role in triggering short-mode take-offs, while other neurons might be involved in long-mode take-offs [186].

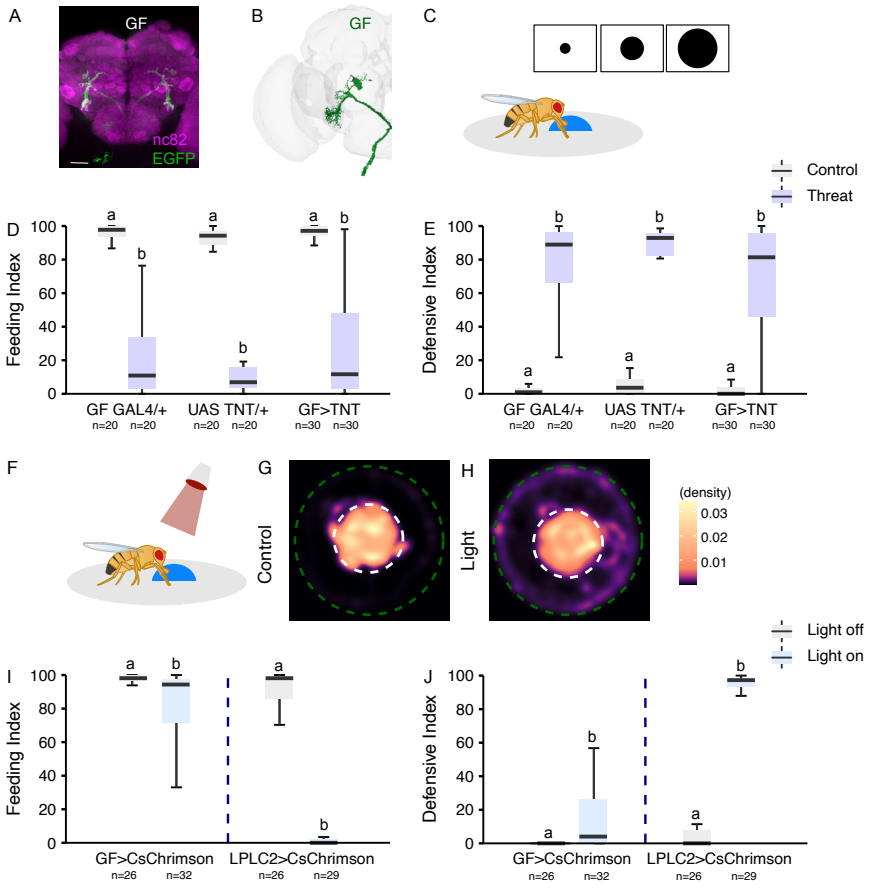


Figure 4.9: Giant Fibre neurons are dispensable the choice between feeding and defensive behaviours. (A) Expression pattern of Giant Fibre neurons. UAS-mCD8-GFP expressed under the control of Giant Fibre splitGAL4 in the adult brain. GFP staining (green); neuropil counterstained with anti-nC82 (white). Scale bar, 50 µm. (B) FlyWire reconstruction of GF neurons [19]. (C-E) GF neurons were inhibited in 24h starved males and tested in the behavioural choice assay. (D) Feeding Index. (E) Defensive Index. (F-J) 24h starved flies were presented with a food source (100mM sucrose). After 5 s of feeding initiation, GF>CsChrimson flies were subjected to continuous 60 Hz red light. (G-H) Occupancy plots of GF>CsChrimson flies in the presence or absence of light. (I) Feeding Index. (J) Defensive Index. Statistical analyses using Kruskal Wallis followed by Dunn Test in D-E and I-J. The sample size (number of flies) is indicated below the boxplots. Box plots labelled with different letters are statistically significantly different.

4.4.9 DNP06 neurons suppress competing behaviours in response to the threat

My findings indicate that the Giant Fibre neurons are not necessary for the choice between feeding and defensive behaviour. While GF may be responsible for initiating jumps, they do not seem to drive sustained escape responses. Therefore, other neurons might be involved in this response. Given the potential connectivity seen in the connectome, I next focused on DNp04 and DNp06. I inhibited both descending neurons using TNT and tested the males in the behavioural choice assay (Fig 4.10A-C). Inhibition of DNp06 neurons, but not DNp04 (data not shown) in 24h starved males tipped the balance towards feeding over defensive behaviours in the behavioural choice assay (DNp06>TNT FI = 93.35%, DI = 5.22%, Fig 4.10D-E). The genetic controls as expected prioritised defensive behaviours instead (FI = 7.51% (DNp06 GAL4/+), 11.66% (UAS TNT/+), Fig 4.10D-E). These results suggest that DNp06 are required for flies to prioritise escape responses over feeding in response to visual threats.

I next aimed to test if activation of DNp06 neurons would result in escape behaviour in feeding flies. I, therefore, activated DNp06 neurons in 24h starved males feeding from a food source. Upon light onset, DNp06>CsChrimson males immediately stopped feeding, left the food patch and showed escape behaviours (FI = 1.47%, DI = 97.92%, Fig 4.10C-D). However, when DNp06 neurons were activated in the absence of food, males did not show a significant increase in walking speed when compared to the controls over 30s of stimulation (Δ speed = 1.019 mm/s(DNp06> CsChrimson), 0.26mm/s(DNp06 GAL4/+), 0.41 mm/s(UAS CsChrimson/+), Fig 4.11B). However, they showed a significant increase in jumps (Number of jumps = 1(DNp06>CsChrimson), 0(DNp06 GAL4/+), 0(UAS CsChrimson/+), Fig 4.11C).

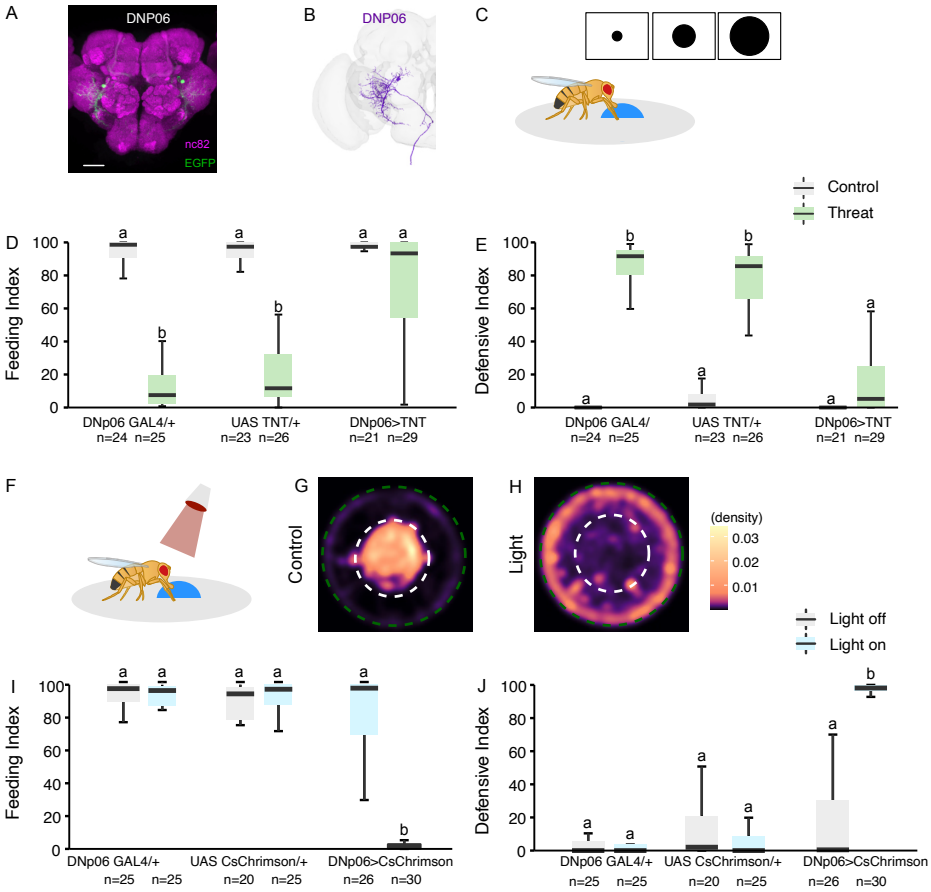


Figure 4.10: DNp06 neurons suppress feeding in response to threat. (A) Expression pattern of DNp06 neurons. UAS-mCD8-GFP expressed under the control of DNp06 split-GAL4 in the adult brain. GFP staining (green); neuropil counterstained with anti-nC82 (white). Scale bar, 50 µm. (B) FlyWire reconstruction of DNp06 neurons [19]. (C-E) DNp06 neurons were inhibited in 24h starved males and tested in the behavioural choice assay. (D) Feeding Index. (E) Defensive Index. (F-J) 24h starved flies were presented with a food source (100mM sucrose). After 5 s of feeding initiation, DNp06>CsChrimson flies were subjected to continuous 60 Hz red light. (G-H) Occupancy plots of DNp06>CsChrimson flies in the presence or absence of light. (I) Feeding Index. (J) Defensive Index. Statistical analyses using Kruskal-Wallis followed by the Dunn Test in B-G. The sample size (number of flies) is indicated below the boxplots. Box plots labelled with different letters are statistically significantly different.

To better understand this inconsistency, I looked at the change in walking speed during successive brief time intervals (5s). DNp06>CsChrimson flies displayed a significant increase in walking speed within the initial 5s after light stimulation, which was not observed in the control groups (Δ speed = 2.67mm/s(DNp06> CsChrimson), 0.029mm/s(DNp06 GAL4/+), 0.25mm/s(UAS CsChrimson/+), Fig 4.11D-E). Moreover, I did not observe any changes in angular velocity (indicative of turning behaviour) during the total stimulation time or right upon light onset (data not shown). These findings suggest that DNp06 neurons cause a transient increase in velocity, but do not entirely recapitulate the sustained escape responses triggered by LPLC2 activation.

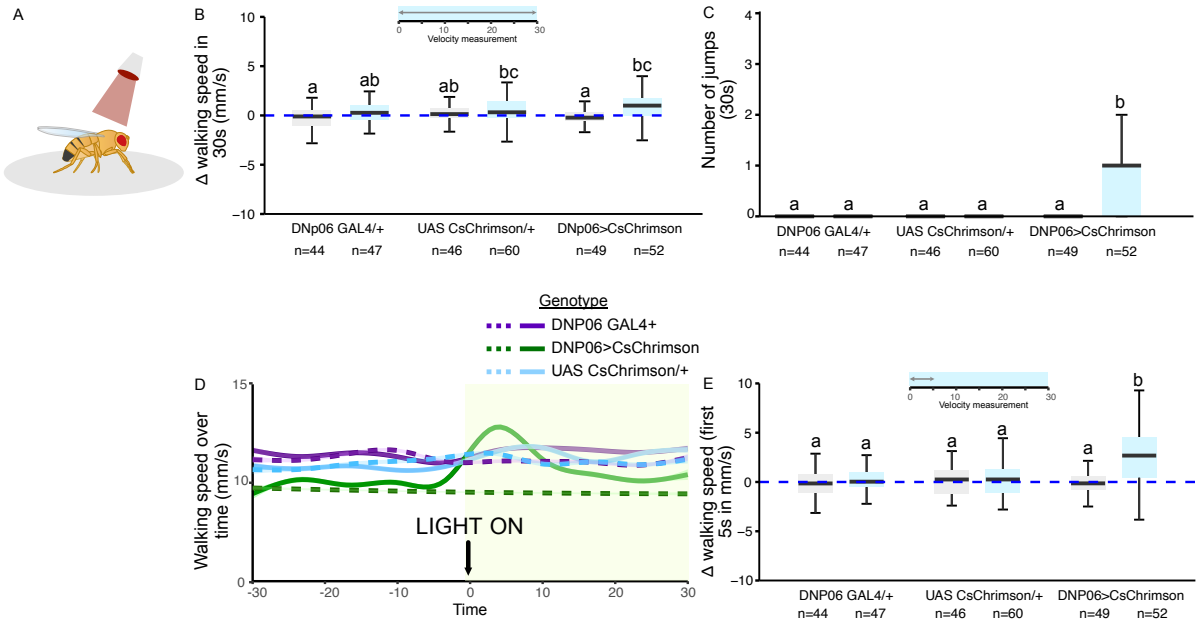


Figure 4.11: Activation of DNp06 neurons leads to a transient increase in walking speed. (A) DNp06 neurons were activated in the absence of a food source in solitary males and their defensive behaviours were automatically classified. (B) Quantification of the total change in walking speed after light stimulation (mm/s) calculated for 30s. (C) The number of jumps after light stimulation. (D) Change in walking speed over time (before and after light stimulation). (E) Quantification of change in walking speed after light stimulation (mm/s) calculated for the first 5 seconds. Statistical analyses using Kruskal-Wallis followed by the Dunn Test in B-E. The sample size (number of flies) is indicated below the boxplots. Box plots labelled with different letters are statistically significantly different.

