SUGAR INTAKE ELICITED LOCOMOTOR BEHAVIOURS IN FLIES AND HONEY BEES

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UNDER THE GUIDANCE OF

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DECLARATION BY THE STUDENT

I declare that this thesis entitled “Sugar intake elicited locomotor behaviours in flies and honey bees” submitted for the award of Doctor of Philosophy to THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY, Bengaluru, is my original work, conducted under the supervision of my guide Dr. Axel Brockmann, National Centre for Biological Sciences, TIFR, Bengaluru. I also wish to inform that no part of the research has been submitted for degree or examination at any university. References, help and material obtained from other sources have been duly acknowledged.

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CERTIFICATE

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Table of Contents

1. Introduction 1

1.1 Foraging Behaviour in Insects 1

1.1.1 Honey Bee Dance 2

1.1.2 Evolutionary origins and neural basis of the honey bee dance 3

1.2 Solitary sugar-elicited behaviours 5

1.2.1 Sugar-elicited search in flies 5

1.2.2 Neural and mechanistic basis of search 7

1.3 Connection between sugar-elicited search and honey bee dance 8

1.4 Aims of the study 9

1.4.1 To understand the connection between solitary sugar-elicited behaviour and social dance communication 9

1.4.2 To study the mechanistic basis of sugar-elicited search in flies 10

1.5 Description of work 10

1.5.1 Chapters 11

2. Sugar intake elicits intelligent search behaviour in flies and honey bees 13

2.1 Introduction 13

2.2 Materials and Methods 15

2.2.1 Fly rearing 15

2.2.2 Fly local search 16

2.2.3 Local search in the absence of visual and chemosensory cues 16

2.2.4 Passive displacement experiments in flies 17

2.2.5 Honey bee colonies 17

2.2.6 Honey bee local search 18

2.2.7 Passive displacement experiments in honey bees 18

2.2.8 Analysis of trajectories for local search 19

2.3 Results 20

2.3.1 Flies and bees initiate a local search after sugar intake 20

2.3.2 Flies and bees use path integration to return to the location of the food24

2.3.3 Location of the sugar reward had no bearing on path integration-based local search in flies and bees 26

2.3.4 Onset of walking after sugar intake starts the path integration system in flies and bees 30

2.4 Discussion 32

3. Solitary sugar-elicited behaviour and social dance communication 36
3.1 Introduction 36
3.2 Materials and Methods 37
3.2.1 Delay experiments in flies 37
3.2.2 Delay experiments in honey bees 38
3.2.3 Honey Bee Dance experiments 38
3.2.4 Dance recording and analyses 40
3.3 Results 41
3.3.1 The heightened motivation to start a path integration-based local search lasted at least for 3 min post-feeding 41
3.3.2 Delaying departure of foragers after sugar intake at the feeder reduced the probability of dancing 47
3.1 Discussion 51
4. Exploring the mechanistic role of walking in path integration in local Search using flies 53
4.1 Introduction 53
4.2.1 Testing Nanchung mutants to test the role of proprioception 54
4.2.2 Inactivating Nan neurons thermogenetically 54
4.2.3 Visualizing Nan expression 55
4.2.4 Testing the behaviour on large arena 55
4.2.5 Leg clipping experiments 55
4.2.6 Quantifying additional homing clusters 56
4.2.7 Testing scale-free walk 56
4.3 Results 58
4.3.1 Nanchung mutants have impaired returns during search 58
4.3.2 Shortening the leg length had no effect on path integration at the origin of the search 62
4.3.3 Local search consisted of smaller clusters of homing at points in addition to the origin, in flies with shorter legs 65
4.4 Discussion 71
5. Conclusion and future directions 73
5.1 Conclusions 73
5.1.1 Connection between solitary sugar-elicited behaviour and honey bee dance 73
5.1.2 Mechanistic basis of sugar-elicited search 74
5.1.3 Summary 74

5.2 Future directions 76
5.2.1 Identifying neurotransmitter and neuromodulator system involved in initiating and regulating search and dance 76
5.2.2 Studying neural and mechanistic basis of path integration 76
5.2.3 Studying neural basis of sugar-elicited search 77
5.2.3.1 Modification to the behavioural set up to achieve neuronal silencing 77
5.2.3.2 Indentifying neural substrates of the behaviour by neurogenetically silencing regions of the brain. 77
6 References 78
7. Appendix 93
7.1 Supplementary figures 93
7.2 Supplementary tables 102

List of Figures

Chapter 1

Figure 1.1: Summary of inputs to honey bee brain..4

Chapter 2

Figure 2.1: Experimental setup to study local search..18

Figure 2.2: Trajectories depicting sugar-elicited search...21

Figure 2.3: Unfed flies and bees did not initiate search...22

Figure 2.4: Hungry flies did not initiate search when there was no sugar stimulus.....22

Figure 2.5: Sugar intake is necessary for initiating local search...................................23

Figure 2.6: Behavioural parameters were significantly lower in unfed flies and bees...24

Figure 2.7: Flies make returns to the location of the sugar drop..................................25

Figure 2.8: Flies made returns and navigate to the origin in the absence of external cues...26

Figure 2.9: Flies return to the location of the sugar drop in the absence of external cues...27

Figure 2.10: Flies and bees started a search involving path integration when displaced to a new location after feeding...28

Figure 2.11: Upon passive displacement post-feeding, flies and bees started a search involving path integration around the position in the arena where they are transferred...30

Figure 2.12 Comparison of behavioural parameters between flies and bees for displacement experiments...31

Figure 2.13 Displaced flies and bees use the end of the pre-search walk as the reference point for path integration-based search...32

Figure 2.14: Scheme of behavioural responses involved in sugar-elicited search.......34

Chapter 3

Figure 3.1 Honey bees at the feeder...41

Figure 3.2 Effect of delay post-feeding on initiation and parameters of path integration-based local search in flies and honey bees. 43

Figure 3.3 Less than 20% of tested flies and bees initiate local search for a delay of 3 min..44

Figure 3.4 Search in flies became less centred towards the origin with the delay duration..45

Figure 3.5 The search in bees was less centred towards the origin of walking as the delay duration increases..46