These results suggest that upon threat-induced activation, DNp06 neurons might immediately interrupt feeding behaviour, prompting flies to leave the food patch, while other neurons might be involved in increasing velocity. I thus reasoned that DNp06 neurons could function as command

neurons that induce flies to cease any ongoing behaviour in response to threats and prompt them to move away. If that were true, activation of DNp06 neurons should inhibit other innate behaviours such as courting or grooming. To test this hypothesis, I presented DNp06>CsChrimson males (or controls) with a wild-type Canton-S female and let the males court. After 5s of courtship initiation, I shone a red light to activate the DNP06 neurons in the males (Fig 4.12A). Interestingly, I found that DNp06>CsChrimson males immediately stopped courting and showed escape responses upon light stimulation while the control flies continued to court through the observation time (Courtship Index after light onset = 2.75% (DNp06>CsChrimson), 93.11% (DNp06 GAL4/+), 65.38% (UAS CsChrimson/+), Fig 4.12B-C). Similarly, DNp06>CsChrimson flies dusted with Brilliant Yellow powder ceased to groom and exhibited escape behaviour in response to light stimulation (Grooming Index after light onset = 3.60% (DNp06>CsChrimson), 76.16% (DNp06 GAL4/+), 88.95% (UAS CsChrimson/+), Fig 4.12D-F). Thus, activation of DNp06 neurons can stop the execution of innate behaviours like courtship and grooming whilst simultaneously triggering escape behaviour.

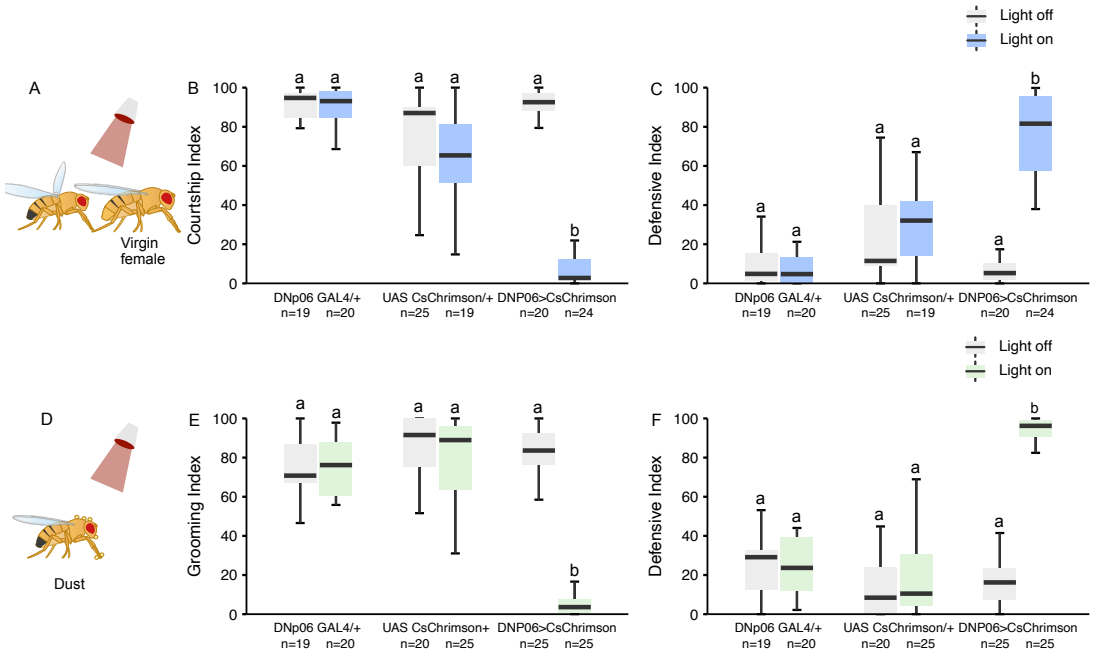


Figure 4.12: Threat-driven DNp06 activation suppresses competing behaviours. (A-C) DNp06 neurons were activated in fed males courting a wild-type Canton-S female. (B) Courtship Index or % time spent courting the wild-type female during the stimulation period. (C) Defensive Index or % of time spent exhibiting freezing or escape behaviour. (D-F) DNp06 neurons were activated in fed males dusted with Brilliant Yellow dye. (E) Grooming Index or % time spent grooming during the stimulation period. (F) Defensive Index or % of time spent exhibiting freezing or escape behaviour. Statistical analyses using Kruskal-Wallis followed by Dunn Test in B-C and E-F. The sample size (number of flies) is indicated below the boxplots. Box plots labelled with different letters are statistically significantly different.

4.4.10 Down-regulation of receptors for neuro-peptides/neurotransmitters involved in feeding in DNp06 neurons does not affect the feeding-escape behavioural choice.

My findings suggest that DNp06 neurons might stop any behaviours the fly is displaying and elicit escape in the face of threats. I hypothesised that DNp06 neurons could be suppressed when facing severe starvation helping flies to prioritise feeding over escape. To test this, I performed a RNAi screen where I down-regulated receptors of neuro-peptides and neuro-transmitters associated with feeding including insulin, serotonin, allatostatin A, NPF and dopamine specifically in DNp06 neurons. As a negative control, I knocked down GFP, a non-specific target in DNp06 neurons using the DNp06-GAL4 driver. DNp06>GFP RNAi control males prioritised defensive responses over feeding behaviour (FI = 7.39%, Fig 4.13B). As a positive control, I down-regulated multiple nicotinic acetylcholine receptor (nAChR) sub-units in DNp06 neurons as they receive cholinergic input from LPLC2 neurons ([192], Fig 4.13A). Expression of nAChRα4, nAChRα5, and nAChRα6 subunit RNAi in DNp06 neurons tipped the behavioural choice towards feeding (FI = 83.18% (nAChRα4), 95.77%(nAChRα5), 98.76%(nAChRα6), Fig 4.13B-C). These results confirm that cholinergic input is necessary for the feeding-escape behavioural choice. However, down-regulation of receptors involved in feeding did not alter the behavioural choice in 24h starved flies (Fig 4.13D-E). Therefore, it is possible that the modulation of DNp06 neurons occurs through an upstream neuron, or other neuro-modulators may convey the hunger signal.

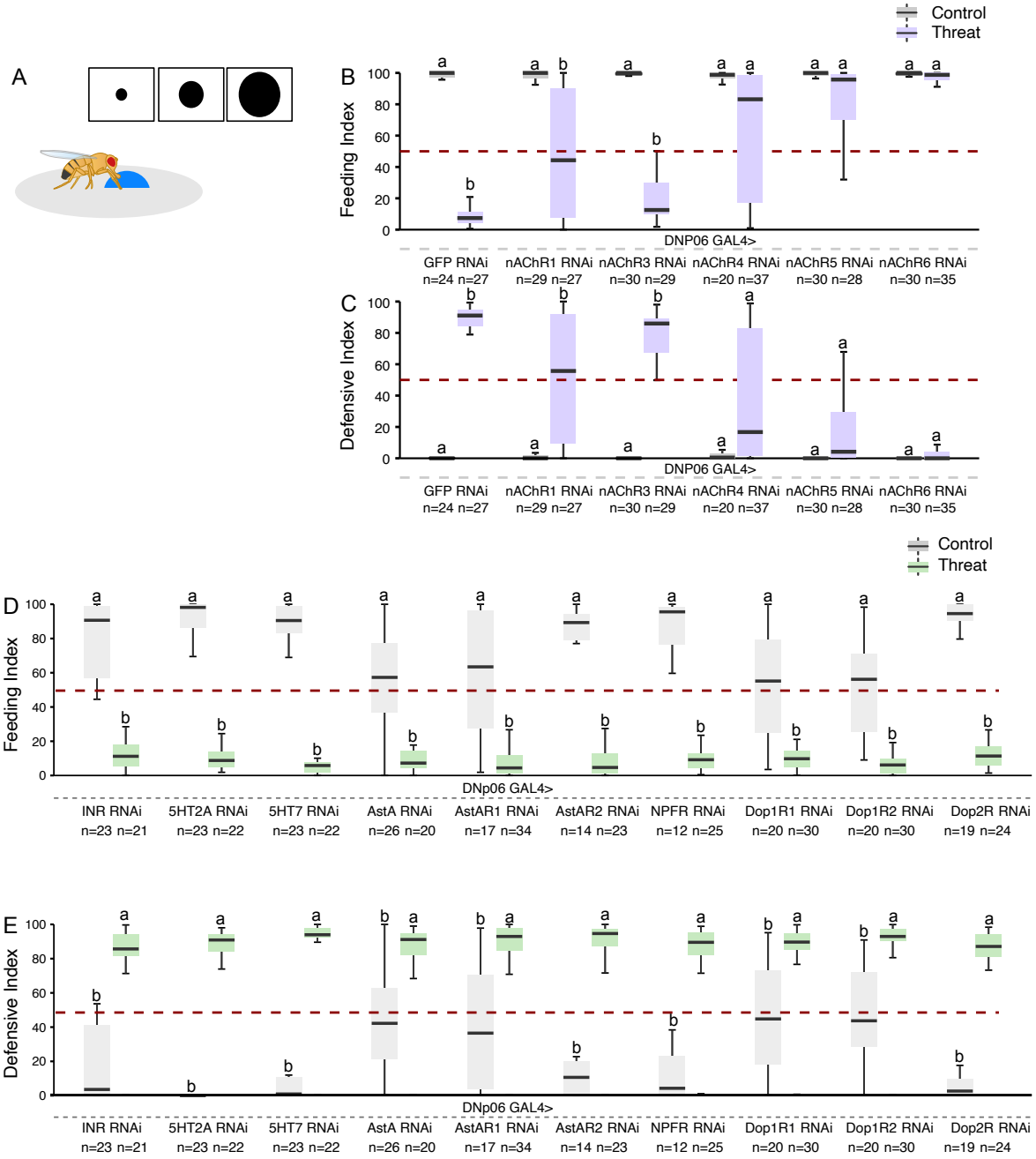


Figure 4.13: Down-regulation of receptors for neuro-peptides/neurotransmitters involved in feeding in DNp06 neurons does not affect the feeding-escape behavioural choice. (A-B) Positive (nAChR RNAi) and negative controls (GFP RNAi) expressed under the DNp06 split GAL4 for the RNAi screen. (B) Feeding Index. (C) Defensive Index. (D-E) Neuro-peptide receptors were down-regulated using RNAis under the control of DNP06 split-GAL4 in 24h starved males and tested in the behavioural choice assay. (D) Feeding Index. (E) Defensive Index. Each experimental group was compared to the control (DNp06>GFP RNAi) using the Wilcoxon test. The sample size (number of flies) is indicated below the boxplots. Box plots labelled with different letters are statistically significantly different.

4.4.11 DNp06 neurons integrate aversive olfactory cues

My findings so far suggest that DNp06 neurons might integrate cholinergic inputs and subsequently induce rapid escape responses when a looming threat is detected. Building on this, I aimed to investigate whether DNp06 neurons could elicit escape responses to other types of threats beyond visual stimuli, such as olfactory or auditory cues. When exploring the Neuprint database, I found DNp06 neurons to receive a substantial number of inputs, amounting to a total of 12670 input connections. Notably, DNp06 neurons receive input from two lateral horn input neurons, LHAV1a1 (21 and 19 connections deceptively) which have been previously implicated in geosmin avoidance [193]. Geosmin is a harmful fungal metabolite and flies avoid this odour in isolation or when presented with food [90].