Figure 3.6 Behavioural parameters of unfed flies and bees compared to 3 min delay duration.. 47

Figure 3.7 Initiation of dance and number of circuits reduce with delay duration in honey bees..49

Figure 3.8 Regression and change-point analysis for number of circuits in honey bee dance experiments..49

Chapter 4

Figure 4.1: Leg clipping experiment...57

Figure 4.2: Flowchart for quantifying additional homing clusters...............................58

Figure 4.3: Nanchung protein has characteristic expression...60

Figure 4.4: Positional heatmaps for Nan36a indicated impaired returns......................61

Figure 4.5: Path integration-based search was disrupted in Nan^{36a} mutants................62

Figure 4.6: The returns were not affected when Nanchung was thermogenetically inactivated using Shibire..63

Figure 4.7: The feeding time did not change for legs with clipped flies, however the speed was lower for flies in light as compared to sham..64

Figure 4.8: The heatmaps did not show a difference when the leg length of CS flies was shortened..65

Figure 4.9: The number of returns to the origin did not show a difference when the leg length of CS flies was shortened. 66

Figure 4.10: Presence of small localized clusters of homing in flies with shorter legs. 67

Figure 4.11: Presence of small localized clusters in flies tested in large arena............68

Figure 4.12: The additional clusters show high density around the origin of the clusters..69

Figure 4.13: Quantifying the presence of additional clusters of homing.................... 69

Figure 4.14: Testing scale-free walk...71

Chapter 5

Figure 5.1: Model summarizing the major conclusions of the study............................76

List of Publications

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3. **Shakeel M.**, Kaushik S., Tanimura T., Brockmann A., Kain P. (**2023**). Tracking Sugar-Elicited Local Searching Behavior in *Drosophila.* Journal of Visual Experiments, e65955, In-press. doi: 10.3791/65955 https://www.jove.com/t/65955/tracking-sugar-elicited-local-searching-behavior-in-drosophila

Synopsis

Foraging behaviour is one of the most successful experimental paradigms to study navigation. Insects are highly capable and efficient navigators (Rüdiger 2020; Menzel 2023). Honey bees are unique in that they evolved the capability to communicate the location of profitable food sources to nest mates through dance communication (Frisch 1967). However, the evolution of dance remains largely unresolved (Price and Grüter 2015; Barron and Plath 2017). Interested in the question of how social behaviour or communication evolved from solitary behaviour, Vincent Dethier drew attention to similarities between a local search behaviour in flies and the honey bee dance (Dethier 1957). He was also the first to describe sugar-elicited search behaviour, a particular form of local search that is initiated after taking in a small amount of food. Typical local search behaviour includes a highly tortuous walk with high turning rate, and frequent returns to the location of the sugar drop. This behaviour was studied in other dipterans: *Musca domestica* and *Drosophila melanogaster* (Bell 1984; White et al. 1985; Pereira and Sokolowski 1993). However, the question whether this search behaviour might comprise path integration to return to the location of the food had not been asked. Use of path integration in navigation and homing has been demonstrated in desert ants, dung beetles and bumble bees navigation; and also in mammals. (Muller and Wehner 1988; Chittka et al. 1999; Dacke et al. 2020; Patel et al. 2022; Mittelstaedt and Mittelstaedt 1980).

Additionally, Dethier's idea had not been taken up by bee researchers. This thesis explores the hypothesis that the innate sugar-elicited local search behaviour of solitary insects - which might also be present in social insect foragers - was co-opted during the evolution of social communication of navigational information in honey bee dance. We have established a set up to study sugar-elicited search behaviour in *Drosophila melanogaster* and the western honey bee *Apis mellifera*. To understand the connection between solitary sugar-elicited behaviour and social dance communication, I studied initiation and regulation of local search in both the species, and compared that to honey bee dance. Furthermore, I tested whether local search involves path integration to guide returns to the location of food reward. The role of self-motion cues in the local search was also explored to understand the mechanistic basis of local search.

1. Introduction

1.1 Foraging Behaviour in Insects

In order to survive, animals need to search and find food in a highly cluttered world. Successful foraging requires multi-modal sensory processing and integration, decision making and memory. Foraging behaviours in insects are intricate adaptations shaped by a myriad of factors including resource availability, energy costs, and environmental conditions. These behaviours are fundamental to the survival and reproductive success of insect populations. Insects are renowned for their remarkable navigational skills with honey bees standing out as eusocial animals capable of not only foraging efficiently but also communicating the location of food sources through dance communication (Frisch 1967; Rüdiger 2020; Menzel 2023).

Insects exhibit a wide range of behaviours, including complex communication systems like the pheromone trails of ants and the dance language of honey bees (Wilson, 1971; von Frisch, 1967). Their ability to navigate diverse landscapes using various sensory cues, such as visual landmarks and celestial cues, further demonstrates their remarkable adaptability and cognitive capabilities (Menzel, 2012). Insects exhibit a diverse array of foraging strategies, ranging from solitary search tactics to complex cooperative behaviours in eusocial species like honey bees (Pyke et al., 1977; Dall et al., 2005).

Insect foraging behaviour has been a subject of extensive research with studies emphasizing the adaptive nature of foraging strategies and their implications. The efficiency of foraging directly impacts an organism's fitness, as it determines the balance between energy gain and expenditure, often referred to as profitability (Pyke, 1984). Optimal foraging theory, pioneered by has provided valuable insights into the trade-offs involved in foraging decisions and the evolution of efficient foraging strategies (Pyke et al., 1977; Charnov, 1976). Understanding the mechanisms underlying foraging behaviour involves investigating both proximate factors, such as sensory stimuli eliciting search behaviour, and ultimate factors related to the evolutionary significance of foraging strategies (Tinbergen, 1963). Furthermore, advances in neurobiology have deepened our understanding of the neural mechanisms underlying foraging behaviours, highlighting the interplay between sensory perception, decision-making processes, and environmental cues (Dall et al., 2005; Menzel & Giurfa, 2001).

1.1.1 Honey Bee Dance

Foragers in social hymenopterans regularly leave their nests to search for food, return to the colony, and signal the location of food to their nestmates. Honey bee foragers navigate complex landscapes to gather nectar and pollen, crucial resources for the entire colony. The waggle dance, a unique form of communication, allows foragers to convey precise information about the location, quality, and distance of food sources to their nestmates (von Frisch, 1967; Seeley, 1995). Two kinds of foragers exist in a bee colony: scouts and recruits. Scouts are the individuals that explore novel food sources, while recruits forage at known food sites by following the dance of the scouts (Seeley 1983; Biesmeijer and De Vries 2001). Scout bees, upon discovering a rich food source, return to the hive and convey information about the location, quality, and distance of the resource throughs the dance.

Karl von Frisch, the Austrian zoologist, dedicated his studies to decoding the intricate language of honey bees. The communication system involves a unique form of dance, known as the "waggle dance," to guide hive members to profitable food sources (von Frisch 1914; 1919). In recognition of his pioneering contributions, Karl von Frisch was awarded the Nobel Prize in Physiology and Medicine in 1973, alongside Konrad Lorenz and Nikolaas Tinbergen, "for their discoveries concerning organization and elicitation of individual and social behaviour patterns". Over the next decades, the waggle dance and foraging were extensively studied in the European honey bee, *Apis Mellifera*.

Honey bees use path integration for spatial orientation during foraging and hive-bound flights. Path integration involves the integration of self-motion cues, allowing the bees to continuously update their position relative to a reference point and navigate directly back to their hive or nest along a precise home vector (Chittka et al. 1999; Wehner 2020). Path integration has been demonstrated in other insects such as desert ants, bumble bees, dung beetles while foraging (Müller and Wehner 1988; 1994; Dacke et al. 2020; Patel et al. 2022). The dance likely results from the interaction between the neural systems responsible for flight and walking path integration, involving the transfer of information between these systems (Brockmann & Robinson 2007).

The dance occurs on the vertical honeycomb and consists of two main components: the waggle phase and the return phase. During the waggle phase, the bee performs a figure-eight dance, with the straight run indicating the direction of the food source relative to the sun's azimuth. Recruits interpret and decode the dance, subsequently fly out to the reported food source based on the communicated information. Foragers employ round dances for close food sources (less than 100 meters from the hive) and waggle dances for more distant locations (von Frisch 1967). The duration of the waggle portion correlates with the distance to the resource, providing precise spatial information (Seeley 1995; Dyer 2002).

1.1.2 Evolutionary origins and neural basis of the honey bee dance

While the honey bee dance was discovered and decoded over a hundred years ago and has since been extensively studied, two crucial questions remain the topic of scientific enquiry. First is the evolution and the underlying neural mechanisms of the dance (Price and Grüter 2015; Barron and Plath 2017). The evolution of eusociality, where individuals in a colony exhibit overlapping generations, cooperative care of the young, and reproductive castes, is considered a major leap in social evolution. Notable examples of eusocial insects include honey bees, certain species of ants, and termites. The capacity of honey bees to convey nuanced information about food source location, quality, and distance through a symbolic dance provides a substantial evolutionary advantage. Social insects, are believed to have evolved from solitary ancestors through a series of transitional stages (Michener 1974; Holldobler and Wilson 2009). However, the evolution of dance remains largely unresolved.

Current knowledge of the neural processes underlying honey bee foraging and dance behaviour has been advanced through neurobiological investigations. Honey bees can learn and remember places, routes and landmarks (Cartwright and Collett 1982; 1983; Gould 1986; Stankiewicz and Webb 2021). As the bees traverse complex landscapes, the visual, olfactory, and proprioceptive cues are integrated forming a comprehensive spatial representation. The polarization pattern of sunlight scattered in the sky is used as a celestial cue during flight. The terrestrial and celestial sensory inputs interact with the path integration system (Collett and Collett 2000b; Collett et al. 2013; Heinze et al. 2018). There is evidence that memory processes might be involved in dance communication (Lindauer 1954; von Frisch 1967; Dyer 1987; Menzel 2011).

The mushroom bodies in the bee brain are integral to sensory processing as well as learning and memory in the bee brain, and play a pivotal role in foraging (Menzel 2011; Farris and Schulmeister 2011; Cabirol et al. 2018). The central complex, a conserved brain region, is associated with motor control, spatial orientation and path integration: essential components for the intricate movements involved in the dance (Menzel and Giurfa 2001; Stone et al. 2017; Collett 2019). Genetic and molecular studies, including the expression of foraging-related genes, have provided an understanding of the genes and pathways involved in dance (Ben-Shahar et al. 2002; Hunt et al. 2007; Robinson et al. 2008). Neuromodulators also play a crucial role in controlling honey bee dance and foraging behaviour (Ai and Farina 2023, Schilcher and Scheiner 2023).