To test if DNp06 neurons mediate geosmin avoidance, I performed an olfactory trap assay where flies were provided with a choice between vinegar and vinegar paired with geosmin [90]. Genetic controls exhibited a preference for the control chamber, indicating their avoidance of geosmin (Preference index(PI) = -0.5 (DNp06 GAL4/+), -0.412 (UAS TNT/+), Fig 4.14A-B). In contrast, inhibition of DNp06 neurons increased the preference index towards geosmin (PI=-0.095). These results suggest that DNp06 neurons play a significant role in aversion towards geosmin. In further connectome analyses, I identified inputs to DNp06 from the antennal mechanosensory and motor centre (AMMC) region. Consequently, I speculate that DNp06 neurons might initiate escape responses in response to multisensory information. Future experiments should delve deeper into whether DNp06 acts as a multisensory hub, integrating aversive auditory, visual, and olfactory cues to prioritise escape responses over other competing behaviours.

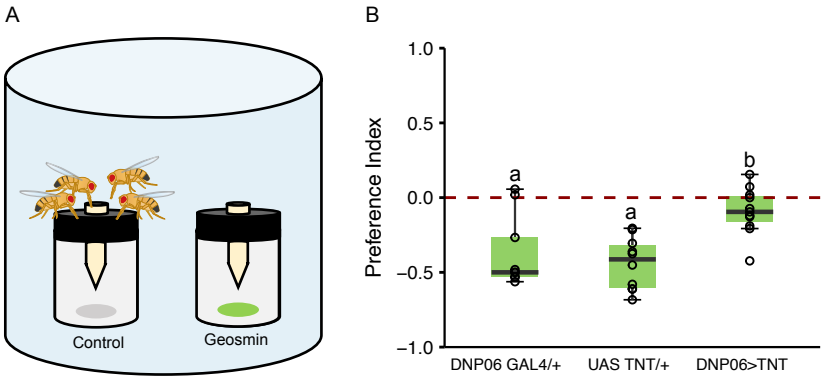


Figure 4.14: Inhibition of DpP06 neurons reduces geosmin avoidance. (A) Fed male flies were given a choice between balsamic vinegar and balsamic vinegar additionally containing 10^{4} geosmin in an olfactory trap assay (schematic on the left). (B) Preference Index (PI) of flies towards geosmin. n=9-11 replicates. Kruskal-Wallis followed by Dunn Test in B. Box plots labelled with different letters are statistically significantly different.

4.4.12 Activation of the feeding motor circuit suppresses the threat response

My findings show that upon threat detection, LPLC2 neurons activate DNp06 neurons, prioritising escape responses over feeding. To further investigate the neural circuit mechanism involved in this behavioural choice, I focused on downstream neurons that might stop feeding in response to the visual threat. In order to do this, I looked into the local feeding circuit in the subesophegal zone (SEZ) downstream of sugar-sensing gustatory receptor neurons [60]. Shiu and colleagues identified 3-4 neuronal relays between GRNs and the feeding motor neurons [60] namely second-order, third-order, and pre-motor neurons. I sought to ask which circuit elements of the feeding program will be inhibited when the flies are exposed to the threat.

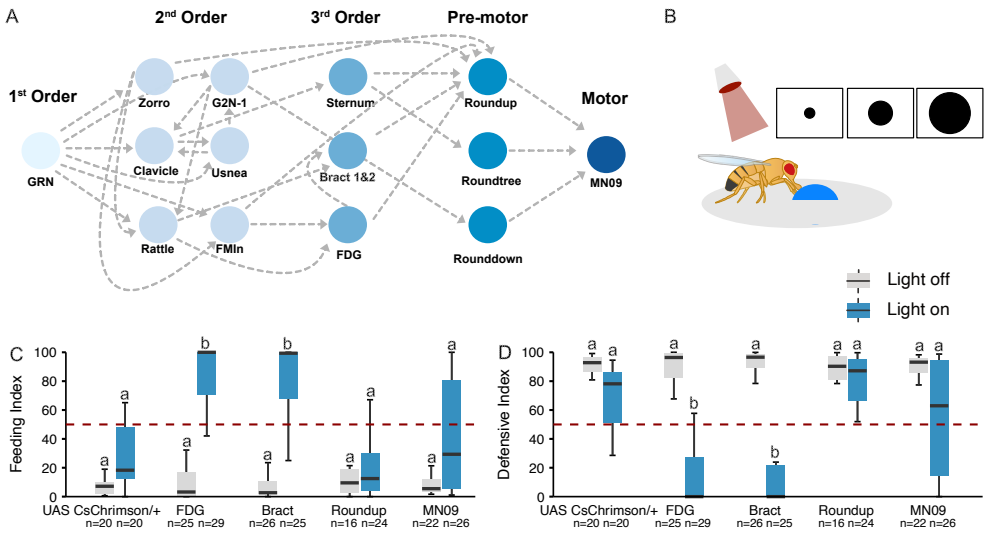


Figure 4.15: Activation of the feeding motor circuit suppresses the threat response. (A) Network graph showing the connections between sugar GRNs and feeding motor neurons (adapted from Shiu et al. [60]). (B-D) Neurons in the feeding motor circuit were activated using CsChrimson in 24h starved males and tested in the behavioural choice assay. (C) Feeding Index. (D) Defensive Index. Each experimental group was compared to the control(UAS CsChrimson/+) using the Wilcoxon test. The sample size (number of flies) is indicated below the boxplots. Box plots labelled with different letters are statistically significantly different.

I hypothesised that the visual threat would suppress the final layers of the feeding motor program to stop feeding behaviour i.e. the motor neurons. If the threat were to inhibit these neurons, artificial activation would overcome this inhibition and the flies would continue to feed during the stimulation period. To test this, I activated the MN09 motor neurons in 24h starved

males in the presence of the visual threat. Surprisingly, activation of MN09 neurons did not alter the response to threat. Specifically, MN09>CsChrimson males did not significantly increase their feeding index when compared to no light controls (FI = 29.34%(light), 5.50%(no light), Fig 4.15B-C). I obtained similar results with activation of pre-motor neurons Roundup (FI = 12.49%(light), 9.54%(no light), Fig 4.15B-C). However, activation of two third-level inter-neurons FDG and Bract was sufficient to trigger feeding behaviour even in the presence of the threat. Bract>CsChrimson males continued to feed and did not show defensive behaviours (FI = 99.36%(light), 2.68%(no light), Fig 4.15). This effect was absent in the genetic controls (Fig 4.16A-E). Considering that FDG neurons send excitatory connections to Bract neurons, it is plausible that the activation of the feeding inter-neurons Bract alone is sufficient to overcome the response to the threat [60].

These findings suggest that Bract neurons might get inhibited upon threat detection, prompting male flies to stop feeding when danger is signalled. To test this idea, I explored the connectome data through the FlyWire Codex platform [19]. I identified the shortest path between LPLC2 and Bract neurons. Here, I discovered that there are two layers of neurons that serve as intermediaries in connecting these neurons. Specifically, I found that LPLC2 neurons send cholinergic input to PVLP137.L neuron (Fig 4.16F). These neurons in turn send cholinergic input to two neurons called horntail (GNG8.11 and GNG.18) in the subesophageal zone (SEZ) (Fig 4.16F). Both horntail neurons send 93 and 97 GABAergic connections to Bract neurons, suggesting that Bract neurons might be inhibited by these Horntail neurons (Fig 4.16F). Therefore, there exists a putative feed-forward inhibitory pathway from LPLC2 to Bract neurons that could suppress feeding behaviour in response to the looming stimulus.

To test if GABAergic inhibition in Bract is necessary for the flies to prioritise feeding over escape, I down-regulated GABA A receptor RDL in Bract neurons in 24h starved males and tested the effects on the behavioural choice (Fig 4.16G-H). Surprisingly, the results indicated that Bract>RDL RNAi flies aborted feeding in response to the threat and their behaviour was in-

distinguishable from that of controls (FI= 21.61%(Bract GAL4/+), 13.63%(UAS RDL RNAi/+), 19.55%(BRACT>RDL RNAi), Fig 4.16G-H). While this could boil down to the inefficiency of the RNAi tool itself, I cannot exclude the role of other GABA receptors in feeding-escape behavioural choice. Future experiments will be essential in identifying whether GABA receptors are at play and exploring the potential functional connectivity between LPLC2 and Bract neurons.

Based on the behavioural data I have collected, I propose a neural mechanism for the feeding-escape behavioural choice in flies. This mechanism involves specific visual projection neurons known as LPLC2 neurons, which are responsible for detecting looming stimuli. These neurons send excitatory cholinegeric signals to two distinct descending neurons called Giant Fibre and DNp06 to trigger escape and jumping behaviour. Additionally, there is a third neural pathway in parallel that inhibits the feeding interneuron known as Bract to suppress feeding behaviour.

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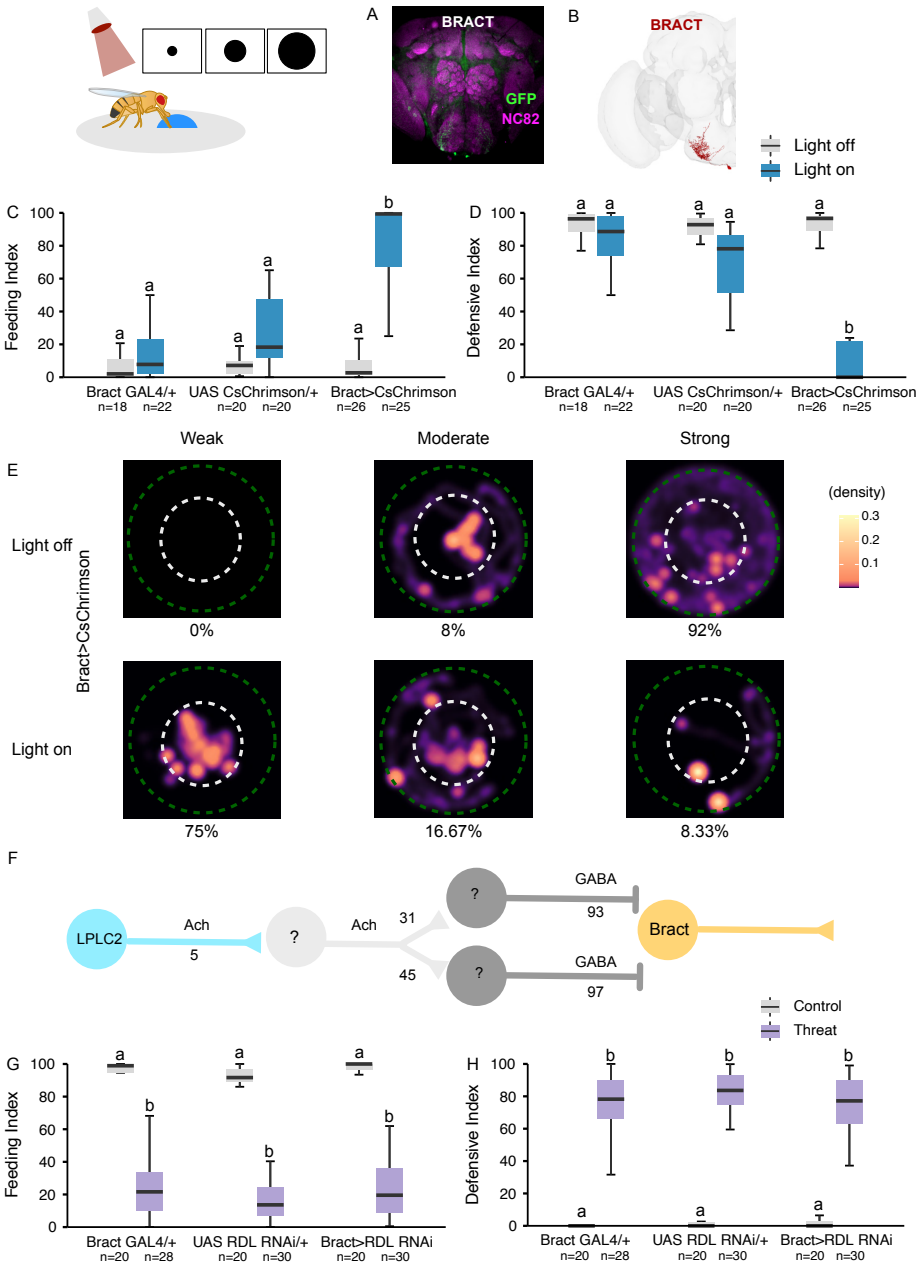


Figure 4.16: Activation of Bract interneuron is sufficient to overcome the threat response. (A-E) Bract neurons in the feeding motor circuit were activated using CsChrimson in 24h starved males and tested in the behavioural choice assay. (A) Expression pattern of Bract neurons. UAS-mCD8-GFP expressed under the control of Bract split-GAL4 in the adult brain. GFP staining (green); neuropil counterstained with anti-nC82 (white). (B) FlyWire reconstruction of Bract neurons [19]. (C) Feeding Index. (D) Defensive Index. (E) Occupancy plots of Bract>CsChrimson flies subjected to threat with and without light. (F) Pathway connecting LPLC2 neurons and Bract neurons. Bract neurons receive inhibitory GABAergic input from two horntail neurons that are downstream of LPLC2. Data from Flywire [19]. (G-H) GABA receptor RDL is downregulated in Bract neurons in 24h starved males and tested in the behavioural choice assay. (G) Feeding Index. (H) Defensive Index. Kruskal-Wallis followed by Dunn Test in B-C and F-G. The sample size (number of flies) is indicated below the boxplots. Box plots labelled with different letters are statistically significantly different.