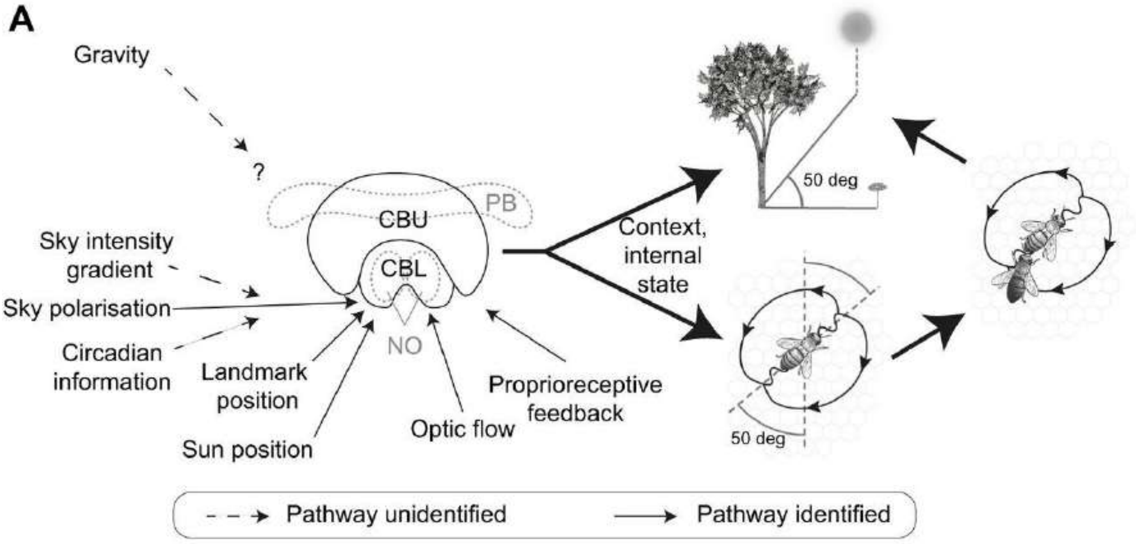


Figure 1.1: Summary of inputs to honey bee brain. Inputs to the central complex involved in orientation, and proposed to also be involved in dance. The central complex is composed of the central body upper unit (CBU), the central body lower unit (CBL), the protocerebral bridge (PB) and the noduli (NO). (From Barron and Plath 2017).

Vincent Dethier's pioneering work in the mid-20th century laid the groundwork for understanding the evolutionary connections between solitary foraging behavior and complex social communication observed in eusocial species, such as honey bees (Dethier, 1957). Dethier proposed that elements of innate search behavior exhibited by solitary insects could have been co-opted and elaborated upon during the evolution of sophisticated communication systems, such as the honey bee dance, which conveys vital navigational information to colony members (Dethier, 1976). This hypothesis underscores the interplay between proximate sensory mechanisms triggering local search behavior and the ultimate evolutionary implications of these behaviors in social insects.

Unfortunately, honey bees are not a very good system to study the mechanisms of behaviour due to lack of neural and genetic manipulation tools. On the other hand, advances in neurogenetics techniques in the fruit fly, *Drosophila melanogaster* makes it a powerful model system. Freely walking and head-fixed behavioural paradigms, whole-cell patch clamp recordings and two-photon imaging with genetically encoded calcium indicators make it a suitable system to study neural and mechanistic details of components of navigation. Additionally, studying the remarkable navigational behaviour of social insects in their natural environment requires monitoring the activity in foraging animals which is experimentally challenging. Studying the mechanisms of honey bee behaviour using *drosophila* posits investigating similarities of the bee behaviour and solitary fly.

This thesis sheds light on the intriguing connection between solitary sugar-elicited behaviour, social dance communication, and navigation in insects, offering valuable insights into the evolution of complex communication systems and navigational strategies in insects.

1.2 Solitary sugar-elicited behaviours

The genesis of this research finds its roots in the insights of Vincent Dethier, who drew attention to a striking resemblance between the local search behaviours of solitary flies and the honey bee dance (Dethier 1957). Foraging in solitary insects involves long and straight locomotion called ranging, and local search which gets initiated on encountering a profitable food resource (Jander 1975; Hansson and Åkesson 2014).

1.2.1 Sugar-elicited search in flies

Sugar-elicited search behaviour, a particular form of local search, was first studied by Dethier in blow fly *Phormia regina* (Dethier, 1957). When a hungry fly is presented with a little amount of sugar solution such that it does not satiate them, it begins a search. This local search behaviour involves a tortuous walk, frequent turns, and repeated returns to the location of the sugar drop. Drawing parallels between this behaviour of solitary flies and the honey bee dance, Dethier made a compelling hypothesis. First, both the behaviours are initiated after sugar intake. Second, he observed the similarities in walking pattern of the fly where it ‘circled’ around the location of the sugar drop to the honey bee dance. Finally, he brought attention to a difference in both the behaviours; the search commences immediately after sugar intake while the dance occurs at the hive, after the bee has ingested sugar and flown back to the hive. He described a passive displacement experiment, where he could feed the fly and then transfer it to a paper, where the fly ‘danced’. Based on these observations, Dethier suggested that the ancestral behavioural locomotor pattern in flies evolved into the complex dance communication observed in honey bees. He proposed that since behaviour doesn’t arise de-novo, the template for organizing local search could have been co-opted for honey bee dance.

Subsequently sugar-elicited search has been studied in other dipterans such as *Musca domestica* and *Drosophila melanogaster* (White et al. 1984; Bell et al. 1985; Pereira and Sokolowski 1993). The initiation, intensity and duration of the search behaviour is regulated by the internal state (deprivation and motivation) of the animal and external factors such as resource availability and quality (Dethier 1957; 1964; Bell 1990). Our collaborators have demonstrated that this path integration-controlled search behaviour is initiated by pharyngeal taste receptors (Murata et al. 2017). Additionally, it has been demonstrated that local searches performed in different behavioural contexts can be triggered by activating various olfactory and gustatory sensory neurons in flies (Corfas et al. 2019; Haberkern et al. 2019; Behbahani et al. 2021; Titova et al. 2023).

However, Dethier's ideas largely remained unexplored by researchers in the field of bee behaviour. This thesis aims to bridge this gap by studying the initiation and regulation of local search behaviours in these species, with comparisons drawn to honey bee dance communication. Additionally, studying the behaviour in flies allowed me to study the mechanistic basis of the behaviour. For instance, a characteristic feature of the sugar-elicited search is frequent and precise returns to the starting point of the search, defined by the location of the sugar drop.

White et al. (1984) were the first to suggest in house flies (*Musca domestica*) that an internal sense of spatial position may assist the fly in returning. This indicates that search behaviour involves idiothetic (self-motion) cues; that is the fly is able to estimate its current position with respect to the position of the drop, the starting position of the search, as a reference and perform path integration. However, it remained unexplored whether this search behaviour incorporated path integration to guide the return to the food source: a navigational strategy demonstrated in various insects and mammals (Muller & Wehner 1988; Chittka et al. 1999; Dacke et al. 2020; Patel et al. 2022; Mittelstaedt & Mittelstaedt 1980). I asked the crucial question: whether flies and honey bees use path integration to guide their way back to the location of the food source? Kim and Dickinson (2017) independently reported that the search behaviour in flies involves path integration, guiding them to return to the location of the sugar drop. Since path integration relies on proprioceptive inputs, I also explored the role of mechanosensory feedback in the behaviour.

1.2.2 Neural and mechanistic basis of search

Local search behavior is influenced by a myriad of sensory stimuli, including visual cues, olfactory signals, and mechanosensory feedback, all of which play crucial roles in guiding an organism's movements during foraging (Collett & Collett, 2000; Srinivasan et al., 2010). The integration of these sensory inputs is orchestrated by neural circuits in the brain, particularly within the central complex, a region known for its role in spatial navigation, sensory integration, and decision-making processes (Heinze & Reppert, 2012). Central complex is the brain region which integrates multimodal senses and regulates locomotion (Strauss and Heisenberg 1993; Strauss 2002). It is involved in several components of navigation: modulating locomotion, visual learning and landmark orientation (Liu et al. 2006; Ofstad et al. 2011; Seelig and Jayaraman 2013; Weir and Dickinson 2015; Turner-Evans and Jayaraman 2016; Stone et al. 2017). Central brain is where visual landmarks and self-motion are encoded and integrated (Shiozaki and Kazama 2017, Tanaka et al. 2023). The role of central complex in navigation seems to provide a frame where a wide range of sensory signals get integrated with compass information and spatial orientation in order to produce outputs that control locomotion.

The mushroom bodies in flies are involved in learning and memory. They receive input from the antennal lobes, integrating sensory information related to odours and other environmental cues. Mushroom bodies are essential for forming associative memories and spatial learning in *Drosophila* (Liu et al. 1999; Zhang et al. 2007; Xi et al. 2008). The coordinated functioning of the mushroom bodies and central complex allows *Drosophila* to process sensory information, form memories, and execute motor responses crucial for navigation and orientation within its environment.

1.3 Connection between sugar-elicited search and honey bee dance

Sugar ingestion leads to an “excited” state resulting in stereotypical locomotion pattern in flies and bees. Earlier studies on the sugar-elicited search behaviour in blow flies and house flies highlighted that the intensity and duration of the search depends on the “central excitatory state”, starvation period, sugar concentration and the volume taken in, supporting the idea that the sugar intake modulates the behaviour (McGuire and Tully 1986; Mayor et al. 1987). Recruitment behaviours of stingless bees and bumblebees after successful foraging have similar relations between the initiation and intensity of the locomotor displays and the food reward but lack communication of navigational information (Lindauer and Kerr 1958; 1960; Dornhaus and Chittka 2001; Hrncir et al. 2004; 2011). The question remains, how flight navigational information got incorporated in these excited behaviours during evolution.

Flies and bees might exhibit similar (ancestral) modulatory circuits regulating the activity of the navigational system (Scheiner et al. 2006; Schröter et al. 2007; Busch et al. 2009; Menzel 1999; 2009; Barron et al. 205; Yang et al. 2015; Jiang and Pan 2022). For instance, octopaminergic signaling has been implicated in the regulation of various aspects of foraging, including responsiveness to sucrose and memory consolidation associated with foraging experiences (Scheiner et al. 2002; Farooqui et al. 2003; Barron et al. 2007). Additionally, research in flies has indicated that navigational information for flight and walking is processed within the same brain neuropils (Baker et al. 2007; Giraldo et al. 2018). The central complex is a conserved brain region associated with motor control and spatial orientation. It plays a pivotal role in coordinating complex behaviours, including those related to navigation and locomotion in both flies and honey bees.

The internal state and the environment of the animal play into exploration-exploitation trade-off (Corrales-Carvajal et al. 2016; Eriksson et al. 2017; Lin et al., 2019; Mahishi and Huetteroth 2019; Weaver et al. 2023). Delayed behaviour post-stimulus and persistent behavioural states are studied mainly in mating behaviour in *Drosophila* but have not been explored in feeding related behaviour (Jung et al. 2020; Thornquist et al. 2020; Deutsch et al. 2020; Devineni and Scaplen 2022).

As a first step towards exploring Dethier's pioneering ideas, which hinted at a possible connection between solitary sugar-elicited behaviours and the evolution of complex social communication in honey bees, this work studies local search in flies and honey bees, and the honey bee dance. Through a series of experiments, I delved into the initiation, temporal dynamics, and regulation of these behaviours. The overarching goal of this thesis is to bridge the gap between honey bee communication and fly search behaviours, and hope to uncover general principles of navigation. The thesis also has broader implications, as it suggests that *Drosophila melanogaster* can serve as a valuable model organism for studying the initiation and control of the path integration system, offering an alternative approach to unravelling the neural pathways involved in dance communication among honey bees, where neurogenetic manipulations pose significant challenges.

1.4 Aims of the study

1.4.1 To understand the connection between solitary sugar-elicited behaviour and social dance communication

I wanted to explore Dethier’s idea that honey bee dance evolved from the walking pattern of local search in solitary flies. Using a comparative approach, local search behaviour in flies and honey bees after sugar intake was studied. I then explored the temporal dynamics of initiation of local search and dance in flies and honey bees, respectively. Following questions were investigated:

a) Do *Drosophila melanogaster* males and *Apis mellifera* foragers initiate local search after sugar intake?

b) What is the importance of the location of food reward in the behaviour?

c) How long does the motivational effect of the food reward last after sugar intake for initiating local search?

d) What are the temporal dynamics of initiating dance after sugar collection in honey bee foragers?

1.4.2 To study the mechanistic basis of sugar-elicited search in flies

Interested in the navigational aspect of the behaviour, the role of path integration and proprioceptive inputs from the leg was explored. This aim had the following objectives:

a) Which navigational strategy do flies use to return to the location of the sugar: external cues vs path integration?

b) What is the role of mechanosensory inputs from the legs during path integration-based local search?