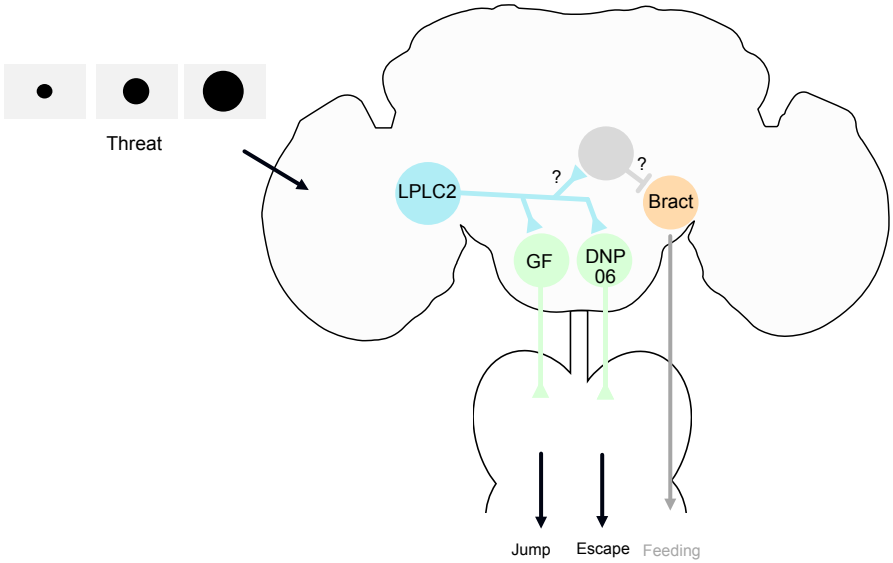


Figure 4.17: Neural mechanism underlying the choice between feeding and defensive behaviours. LPLC2 visual projection neurons detect looming stimuli and subsequently activate two distinct descending pathways involving Giant Fibre and DNP06 neurons. This coordinated activation leads to an escape and jump response. Additionally, these neurons inhibit the feeding interneuron Bract, effectively suppressing feeding behaviour.

4.5 Discussion

4.5.1 The choice between feeding and escape

Maslow's hierarchy of needs theory posits that basic physiological needs (like hunger, thirst, and sleep) must be fulfilled before higher safety needs (threat detection and avoidance) can be met [179]. However, my results showed that in *D. melanogaster* males starved for 24 hours, defensive behaviours take precedence over feeding. Both 24h starved mated and virgin females exhibited a similar response to males in the presence of a visual threat, indicating that behavioural choices are not sex-specific in this context. The primary defensive behaviour exhibited by the flies in this choice scenario is escape, rather than freezing. Males spent 68.76% of their time escaping, whereas mated and virgin females dedicated 71.50% and 70.49% of their time to escape, respectively. This is in contrast to the findings of Zacarias et al. 2017, who reported that flies spent approximately 75% of their time freezing when confronted with a looming stimulus [73]. The authors argue that flies only exhibit freezing when they learn that escape is not possible within the arena. Therefore, it is crucial to consider the disparities in experimental design between their study and mine. Zacarias and colleagues allowed fed flies a 5-minute habituation period before exposing them to looming stimuli over 5 minutes, in the absence of any sensory cues. In contrast, the looming protocol I employed was acute, lasting only 30s, and the habituation period varied due to differences in feeding latency among individual flies (between 5s-120s). It is possible that in a shorter habituation time, flies do not become aware that the chamber is inescapable and hence do not freeze. Consistent with this, my experiments involving solitary flies indicate that providing a brief habituation time of 10 seconds does not result in an increase in freezing behaviour. However, when a habituation time of 180 seconds is given, the flies exhibit freezing behaviour.

The prioritisation of defensive behaviour over feeding is not unique to the looming stimulus. Gibson et al. found that 24h starved flies leave the food patch in response to a translational visual threat [74]. Similarly, flies avoid food laced with geosmin, a harmful fungal metabolite [90]. This suggests that fruit flies prioritise defensive behaviours when facing threats, regardless of whether

they are visual or olfactory in nature.

To explore how different species respond to the behavioural choice, I used three different *Drosophila* species. I found that all the species stopped feeding in response to the threat and showed defensive responses suggesting that the prioritisation is not specific to *D. melanogaster.* However, *D. simulans* showed a slightly higher feeding index when compared to the other species. Given the small size of *D. simulans*, it is possible that they might be less tolerant towards starvation pressure when compared to *D. melanogaster*. However, this needs to be empirically tested.

When examining the defensive responses in the choice context, I observed that *D. melanica* and *D. erecta* flies mainly showed freezing behaviour in response to the loom. This is in contrast with *D. melanogaster* which displays escape behaviour. Zacarias et al. showed that the walking speed before the threat delivery is an also important determinant of the kind of defensive behaviour elicited [73]. Increased walking speed is associated with escape behaviour while slower speeds with freezing [73]. In line with this, I found *D. erecta* and *D. melanica* to be slow when measuring their baseline locomotion. The root cause of this lethargy remains unknown—whether it stems from the quality of the food provided in laboratory conditions that is optimised for *D. melanogaster* or from real behavioural differences. For instance, *D. erecta* specialises in feeding on *Pandus* fruits and may not thrive well on cornmeal agar media conventionally used for *D. melanogaster*. Future experiments will need to focus on developing new recipes optimised for each species to validate these observed differences and confirm their species-specific nature.

4.5.2 The neural mechanism underlying the behavioural choice

My findings suggest LPLC2 visual projection neurons (VPNs) act as the key players in the feeding-escape behavioural choice. In response to threat, activation of LPLC2 neurons triggers escape and jumping helping the flies to prioritise defensive behaviours over feeding. This is consistent with the previous studies done by Card lab [82, 83] which found an increase in the execution of jumps upon LPLC2 activation. While there are multiple VPNs that can detect loom-

ing stimuli, inhibiting these other VPN candidates did not produce any observable changes in behaviour. This suggests that LPLC2 neurons may be particularly important for detecting threats, while the other VPNs may not be essential for this specific context.

Given that LPLC2 activation triggers two kinds of defensive behaviours, I predicted that there are two downstream neural pathways that trigger each behaviour. In line with this, I found two descending neurons with distinct roles: (1) GF neurons that mainly trigger jumping and (2) DNp06 neurons that trigger escape. Inhibition of the DNp06 neurons, but not the GF neurons, led to a shift in behaviour towards feeding, suggesting that DNp06 neurons play a bigger role in the behavioural choice. Conversely, activation of DNp06 neurons caused the flies to stop feeding (and other innate behaviours such as courting and grooming) and initiate an escape response. A recent study by Kim et al. further supports the role of DNp06 neurons, particularly in regulating escape turns in flies in response to a loom when tethered to a flight simulator[194]. However, it's important to note that when no food was present, DNp06 neuron activation resulted in only a temporary increase in velocity, contrasting with the sustained effect observed with LPLC2 activation. It is possible that the concurrent activation of both Giant Fibre and DNp06 neurons may be necessary to produce a sustained increase in velocity. Additionally, it's worth considering the potential involvement of other descending neurons, such as DNp04, in bringing in the velocity component during escape behaviours.

Downstream of the escape circuit, my data shows that third-order feeding inter-neurons could be inhibited in response to threat allowing the animal to stop feeding and engage in escaping. Moreover, inhibition of neurons expressing neuropeptides such as DILPs, MIPs and Lk had no effect (data not shown), suggesting that inhibition of feeding is likely to happen at the motor circuit in the SEZ. Specifically, activation of Bract or FDG neurons in 24h starved flies triggered feeding behaviour and prevented the execution of defensive behaviours in the presence of a threat. Given that FDG neurons directly connect to Bract neurons (38 connections to Bract 1 and 11 connections to Bract 2), I predict that activation of Bract neurons is sufficient to overcome the

threat response. Moreover, FlyWire data indicates that Bract neurons receive GABAergic input from two neurons called "horntail" downstream of LPLC2 neurons, further supporting their role in the feeding-escape behavioural choice [19]. One way to test if this feed-forward inhibitory pathway is part of the mechanism is to activate Horntail neurons or their upstream neurons and see if that decreases feeding in flies. Interestingly, Bract neurons are descending neurons that project to the ventral nerve cord (VNC) [60]. As Bract neurons send information to feeding pre-motor neurons and the VNC, I hypothesise that they contribute to the coordination of the feeding motor program by reducing locomotion in flies whilst simultaneously triggering proboscis extensions. When presented with a visual threat, it is possible that Bract neurons are initially inhibited before the escape pathway via DNp06 is activated.

To validate the connections between these neurons, functional connectivity experiments need to be performed where LPLC2 neurons are activated and the resulting changes in activity levels in Bract neurons are measured. This can be achieved by two-photon live imaging where LPLC2 neurons are activated using CsChrimson whilst simultaneously measuring GCaMP signal in Bract neurons. In a complementary approach, the activity levels of Bract neurons can be measured when flies are presented with a looming stimulus. A new tool established by the Haitori lab will allow us to monitor changes in neural activity using immunostaining and confocal microscopy [195]. They used a GFP-tagged version of CRTC protein that translocates to the nucleus upon activation [195]. By comparing the signal in the nucleus and the cytoplasm, we can test if a neuron is activated by an upstream neuron or a sensory cue [195]. By combining connectomic approaches with the above-mentioned imaging techniques, we will be able to test if the Bract/ DNp06 neurons are indeed functionally connected to LPLC2 neurons and help us connect the sensory systems to the motor output.

4.5.3 The integration of the hunger signals

My findings show that the choice between feeding and defensive behaviour is flexible. As the time of starvation increases, both male and female flies are more likely to continue to feed and

ignore the visual threat. This suggests as the need to feed becomes more urgent, flies are able to modulate their behaviour to tip the balance towards feeding. In line with this, a study by Burnett and colleagues showed that hungry mice overcome their fear of a predatory odour to start feeding [185]. Similarly, hungry gerbils continued to feed in the presence of their predator owl while fed gerbils did not [196]. This suggests that behavioural hierarchies are not necessarily fixed, and depend on the internal state of the animal.