1.5 Description of work

To investigate the behavioural response of male hungry flies and honey bee nectar foragers to sugar stimulus, I studied their response to sugar in a lab set up. Trajectories were recorded and analyzed, including control experiments with no sugar stimulus. The findings revealed sugar-elicited local search behaviour involves path integration and increased turning. I then did passive displacement experiments, involving feeding flies or bees at one location and transferring them to a new arena before they commenced walking. This experiment tested whether the stimulus location could be decoupled from the search behaviour. Upon transfer, both flies and bees initiated a search around the location of release, and the search's origin was independent of the sugar location. To investigate the temporal dynamics of path integration-based search initiation, a series of delay experiments were performed. These experiments revealed that the motivation to initiate such a search persisted for 3 minutes in both species. After 3 min, the turning and looping was still observed suggesting that the search itself is composed of two independent behavioural responses: an increased turning behaviour, and path integration guided for limited duration of 3 min. A similar delay experiment in honey bee foragers demonstrated a significant reduction in the probability of initiating dance behaviour after 15 minutes, with a decline in the number of circuits at 3 minutes. This work will constitute chapters 2 and 3 of the thesis.

To distinguish whether flies relied on external sensory cues or self-motion cues to return to the sugar source, behaviour was recorded in the absence of visual and chemosensory cues. The flies were able to initiate a search under all the experimental conditions, indicating the utilization of path integration for navigation. Passive displacement experiments described above corroborated this finding. Additionally, mechanosensory receptor (Nanchung) compromised flies were employed to explore how mechanosensory inputs from the legs influenced path integration during local search. Mutant flies exhibited defects in returning to the origin. A manipulation experiment involved clipping the legs of flies and recording their behaviour after sugar intake to study the role of impaired feedback from the legs. Search trajectories of leg-clipped flies did not impact returns but displayed two phenotypes: small clusters of searches around the origin and a scale-free walking pattern known as Lévy walk. These experiments will constitute part of chapter 2 and entire chapter 4 of the thesis.

In the following chapters, we will look into the intricate details of our experimental procedures, results, and analyses, providing a comprehensive narrative of the insights from the study of sugar-elicited locomotor behaviours in flies and honey bees.

1.5.1 Chapters

Chapter 2: Sugar intake elicits intelligent search behaviour in flies and honey bees

This chapter describes the lab set up to study the behaviour in both the species. The paradigm is published in JoVE (Journal of Visualized Experiments). The response of flies and honey bee foragers to sugar intake is studied. Sugar intake leads to a local search which involves complex spatial orientation mechanisms, including path integration and increased turning. Displacement experiments demonstrated that stimulus (sugar) and behavioural response (local search) can be spatially dissociated. These experiments also corroborate the involvement of path integration in the behaviour. The major finding is that sugar intake increases the probability of initiating a path integration-based search, but the search begins at the onset of walking in flies and bees. This chapter will contain results from Brockmann et al. 2018, published in Frontiers of Behavioural Neuroscience.

Chapter 3: Solitary sugar-elicited behaviour and social dance communication:

This chapter will focus on the temporal dynamics of local search in flies and honey bee dance. Using a series of delay experiments where the flies and bees were impeded from walking post-ingestion, I studied the persistence of motivation to start a path integration-based search. I found that the motivation to initiate path integration-based search lasted for 3 min in both flies and bees. In addition, the parameters of the search were also reduced after 3 min in flies. Similar experiments in honey bee dance demonstrated that the motivation to initiate dance lasted for 15 min, while the number of circuits declined after 3 min. Initiation of both these behaviours showed a similar linear decline when immobilized after sugar intake This work is published in Journal of Comparative Physiology A as Shakeel and Brockmann 2023.

Chapter 4: Exploring mechanistic role of walking in path integration in local search using flies

To explore how mechanosensory inputs would feed into path integration-based local search, we used Nanchung compromised flies. Nan mutants showed impairment in returning to the origin. I also did a manipulation experiment where the legs of the flies were clipped to impair feedback. While the returns to origin were not affected in flies with shorter legs, I found additional clusters with path integration and high turning, at arbitrary positions in the arena. These flies also displayed scale-free walking pattern known as Lévy walks. Interestingly, we found the same phenotype for flies that were tested on large arena. Together, these results indicate that sugar intake provides the motivation to start a search, which may include smaller localized clusters.

Chapter 5: Conclusion and future research

In the final chapter, I discuss the findings of my studies with respect to the Dethier's original hypotheses and the connection between fly local search and honey bee dance. This chapter also outlines the future prospective of this research.

2. Sugar intake elicits intelligent search behaviour in flies and honey bees

2.1 Introduction

Sugar-elicited search behaviour, a particular form of local search, was first studied by Dethier in blow flies (Dethier 1957). Local search involves utilization of the resource followed by convoluted paths, circling around the patch and thus is also called ‘success motivated search’ (Vinson 1977). When hungry flies are presented with a little amount of sugar such that it does not satiate them, they begin a local search in the vicinity of the sugar drop. Typical search behaviour includes a highly tortuous walk with low locomotory and high turning rate, and making returns to the location of the sugar drop. In this seminal paper, Dethier suggested that the locomotory pattern of this behaviour from solitary flies could have been co-opted into the honey bee dance communication.

The honey bee dance was discovered a hundred years ago but its evolutionary origins remain unclear (Barron and Plath 2017). One of the approaches to understand the evolutionary path to this complex behaviour is to look at the building blocks of the dance. While Dethier’s hypothesis have not been taken up by honey bee researchers, sugar-elicited search in flies been studied in great detail. Local search studies using sugar in house flies and fruit flies unraveled many questions about the behaviour (Bell 1984; 1985). The initiation, intensity and duration of the search is regulated by the internal state (deprivation and motivation) of the animal and external factors such as resource availability and quality (Dethier 1957; 1964, Bell 1990; Murata et al. 2017).

We have developed a lab set up to study sugar elicited search behaviour in flies and honey bees (Shakeel et al. 2023). To test whether bees show sugar-elicited search behaviour and whether this assay could be useful to study molecular or neuronal mechanisms involved in navigation we performed a series of comparative studies with honey bees and fruit flies. We focused on three main questions. First, do honey bees actually show sugar-elicited search behaviour, second if so, how similar is this search behaviour in solitary flies and social honey bees, and third, is the search behaviour based on a simple increase in turning frequency or does it involve more complex mechanisms of spatial orientation.

To address the first two questions, behavioural response of male hungry flies and honey bee nectar foragers to sugar stimulus was studied by video recording. As a negative control, no sugar stimulus was provided to test animals and their response was recorded. Our experiments showed that social honey bees initiated a search behaviour after ingesting a drop of sugar which is quite similar to that of solitary flies. We propose that sugar-elicited search behaviour promises to be a fruitful behavioural paradigm to study general neural and molecular mechanisms of navigation.

I was also interested in the navigational aspect of the behaviour, particularly in understanding how do flies and bees precisely return to the location of the sugar drop. There are two possible mechanisms which flies and bees might use to return to the location of the food source without using any environmental cues: (a) the turning behaviour increases the probability to return to the starting position of the search trajectory (Wehner and Srinivasan, 1981), or (b) flies and bees use self-motion (idiothetic) information and path integration to intentionally return to the location of the food source (Mittelstaedt and Mittelstaedt, 1980; 2001; Seyfarth et al. 1982; Chittka et al. 1999; Thiélin-Bescond and Beugnon 2005; Zeil et al. 2013). Path integration is a navigational strategy in which an animal continuously tracks its distance and direction covered, as it moves away from a reference point. In order to generate idiothetic information the animal needs to estimate its own position as a function of body movements in space i.e. distance and direction.

To distinguish between these two mechanisms, I recorded the response of flies in the absence of visual and chemosensory signals or cues, i.e., footprint pheromones or minuscule amounts of sugar-water or dried sugar crystals (Cederberg 1977; Ferguson and Free 1979; Giurfa and Núñez 1992; Witjes and Eltz 2009). These experiments how that that search behaviour involves idiothetic (self-motion) cues; that is the fly is able to estimate its current position with respect to the position of the drop, the starting position of the search, as a reference and perform path integration.

Passive displacement experiments- where a locomoting animal is displaced by the experimenter are successfully used to study path integration in navigation (Müller and Wehner 1988; 1994; Dacke et al. 2020; Patel et al. 2022). By physically separating the stimulus and behavioural response in passive displacement experiment, wherein we let the flies and nectar foragers feed on a small amount of solution and transferred them to a new arena without any food reward, I confirmed that flies and bees use path integration.

Additionally, we were interested in understanding the initiation of the behaviour. Using displacement experiments, we provide evidence that flies and bees initiate a path integration-based search around the location where they started walking and not around the location of the sugar. The ingestion of sugar served as an initiator of the behaviour in both flies and bees, as it increased the likelihood of initiating a local search. However, we found that the path integration-based search commences upon the onset of walking and not the finishing of food intake.

These findings suggest that this small-scale spatial orientation behaviour involves capabilities and strategies present in large-scale navigation (Frisch 1967; Wehner and Srinivasan 2003; Collett et al. 2013; Wehner et al. 2016). We propose that sugar-elicited search behaviour can be used as a paradigm to study initiation and control of path integration system. Additionally, this study supports the idea that we can use local search in flies to understand the mechanisms of honey bee dance communication.

2.2 Materials and Methods

2.2.1 Fly rearing

Male flies of *Drosophila melanogaster* Canton-S (CS) strain were used in all the experiments. Both male and female flies show sugar-elicited search behaviour but starvation time is more consistent among male flies. Additionally, female flies change their feeding preference after mating (Carvalho et al., 2006). Hence, we used only male flies. Flies were reared and maintained on standard fly media (Table S1) at 25°C in a 75% relative humidity in a 12-hour light/dark cycle. Flies eclosing within a 12-hour period were collected and maintained in fresh medium for two days. The flies were starved of food (with access to water) before the behavioural experiments. To standardise the hunger state across trials, flies were starved for the duration of 90% survival of the population under food-starved conditions.

Food-starvation tolerance was calculated by depriving two-day old flies of food with access to water with soaked Kimwipe paper at the bottom. 15 flies were placed in 3 vials each. The number of surviving flies were counted every hour and survival curves were plotted. The duration at which 90% of the starved flies survived was used as the starvation period. This starvation period for CS flies ranged from 26-29 hours in tested flies, over the course of the experiments. Behavioural experiments in flies were performed between 1400-1700 hrs.

2.2.2 Fly local search

Flies were individually tested for local search after starvation. 90 mm Petri dishes were used as an arena for behavioural assays. The arena was illuminated from the bottom by a panel of surface-mounted cool white LEDs. The arena was made of opaque milk glass which diffused the light generating an almost homogeneous light distribution. The light intensity was 320 lux at the centre of the arena, measured using TENMARS TM-203 Data Logging Light Meter. 2-D position of the flies was recorded with an overhead camera at 40 fps (Flea3, Point Grey, 1214 mm lens, Azure). The Petri dish was surrounded by a white cylinder (51.5 mm height, 114 mm inner diameter) made of polyvinyl chloride resin to contain the visual field of the flies (Fig. 2.1). Petri dishes were wiped with 70% ethanol between trials.

A drop of sucrose solution (0.2 µl, 500 mM) was positioned in the centre of the arena. A 2 ml microcentrifuge tube (inner diameter 8.7 mm, length reduced to 5 mm by stuffing cotton at the bottom) housing a single fly was inverted over the sugar drop. The experimenter waited until the fly found the sugar and started feeding before removing the tube. We used this protocol to record the behaviour of control flies (N=52), who were fed with sugar and allowed to search without any displacement. These flies were used as undisplaced control and control against unfed flies (N=11), who were not given any sugar. We observed the trial till the time the fly reached the periphery, climbed over the edge, and left the arena.

2.2.3 Local search in the absence of visual and chemosensory cues

To test if flies use external cues to guide their returns to the starting position, we did two sets of experiments in complete darkness. Experiments under dark conditions were performed in a dark room (<1 lux, measured by CENTER 337 digital mini luxmeter; Center Technology Corp., Taiwan) and the arena was illuminated with infrared (IR) LEDs (850 nm, S8100-60-B/C-IR, Scene Electronics, China). Preparation and positioning of the flies were done under the dim deep red light. When the experimental fly commenced ingestion of the solution, the deep red light was turned off and the video was recorded in only IR.