Unlike the male flies, both virgin and mated females required longer starvation time to alter their behavioural prioritisation towards feeding. Female flies consistently prioritised defensive behaviours over feeding up to 48 hours of starvation but showed an increase in feeding behaviour after 60 hours. This time lag in modulation could be partly due to the differential effects of starvation on both sexes. Female *D. melanogaster* flies have more fat reserves than males and metabolise them slower, making them more resilient towards starvation [191]. Previous research has identified adipokinetic hormone (Akh) as a crucial regulator of fat metabolism [191]. Akh is produced by Adipokinetic-producing cells in the central brain (APCs) and released into the hemolymph [191]. It further binds to receptors in distant targets like fat bodies to promote fat breakdown [191]. Wat et al. found that the expression of female-specific transcription factor TRA^F protein in APCs is necessary for increased fat storage in females [191]. This prompts an intriguing question: could the feminisation of APCs in males lead to a shift in their behavioural prioritisation similar to that of females? Conversely, would masculinisation of APCs (via Dsx^M) would make females behave like males? One hypothesis is that differences in fat storage eventually lead to differences in circulating sugar levels in the hemolymph, which could be sensed by neurons in the brain. When the sugar levels are low due to starvation, the nutrient deficit can be detected by "sensor" neurons that can suppress the escape pathway in the cases of extreme starvation, activate the feeding pathway or both. In line with this, Miyamoto et al. discovered gustatory receptor 43a in the fly brain, which responds to fluctuations in fructose levels in the hemolymph [197]. Alternatively, gut endocrine neurons can sense the decrease in nutrient levels and send the signal to the central brain [59]. Future experiments will determine which neurons in the central

brain receive the hunger signal and further relay it to the feeding-escape neural circuitry.

A second hypothesis is that starvation levels alter the synthesis of neuro-modulators through specific enzymes. For instance, Tdc2 and Tbh enzyme levels change in response to starvation leading to decreased tyramine and increased octopamine in the fly brain [2, 70]. These neuro-modulators then can differentially activate downstream targets and change their activity levels. Using FlyWire, I have identified a promising candidate, AD1g1 lateral horn output neuron which could be modulated by these neuromodulators and play a pivotal role in conveying hunger status [19]. FlyWire predictions suggest that AD1g1 neurons are GABAergic in nature (NT predictions ACH: 0.07, DA: 0.02, GABA: 0.68, GLUT: 0.21, OCT: 0.02, SER: 0.01) (Fig 4.18). These neurons receive input from LPLC2 neurons (totalling 716 connections) and subsequently relay information downstream to both Giant Fiber and DNp06 neurons (with 130 and 424 connections, respectively) (Fig 4.18). Notably, they also receive input (56 connections) from octopaminergic neurons, specifically 13 connections from OA-VPM2, 13 from OA-VUMa8, 17 from OA-AL2b1, and 5 from OA-VUMa1 (Fig 4.18). Compiling all this information together, I hypothesise that activity levels of AD1g1 neurons could be modulated by hunger and thereby change the valence of circuitry. In 24h starved flies, the activity levels of AD1g1 neurons are low presumably due to low levels of octopamine, therefore GF and DNP06 neurons receive less inhibition from AD1g1 in response (Fig 4.18). In 48h starved males, AD1g1 activity levels increase, therefore both descending neurons are less likely to be active (Fig 4.18).

To investigate the role of AD1g1 neurons further, I used the BrainCircuits plugin within FlyWire to identify potential GAL4 drivers that could label these neurons [19]. The results indicate that VT041688-GAL4 may label LHAD1g1 neurons, among others. To further narrow down these neurons, VT041688-DBD can be combined with GAD-AD to create a split GAL4 that intersects GABAergic neurons and neurons labelled by VT041688-GAL4. Once this split GAL4 line is established, we can assess the neurons' impact on behaviour through inhibition or activation experiments. We can further test how the activity levels of AD1g1 neurons or the downstream

descending neurons (GF, DNp06) change in response to starvation using functional imaging or the CRTC:eGFP tool [195]. Importantly, these experiments can be done using both males and females to understand whether their activity levels are differentially modulated in both sexes.

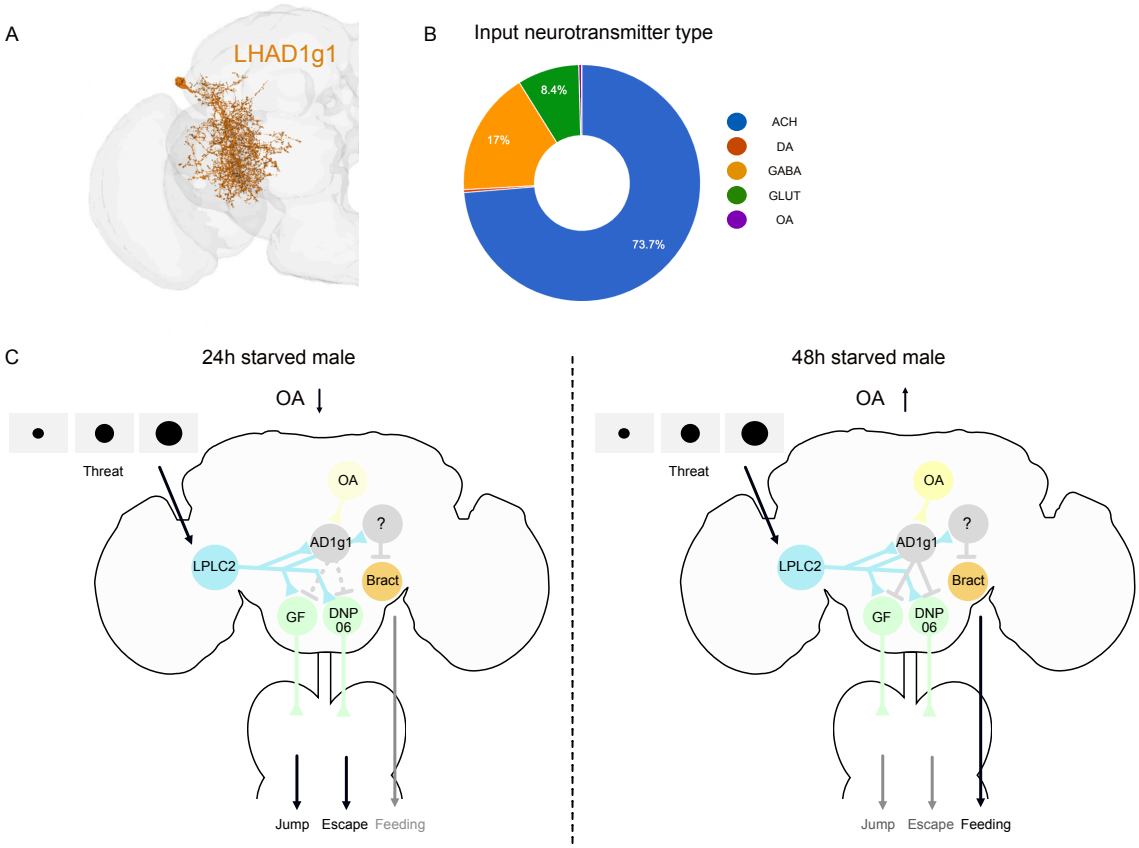


Figure 4.18: Proposed model for integration of hunger state in the neural circuit underlying the behavioural choice. (A) FlyWire reconstruction of AD1g1 neurons [19]. (B) Neurotransmitter type predictions for AD1g1 neurons. (C) The activity levels of lateral horn output neurons Ad1g1 could be altered by octopaminergic neurons, which may subsequently impact the overall valence of neural circuitry. Ach = Acetylcholine, DA = Dopamine, GABA = Gamma-aminobutyric acid, GLUT = Glutmate, OA = Octopamine.

Studies using mammalian models have provided valuable insights into how metabolic signals conveying the hunger status can reach the brain and influence the activity of the neurons. In mice, AGRP neurons have been identified as crucial mediators of hunger-related behaviours like increased food intake. Kong et al. showed that starvation induced an increased number of dendritic spines, accompanied by elevated firing levels in AGRP neurons [198]. The authors further demonstrate that the activation of AMP-activated protein kinase (AMPK) is sufficient to induce the observed morphological and physiological changes in these neurons [198]. They hypothesise

that hunger increases the levels of neuro-hormone ghrelin in the blood which leads to increased calcium levels in AGRP neurons, thereby activating AMPK via Ca(2+)/calmodulin-dependent protein kinase kinase-β. Similarly, *Drosophila* AMPK (dAMPK) has been shown to play a role in metabolism and feeding behaviour [199]. Reduction in dAMPK levels using RNAi or null mutants has been shown to increase persistent feeding behaviour in adult flies [199]. However, whether dAMPK is expressed in the neurons crucial for the feeding-escape behavioural choice remains to be explored.

4.5.4 DNp06 as an integrative node for escape

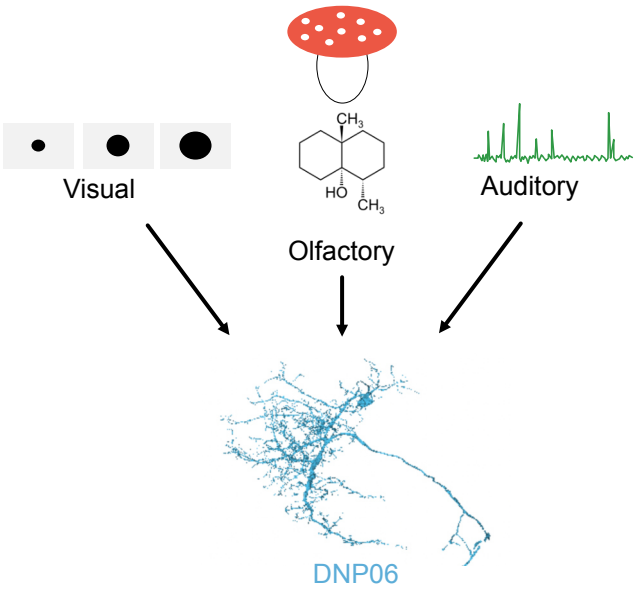


Figure 4.19: DNp06 as an integrative node for escape. DNp06 neurons receive information from visual, olfactory and auditory neurons. Data from FlyWire [19].

My findings indicate that DNp06 receives input from visual projection neurons specialised in detecting visual threats. These neurons receive input from lateral horn output neurons, specifically AV1A1, which bring in information about geosmin, a noxious fungal metabolite [193]. Upon further exploration, I found that DNp06 neurons receive input from neurons in the primary auditory centre, AMMC A1 and AMMC B1 (Fig 4.19). This intriguingly raises the possibility that DNp06 functions as an integrative node capable of eliciting escape responses across various sensory modalities (Fig 4.19). While Arez et al. showed that male courtship songs can trigger escape

behaviour in *Drosophila* females, it remains unknown whether acoustic cues can be aversive in other contexts [200]. Future experiments will shed light on how multi-modal integration happens in DNp06 neurons to trigger defensive behaviours. An analogous circuit is found in Zebrafish, where Mauthner cells trigger the "C-start" escape response by integrating auditory, tactile, and visual stimuli [201]. Similarly, VTA-glutamate neurons in mice respond to both looming stimulus and TMT, a predator odour to trigger escape responses [202]. These studies highlight the existence of analogous neural nodes that integrate multiple aversive cues across different species.

4.5.5 Limitations

To investigate the choice between feeding and escaping, I employed a looming stimulus as a visual threat. This stimulus allowed me to measure defensive behaviours in solitary flies automatically. However, I encountered challenges when attempting to quantify feeding and escape behaviours in the presence of a food source. The quantification of feeding behaviour can typically be accomplished using electrical measurements, such as those obtained with the flyPAD system [58]. Unfortunately, the flyPAD devices use metallic electrodes in the chamber's base, making it difficult to simultaneously track the fly's movements, which are crucial for accurately quantifying defensive behaviours. Therefore, I manually annotated behaviour using BORIS software which is time-consuming. In order to classify feeding and defensive behaviours automatically, the development of a modified flyPAD device is necessary. In this modified setup, the electrodes would be positioned on the side walls of the chamber, while the base would be transparent, enabling the tracking of fly locomotion. Due to time constraints during my PhD research, I was unable to implement this modified setup. Nonetheless, future studies can take advantage of this modified apparatus to obtain automated and unbiased behavioural data.

Secondly, the flies were exposed to 10 looming stimuli within a 30s time frame in my behavioural choice assay. Each stimulus expanded rapidly at a rate of 25 cm/s, which can be considered relatively fast. This gave us a robust behavioural response where the feeding index of flies that had been starved for 24 hours drastically decreased to approximately 20% in the presence of a

threat. It is plausible that the visual stimuli used in this experiment were excessively intimidating for the flies, potentially causing us to overlook subtle behavioural changes.

I used connectomic approaches to identify downstream targets of LPLC2 visual projection neurons: GF and DNp06 and delineate feed-forward inhibitory pathways between LPLC2 and Bract neurons. It's important to note, however, that our current connectomic dataset is derived from the female brain. Therefore, I anticipate the need for validation of my findings once corresponding data from the male brain becomes available. Moreover, in order to gain a comprehensive understanding of the neural circuitry underlying feeding-escape behavioural choice, future experiments will need to confirm the connectomics data with functional imaging.

4.5.6 Summary

In summary, here in this Chapter, I have developed a new behavioural assay to study the choice between feeding and escape. I have shown that 24h starved males and females prioritise defence over feeding. Moreover, the hunger state of the fly is an important determinant of this choice. In response to starvation, female flies take longer times to modulate their behaviour when compared to males. Finally, I have identified a neural circuit connecting the visual projection neurons LPLC2 with feeding and escape motor programs.

5 Discussion

5.1 Behavioural hierarchy in flies

When faced with conflicting options, animals need to prioritise one behaviour over the other. To effectively resolve such conflicts, an animal's behaviour is organised in a series of priority sequences or a behavioural hierarchy [203]. Based on both existing evidence in the literature and the results of my own experiments, I have attempted to establish a behavioural hierarchy for flies. In *D. melanogaster*, defensive behaviour takes precedence over all other behaviours (Fig 5.1). In Chapter 4, I demonstrated that when flies are exposed to a looming stimulus, defensive behaviours are prioritised over feeding (Fig 5.1). Additionally, work done by Dr Laurie Cazale Debat in our laboratory has shown that defensive behaviours also take precedence over mating in male flies (unpublished data). This suggests the avoidance of immediate harm takes precedence over the fly's drive to feed or mate.

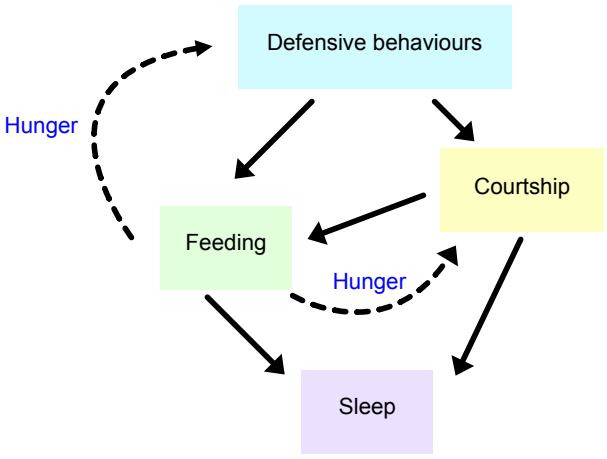


Figure 5.1: Behavioural hierarchy in flies. Unidirectional arrows show the dominance of one behaviour over another. Dotted arrows indicate context-specific dominance (i.e. hunger state)

Upon reaching reproductive maturity, adult flies focus all their energy on finding a mate. Consistent with that, I observed that fed flies prioritise courtship over feeding (Fig 5.1). Even when confronted with a deadly infection, male flies continue to court a female until they are physically

unable to continue. Additionally, previous studies have indicated that male flies sleep less in the presence of a potential mate [204]. However, these behavioural hierarchies are not fixed, as starvation tends to shift the prioritisation towards feeding. In mice, feeding and sleep take precedence over threat avoidance and social behaviours like mating, parenting and cooperation [179]. Thus, different species have evolved unique behavioural hierarchies, shaped by their evolutionary history and ecological niches.

5.2 Effects of internal state on behavioural prioritisation

To produce the most appropriate behavioural output, an animal must take into account not only its external environment but also its own internal state, which includes factors such as hunger, mating status, overall health, stress and sleep patterns. In Chapter 2, I manipulated the health status of the fly and examined its impact on reproductive behaviours. I exposed the flies to six distinct bacterial infections, three of which led to rapid mortality. My findings reveal that infection with bacterial strains, whether pathogenic or not, does not decrease courtship behaviour among males from both wild-type strains, Canton-S and Dahomey. These results are particularly interesting considering that infection is known to disrupt fly metabolism and nutrient reserves [173]. To compensate for the energy decline, it is plausible that resources are allocated only to vital behaviours, with certain behaviours being prioritised while others are compromised. For instance, bacterial infections are shown to reduce sleep in flies, yet courtship behaviour remains unaffected. This highlights the importance of courtship behaviours which is necessary for flies to pass on genes and increase their fitness.

In Chapters 3 and 4, I manipulated the hunger status of the fly and assessed the effects on behavioural choices. As the degree of starvation intensified, the flies demonstrated a clear shift in prioritising feeding-related behaviours over courtship or defensive behaviours. Feeding overrides courtship behaviour when male flies are starved for 15h or longer. Similarly, male flies starved for 48 hours prioritise feeding over escape. It is worth noting that the shift in behaviour towards feeding occurs at different time points depending on the conflict. Overcoming responses to aver-

sive stimuli, such as visual threats, may require a more prolonged period of starvation pressure compared to responses to appetitive stimuli, such as virgin females. This phenomenon is not unique to flies and is observed in other animals. For instance, starved mice prioritise feeding over mating with females and even remarkably overcome avoidance of fear-inducing predatory odours to initiate feeding [179]. Hungry Zebrafish larvae exhibit heightened sensitivity towards prey-like objects while reducing their avoidance of potential threats [205]. Therefore, my results show that the internal state of an animal, particularly hunger, plays a huge role in shaping their behavioural choices.

Throughout my thesis, I focused on manipulating one internal state at a time. However, an intriguing question arises when we consider the interaction of multiple internal states. For instance, how do males who are both starved and infected respond when faced with the choice between feeding from a nutritious food source and courting a female? While uninfected starved males tend to prioritise feeding, it raises the question of whether infected males, facing a potentially shorter lifespan, might opt for courtship as their first choice. Will infected males display more risky behaviours when presented with a choice between feeding and escape? Future experiments will provide us with insights into the complex interactions between these internal states.

5.3 Circuit Architecture

One of the key objectives of my thesis was to understand the structural organisation of neural circuits underlying behavioural choices. In the introduction of my thesis, I presented two competing models of decision-making: the reflexive model and the dynamical model. The reflexive model suggests that inhibitory connections between command neurons dedicated to each behaviour dictate the behavioural prioritisation [8]. In contrast, the dynamical model posits that there are no designated neurons for each behaviour, instead, activity patterns within a set of attractor neurons govern the selection and execution of a particular behaviour [8]. Based on the behavioural data I have collected, I can conclude that the evidence mainly supports the reflexive model over the dynamical model. In particular, *Drosophila* has dedicated command neurons that trigger specific

behaviours. These command neurons exhibit activity patterns that align with the execution of a particular behaviour while remaining inactive during the execution of other behaviours. For example, P1 command neurons respond to female chemical cues and trigger courtship behaviours. These neurons are active during courtship but not while feeding. Similarly, flies have dedicated descending neurons for escape responses and feeding behaviours. Activation of DNp06 neurons triggers escape behaviours while preventing the execution of other innate behaviours like feeding, courtship and grooming. While the exact inhibitory connections between these command neurons remain to be identified, future studies will provide valuable insights into the precise mechanisms underlying the reflexive model.

Tye 2018 proposed several kinds of neural motifs for resolving conflicting stimuli namely: Labelled Lines, Opposing Components, and Divergent Path motifs [206]. Within the Labelled Lines motif, two sensory stimuli undergo processing through separate parallel pathways that operate independently to drive distinct behaviours [206]. In contrast, the opposing components motif involves different sensory stimuli projecting to the same downstream target [206]. Lastly, the divergent path motif receives identical sensory input and subsequently diverges this information towards two or more distinct downstream targets [206]. My data suggest that divergent path motif is a key feature in both of the decision-making processes discussed in Chapters 3 and 4 (Fig 5.2). In line with this concept, my data suggests that the visual threat information detected by LPLC2 neurons is transmitted to two descending neurons, Giant Fiber and DNp06, forming a feed-forward excitation motif (Fig 5.2). Additionally, my findings suggests that the information is also conveyed to Bract feeding inter-neurons through feedforward inhibition (Fig 5.2). The divergent path motif provides flexibility, as the weights or strengths of connections between the downstream targets can be modified. For instance, the weights of the descending neurons can be decreased when an animal is hungry, thereby suppressing the escape pathway. A similar pattern is observed in the neural circuitry responsible for the choice between feeding and courtship behaviours. The perception of sugar activates the feeding-promoting $TyrR^{PLP}$ neuron while simultaneously inhibiting the P1 neurons (Fig 5.2). Therefore, the divergent path motif is instrumental

in triggering one behaviour while actively preventing the execution of another.

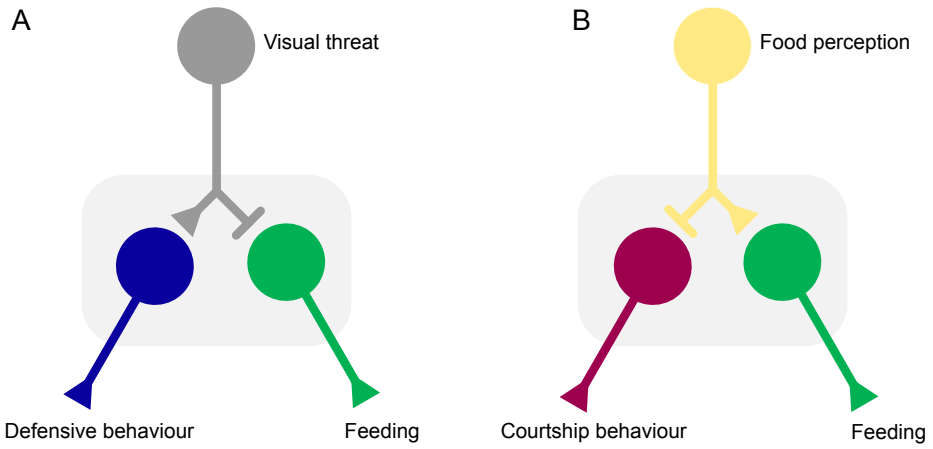


Figure 5.2: Divergent path motif among multiple behavioural conflicts, this motif receives identical sensory input but diverges this information towards two or more distinct downstream targets [206].

Furthermore, it's worth noting that flies continue to exhibit escape behaviour even after the looming stimulus presentations have concluded. Notably, female flies require approximately 20 seconds to resume feeding after the stimulation period has ended suggesting that the response to the looming stimulus is not a simple stimulus-reflex reaction, but leads to a sustained defensive state. This raises an intriguing question: what are the neural mechanisms responsible for maintaining this prolonged defensive state? To test this, I used the FlyWire database and looked for recurrent loops within the DNs [19]. For instance, the Giant Fibre (GF) neuron communicates with the GF input neuron, which in turn sends 210 connections with DNp06 neurons (Fig 5.3). Conversely, DNp06 neurons transmit information back to the GF input neuron, which subsequently relays information to the GF neuron itself (Fig 5.3). While the GF input neuron does not receive visual input from LPLC2 neurons themselves, this intricate network will allow threat-related information originating to form a feedback loop within the descending neurons, potentially enabling flies to sustain their persistent defensive state (Fig 5.3). Gibson et al. argue this prolonged defensive state can be likened to primitive fear-like behaviour [74]. In a state resembling fear, animals often display heightened sensitivity to sensory cues, reduced sleep duration, and decreased social interactions [72]. Future experiments will shed light on whether exposure to threats has a lasting

impact on these critical behaviours in flies, akin to the effects observed in humans.

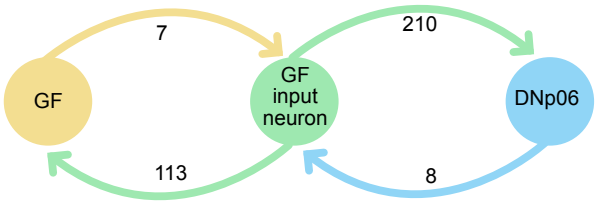


Figure 5.3: Recurrent loop between GF and DNp06 via GF input neuron. Data from FlyWire [19].

My data also raises the possibility of reciprocal inhibition motifs within neural circuits. These motifs involve mutually inhibitory connections between two neuronal populations, either directly or through the involvement of a third intermediary population of inhibitory neurons. Furthermore, reciprocal inhibition prevents the expression of one behaviour while facilitating the execution of another. For instance, Mohamed et al. observed that GABAergic lateral horn local neurons in flies play a crucial role in directing the balance between approach and avoidance when presented with a mixture of attractive and repulsive odours [207]. When the concentration of the attractive odour is high, flies tend to approach it [207]. However, when the concentration of the attractive odour is low, the flies choose to avoid the mixture [207]. This flexible behavioural choice is influenced by the inhibitory nature of the lateral horn local neurons, which modulate the behavioural response accordingly. A similar mechanism could operate in the choice between feeding (an attractive cue) and escaping from a threat (an aversive cue). To test this, I explored the shortest paths between DNp06 and Bract using FlyWire [19]. Interestingly, I found both Bract and DNp06 received GABAergic connections via intermediary neurons (Fig 5.4). These findings indicate that the feeding interneuron Bract, and escape descending neuron DNp06, may engage in reciprocal inhibition (Fig 5.4).

My findings reveal that flies rely on the LPLC2-DNp06 feed-forward excitatory connections to initiate escape when detecting looming objects. Upstream of LPLC2 neurons are T4/T5 neurons that receive information about the changes in luminance [208]. Furthermore, Cheong et al. showed that DNp06 neurons send projections to the lower tectulum (LTct) and hindleg neuropils

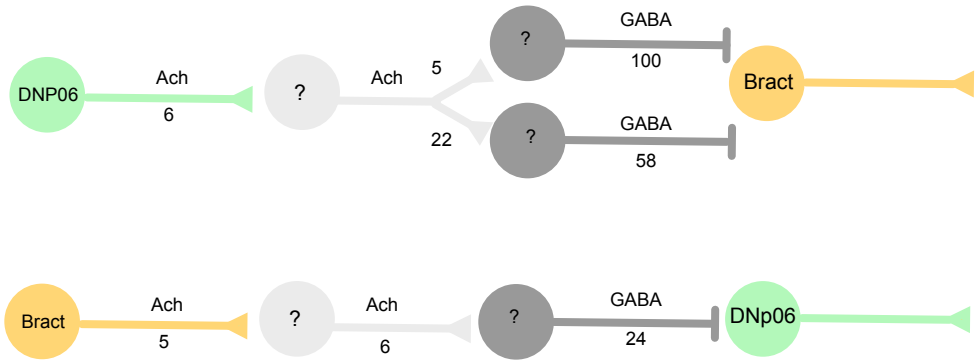


Figure 5.4: The reciprocal inhibition motif between Bract and DNp06. Reciprocal inhibition motif involves mutual inhibitory connections between two neuronal populations, either directly or through the involvement of a third intermediary population of inhibitory neurons. There are putative pathways between DNp06 and Bract suggesting activation of one neuron inhibits the other neurons. Data from Flywire.

(LegNpT3) in the VNC [209]. How these neurons connect with $\sim$ 70 motor neurons in the legs to trigger escape responses remains unknown. The information from optic lobes to motor neurons in the VNC must form long-chain feed-forward networks to trigger quick defensive responses. This data adds to an interesting similarity in the escape pathway across species. For instance, in Zebrafish, the detection of a threat activates a feed-forward circuit in which the Mauthner cells relay the signal to nMLF cells through Cranial Relay Neurons (CRNs), ultimately triggering C-starts as an escape response [201]. In locusts and crickets, a similar pattern is observed in their descending escape pathways [186]. Mammalian brains also employ feed-forward circuits in threat detection, where information flows from photo-receptors to retinal ganglion cells via bipolar cells. Additionally, in mice, the excitatory connections from the medial superior colliculus to the dorsal periaqueductal help in orienting towards safety and facilitating escape [210]. Therefore, long-chain feed-forward loops are crucial for the rapid transmission of information following the perception of a threat and execution of defensive behaviours.

5.4 Conclusion

To conclude, I have thoroughly examined behavioural choices in conflicting scenarios in my thesis. My findings indicate that behavioural choices are flexible and dependent on the internal state of the fly. In particular, the hunger state tips the behavioural prioritisation towards feeding-related behaviours across multiple conflicts. By deciphering the neural circuitry connecting sensory input to motor output, my work identifies common neural motifs in the fly brain prioritising actions. Nevertheless, it's crucial to bear in mind that while there may be commonalities, a singular mechanism may not necessarily exist to resolve various behavioural conflicts. Together, these three chapters provide comprehensive insights into the intricate neural mechanisms governing behaviour in response to varying internal states and external challenges.

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6 Appendix

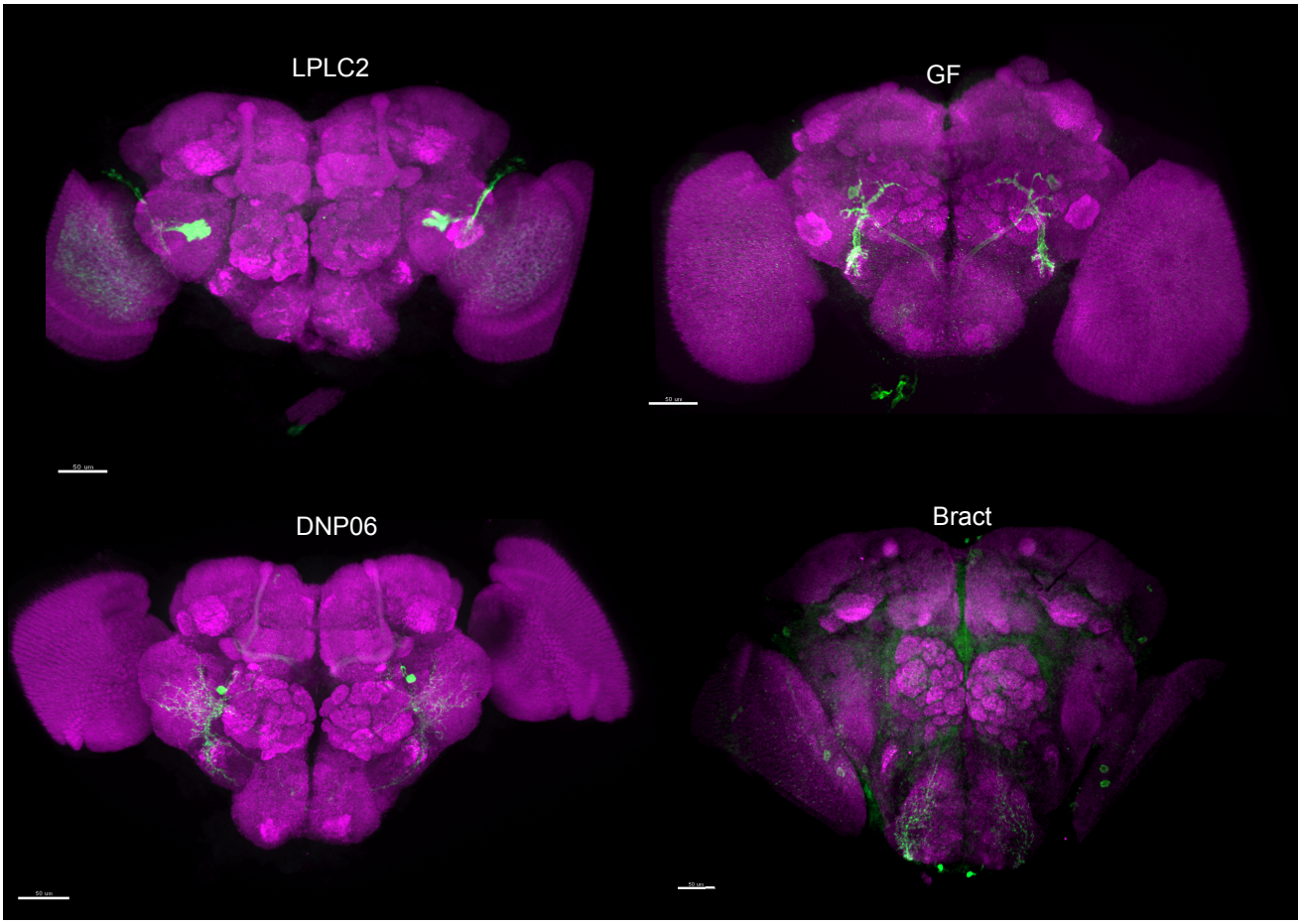


Figure 6.1: Confocal images from Chapter 4. GFP staining (green); neuropil counterstained with anti-nC82 (white).

6.1 R code for analysis

```
library(readxl)
library(plyr)
library(fmsb)
library(tidyr)
library(reshape2)
library(dplyr)
library(readxl)
library(FSA)
```

```
library(betareg)
library(multcomp)
library(gamlss)
library(DHARMa)
library(rcompanion)

# Importing data sets and non-parametric statistics
df <- read_excel("Documents/Feedscape/Statistics/Raw_Data_Feedscape_total.xlsx",i)
summarydf <-df %>% group_by(Group) %>%  summarise(Median = median(FI,na.rm = TRUE),
IQR=IQR(FI,na.rm = TRUE),Sample_size=length(FI))%>% as.data.frame()

df %>%
  group_by(Group) %>%
  do(tidy(shapiro.test(.$value)))

kruskal.test(FI ~ Group,data = df)
a1<-dunnTest(FI ~ Group,data = df,method="bonferroni")
a2<-a1$res

#logistic regression for comparing the effects of staravtion
df1 <- df[df$Treatment=="Threat",]
df1$`Feeding Index` <-as.numeric(df1$FI)/100
model1 <- glm(`Feeding Index` ~ T+ Condition+ T*Condition,
family=quasibinomial(link='logit'), data=df1)
summary(model1)
library(emmeans)
em <- emmeans(model2, "Condition")
contrast(em, "pairwise", adjust = "Tukey")

#Geom scatter plot for velocity
averaged_dataset <- aggregate(Velocity ~ x + y, data = final, FUN = mean)
plot1 <- ggplot(data=averaged_dataset,aes(x=x,y=y,colour=Velocity))+
geom_point(size=1)+theme_classic()+ theme(axis.ticks=element_blank())+
scale_color_gradientn(colours = c("#253494","#3690c0","#41ab5d","#ffeda0","#ffffcc"),
limits = c(0, 30),oob = scales::squish)+xlim(-10,10)+ylim(-10,10)

#Geom density plot for occupancy
center_x <- 0
center_y <- 0
```

```
radius <- 10.0
theta <- seq(0, 2 * pi, length.out = 100)
circle_x <- center_x + radius * cos(theta)
circle_y <- center_y + radius * sin(theta)
circle_data <- data.frame(x = circle_x, y = circle_y)

ggplot(cs_fem96hrs, aes(x=x, y=y) ) +
stat_density_2d(aes(fill = (..density..)), geom = "raster", contour = FALSE,h = 2,n=100) +
scale_fill_distiller(direction = 1)+ scale_x_continuous(expand = c(0, 0)) +
scale_y_continuous(expand = c(0, 0)) +theme(legend.position='none')+
theme_classic()+facet_wrap(~a2)+ scale_fill_gradientn(colors = magma(5),values=c(0,0.01,0.03,0.1,
geom_path(data = circle_data, aes(x, y), color = "darkgreen")+xlim(-11,11)+ylim(-11,11)

# analysis of solitary data
setwd("/Users/ssr978/Documents/solitary/uas CsChrimson+loom+light")



t1 <- c(9000:10799) # pre-stimulation time frame
t2 <- c(10800:12599) # stimulation time frmae

frame_rate <- 60 # in fps
dt <- 1 / frame_rate # in secs
temp = list.files(pattern="*.csv") # get all the files in the folder

Mode <- function(x) {
  ux <- unique(x)
  ux[which.max(tabulate(match(x, ux)))]
}

p <-list()
q <-list()
fib <-list()
fia <-list()
r <-list()
s <-list()
vf_before <- c()
vf_after <- c()
mean_vel_before <-list()
mean_vel_after <-list()
```

```
jumps_before <-list()
jumps_after <-list()
peaks <- list()


for (i in 1:length
     (temp)) {
  df <- read.csv(temp[i],header=TRUE)
  before <- (df[ df$Item5 %in% t1,])
  after <- (df[ df$Item5 %in% t2,])
  pixel <- Mode(df[,1])
  p[[i]] <- ggplot(data=before,aes_string(x=before$Item3.X,y=before$Item3.Y))+
  geom_path()+theme_classic()
  q[[i]] <- ggplot(data=after,aes_string(x=after$Item3.X,y=after$Item3.Y))+
  geom_path()+theme_classic()

  dx_before <- na.omit((before$Item3.X))
  dy_before <- na.omit((before$Item3.Y))
  dx_after <- na.omit((after$Item3.X))
  dy_after <- na.omit((after$Item3.Y))
  a1<- as.matrix(before$Item4)
  a1 <-na.omit(a1)
  sec <- colSums(matrix(a1, nrow=60))

  fib[[i]] <- length(sec[sec<300])*100/30

  a2<- as.matrix(after$Item4)
  a2 <-na.omit(a2)
  sec1 <- colSums(matrix(a2, nrow=60))
  fia[[i]] <- length(sec1[sec1<300])*100/30

  for (k in 2:length(dx_before)){
    xdiff <- dx_before[k]-dx_before[k-1]
    ydiff <- dy_before[k]-dy_before[k-1]
    v_before <- (sqrt((xdiff)^2+(ydiff)^2))/dt
    vf_before[k] <- v_before*20/pixel
```

```
  }

  mean_vel_before[[i]] <-mean(vf_before[vf_before>4],na.rm = TRUE)

  for (s in 2:length(dx_after)){
    xdiff1 <- dx_after[s]-dx_after[s-1]
    ydiff1 <- dy_after[s]-dy_after[s-1]
    v_after <- (sqrt((xdiff1)^2+(ydiff1)^2))/dt
    vf_after[s] <- v_after*20/pixel

  }
  mean_vel_after[[i]] <-mean(vf_after[vf_after>4 ],na.rm = TRUE)

 vel1 <-data.frame(time=c(1:length(vf_before)),velo=vf_before)
# r[[i]] <- ggplot(data=vel1,aes_string(x=time,y=vel1$velo))+geom_line()+theme_classic()

 vel2 <-data.frame(time=c(1:length(vf_after)),velo=vf_after)
# s[[i]] <- ggplot(data=vel2,aes_string(x=time,y=vel2$velo))+geom_line()+theme_classic()


 ju1 <- na.omit(vel1[vel1$velo > 75,])

 ju2 <- na.omit(vel2[vel2$velo > 75,])

 if(dim(ju1)[1] == 0){jumps1<-0
 } else if(dim(ju1)[1] == 1){jumps1=1
 }else
 {ju1$no <- NA
 ju1$no[[1]] <- ju1$time[1]
 for (j in 2:nrow(ju1)){
   ju1$no[[j]] = ju1$time[j] -ju1$time[j-1]
   jumps1<- nrow(ju1[ju1$no>2,])}}


 if(dim(ju2)[1] == 0){jumps2=0
 } else if(dim(ju2)[1] == 1){jumps2=1
 } else
 {ju2$no[[1]] <- ju2$time[1]
 for (j in 2:nrow(ju2)){
```

```
   ju2$no[[j]] = ju2$time[j] -ju2$time[j-1]
   jumps2<- nrow(ju2[ju2$no>2,])}}


 jumps_before[[i]] <-jumps1
 jumps_after[[i]] <- jumps2




}

do.call(grid.arrange,p)
do.call(grid.arrange,q)

do.call(grid.arrange,r)
do.call(grid.arrange,s)


fib.df <- do.call("rbind", lapply(fib, as.data.frame))
fia.df <- do.call("rbind", lapply(fia, as.data.frame))
mean_vel_before.df <- do.call("rbind", lapply(mean_vel_before, as.data.frame))
mean_vel_after.df <- do.call("rbind", lapply(mean_vel_after, as.data.frame))
jumps_before.df <- do.call("rbind", lapply(jumps_before, as.data.frame))
jumps_after.df <- do.call("rbind", lapply(jumps_after, as.data.frame))


diff_velocity.df <- mean_vel_after.df - mean_vel_before.df

final <- cbind(fib.df,fia.df,mean_vel_before.df,mean_vel_after.df,diff_velocity.df,jumps_before.
colnames(final) <- c("Freezing index before threat","Freezing index after threat",
"Walking speed before threat","Walking speed after threat","Change in walking speed",
"Jumps before threat","Jumps after threat")
final
write.csv(final, file = "compiled_data.csv")

#Calculation of Angular velocity
t1 <- c(9000:10799) # pre-stimulation time frame
t2 <- c(10800:12599) # stimulation time frmae
```

```
frame_rate <- 60 # in fps
dt <- 1 / frame_rate # in secs
temp = list.files(pattern="*.csv") # get all the files in the folder

Mode <- function(x) {
  ux <- unique(x)
  ux[which.max(tabulate(match(x, ux)))]
}



dx_after <-c()
dy_after <-c()
angle_list <- c()
angle_list1 <- c()
angle_list2 <- c()
Heading <- c()
deltaHeading <- c()

for (i in 1:length
     (temp)) {
  df <- read.csv(temp[i],header=TRUE)
  before <- na.omit(df[ df$Item5 %in% t1,])
  after <- na.omit(df[ df$Item5 %in% t2,])
  dx_after <- na.omit((after$Item3.X))
  dy_after <- na.omit((after$Item3.Y))


  for (k in 2:length(dx_after)){
    xdiff <- dx_after[k]-dx_after[k-1]
    ydiff <- dy_after[k]-dy_after[k-1]
angle_rad <- acos(abs(xdiff)/(sqrt((xdiff)^2+(ydiff)^2)))
      angle <- angle_rad* (180.0 / pi)

    if (xdiff > 0 & ydiff >= 0){
      angle1<- angle
    } else if (xdiff >= 0 & ydiff < 0){
      angle1<- -angle
    } else if (xdiff < 0 & ydiff <= 0){
      angle1<- -(180- angle)}
```

```
      else if (xdiff == 0 & ydiff == 0){
        angle1<- 0
    }else {
      angle1<- (180 - angle)}

      angle_list[k] <- angle1

      if(k>2){
        angle_list1[k] <- angle_list[k]- angle_list[k-1]
        if((angle_list1[k]) <  -180){
          angle_list2[k] <- angle_list1[k]+360}
        else if (angle_list1[k] >=  180){
          angle_list2[k] <- 360- angle_list1[k]
        } else
        {angle_list2[k] <- abs(angle_list1[k])}


  }

  Heading[i] <- mean(angle_list,na.rm=TRUE)
  deltaHeading[i] <- mean(angle_list2,na.rm=TRUE)
}




final <- cbind(Heading,deltaHeading)
median(deltaHeading,na.rm = TRUE)
IQR(deltaHeading,na.rm = TRUE)
write.csv(final, file = "compiled_angle.csv")
```

Neuron	Flywire IDs for the neurons
LPLC2	720575940627902979,720575940626375172,720575940619654149, 720575940628197892,720575940622769159,720575940608875531,720575940614738444, 720575940623925774,720575940611567638,720575940622680088,720575940616587803, 720575940631582751,720575940624919077,720575940612045352,720575940630302250, 720575940618608177,720575940645328436,720575940637958709,720575940617289782, 720575940628552247,720575940630650936,720575940645699124,720575940628847164, 720575940617569344,720575940618450496,720575940619900993,720575940630209603, 720575940637193798,720575940630731847,720575940628778058,720575940620783180, 720575940620946508,720575940637702222,720575940629384271,720575940638161486, 720575940630831185,720575940639884878,720575940638835288,720575940625477721, 720575940608617564,720575940607288924,720575940633588320,720575940634896992, 720575940635040357,720575940634260079,720575940635576432,720575940612605554, 720575940619919476,720575940615055989,720575940635307639,720575940648418425, 720575940627902078,720575940605825666,720575940622312072,720575940626236041, 720575940615967377,720575940613219474,720575940610116757,720575940610855061, 720575940615332502,720575940627663512,720575940610946713,720575940634146967, 720575940627683483,720575940632779932,720575940653666465,720575940653742753, 720575940639109795,720575940614631079,720575940626873000,720575940611352234, 720575940613145258,720575940631397549,720575940635234478,720575940613763759, 720575940607883442,720575940634700980,720575940624359093,720575940619981494, 720575940622425271,720575940623769783,720575940635516084,720575940624244922, 720575940624611002,720575940624014524,720575940614526974,720575940608088259, 720575940623551684,720575940626414279,720575940624931530,720575940625216202, 720575940629368015,720575940639878352,720575940636539102,720575940632512226, 720575940612462307,720575940613470947,720575940636999401,720575940622093546, 720575940623652083,720575940620575476,720575940626617084,720575940623262461, 720575940622174463,720575940616735489,720575940628990214,720575940626161930, 720575940623543565,720575940618927892,720575940611821334,720575940611891990, 720575940621914904,720575940620887834,720575940616166685,720575940643740964, 720575940645180196,720575940612560680,720575940612874536,720575940622258473, 720575940624417584,720575940624755504,720575940632129848,720575940628976956, 720575940615957314,720575940615920453,720575940638626118,720575940626384200, 720575940622992205,720575940629967703,720575940630719831,720575940638857048, 720575940636878682,720575940637088602,720575940621634913,720575940609592674, 720575940610536290,720575940625399652,720575940606126438,720575940613804906, 720575940613821290,720575940634684267,720575940635004271,720575940635572592, 720575940636529520,720575940631095158,720575940638922624,720575940606855554, 720575940607845250,720575940625700739,720575940627685765,720575940619197318, 720575940624016778,720575940615288203,720575940615907723,720575940617294219, 720575940632711052,720575940616083345,720575940623827348,720575940612159381, 720575940624903064,720575940611740569,720575940617480094,720575940614373279, 720575940619627934,720575940632777124,720575940619750822,720575940620115878, 720575940626735016,720575940603989420,720575940606217138,720575940610953651, 720575940620001718,720575940622455223,720575940624208824,720575940617997241, 720575940615116731,720575940619263937,720575940628603842,720575940607683011, 720575940607854531,720575940622009284,720575940614502854,720575940627876807, 720575940642397128,720575940605669321,720575940623047629,720575940612096462, 720575940622253526,720575940628921303,720575940620306904,720575940616116697, 720575940616377817,720575940616491993,720575940639895003,720575940640510427, 720575940641552347,720575940614067167,720575940612766691,720575940610681829, 720575940626447848,720575940636950505,720575940612254701,720575940619935214, 720575940618711535,720575940619398127,720575940612944382,720575940622156799, 720575940628039034,720575940625695269,720575940623333770
DNP06	720575940611644529, 720575940622673860
GF	720575940622838154, 720575940632499757
Bract	720575940610001220, 720575940626557442, 720575940627285267, 720575940645045527

Table 10: List of flywire IDs for the neurons used for connectomic analysis

6.2 Published Manuscripts

Given below are the two manuscripts published during the course of my PhD:

- Cheriyamkunnel SJ, **Rose S**, Jacob PF, Blackburn LA, Glasgow S, Moorse J, Winstanley M, Moynihan PJ, Waddell S, Rezaval C. A neuronal mechanism controlling the choice between feeding and sexual behaviors in Drosophila. Curr Biol. 2021 Oct 11;31(19):4231-4245.e4. doi: 10.1016/j.cub.2021.07.029.

- **Rose S**, Beckwith EJ, Burmester C, May RC, Dionne MS, Rezaval C. Pre-copulatory reproductive behaviours are preserved in Drosophila melanogaster infected with bacteria. Proc Biol Sci. 2022 May 11;289(1974):20220492. doi: 10.1098/rspb.2022.0492.