To test the possibilities of chemosensory cues guiding the returns, the sugar drop was provided on a disc (17 mm diameter) in the dark. We then waited for the fly to start the search. The discs were 0.175 mm in thickness and made of clear polyester sheet. I had also tested a smaller disc (5.6 mm diameter). However, I observed qualitatively similar behaviour and hence included the analysis for only the larger radius. The disc was gently removed as soon as the fly stepped out of the disc during its search trajectory. Therefore, the area occupied by the disc should be devoid of any physical and chemical cues. All experiments belonging to one set were over several days.

2.2.4 Passive displacement experiments in flies

For passive displacement experiments, the fly was allowed to feed on a drop of sugar for 40 s. This time was fixed based on the mean feeding time of fed flies in control experiments. The fly was then gently picked with a mouth aspirator (diameter<0.5 mm) contacting the thorax and we removed the Petri dish. The fly was then transferred to an arbitrary position on a fresh Petri dish and allowed to walk freely. This transfer procedure took 3-4 seconds and was referred to as instant transfer (N=40).

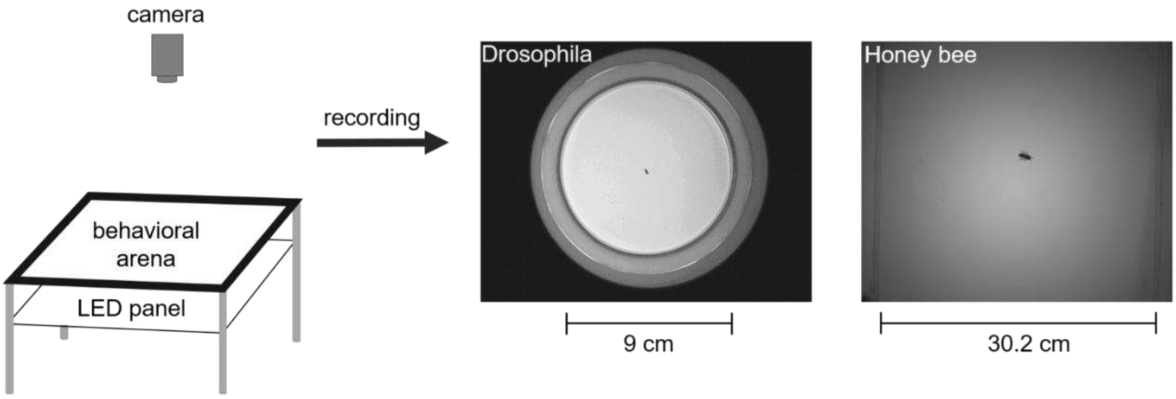


Figure 2.1: Experimental setup to study local search. The arena with overhead view of the arena for flies and honey bees

2.2.5 Honey bee colonies

Apis mellifera colonies were obtained from a local beekeeper. The colonies consisted of 4-frames containing approximately 2000 individuals per frame. The colonies were housed on a lawn inside the campus of the National Centre for Biological Sciences, Bangalore, India. Foragers were trained to an unscented feeder containing 1 M sucrose kept 5 m from the hive entrance. The colonies were also given pollen on an artificial feeder, but the pollen feeder was removed during the experimental hours. The bees were trained for two hours from either 1-3 PM or 2-4 PM depending on the weather conditions.

2.2.6 Honey bee local search

Nectar foragers arriving at the feeder were individually tested for local search. A single bee was collected in a tube (inner diameter: 14.9 mm) before it started collecting sugar at the feeder. It took us under a minute (an average walking time 55.27 s was recorded over days) to reach the behaviour room from the feeder location. We used a larger square arena (30.2 cm x 30.2 cm) for honey bee search experiments. The arena made of transparent acrylic sheet was backlit by a panel of surface mounted cool white LEDs (Fig. 2.1). The tube housing the honey bee was placed on the illuminated arena for 1 min so that it could get acclimated to the change in lighting conditions from outside.

A drop of sucrose solution (3 μl, 2M) was placed in the centre of the arena. The bee was quickly transferred to a bigger tube (inner diameter: 27 mm, length reduced to 8 mm by stuffing cotton at the bottom). This bigger tube was then inverted over the sugar drop and the experimenter removed the tube after the bee found the sugar and started feeding. We used this method to record the search of control fed bees while no sugar was provided for unfed controls. We observed the trial till the bee flew away or reached the periphery of the arena, climbed over the edge and left. The light intensity was 520 lux as measured by TENMARS TM-203 Data Logging Light Meter, at the centre of the arena. Search was recorded with an overhead camera at 40 fps (Flea3, Point Grey, 1214 mm lens, Azure). The floor of the arena was wiped with 70% ethanol between trials.

2.2.7 Passive displacement experiments in honey bees

For passive displacement experiments, we provided the sugar in a 90 mm Petri dish placed in the centre of the arena. This helped us save time since we could quickly remove the Petri Dish and introduce the bee in the arena. The bee was first allowed to feed on a drop of sugar for 8 s. This time was fixed based on the average feeding time of the fed group of control bees. The bee was gently picked with a mouth aspirator (diameter ~2.5 mm) contacting the thorax and the Petri dish was removed. The bee was then transferred to an arbitrary position in the arena and allowed to walk freely. This transfer procedure took about 4 s and was called instant transfer (N=16).

2.2.8 Analysis of trajectories for local search

Ctrax - The Caltech Multiple Walking Fly Tracker (Branson et al., 2009; http://ctrax.sourceforge.net/) was used for tracking the flies. MATLAB 2019b, Rstudio (Version 1.4.1106) and Python are used for analysis.

For control experiments, we defined the end of feeding and start of walking as flies moving at a speed>4 mm s-1 and bees moving at a speed>3 mm s-1 in three consecutive frames. We used the same criterion to define walking in the transfer experiment. We calculated the time it took the insect to start walking after being transferred to the new plate as ‘latency in walking’. The distance covered in this time by flies and bees was called ‘distance before walking’.

We used the following parameters: meander, stay time, path length and number of returns, to quantify the search behaviour. Path length (distance walked during the search, in mm) and stay time (time spent during search, in s) were calculated from search trajectory. The insects ‘meander’, the inverse of path straightness, was calculated by dividing the beeline of the path (the distance between the first and the last point of the path) to the total path length, and subtracting from 1. High values of meander indicate more tortuosity in the walking path.

We developed an algorithm to identify and count the number of returns using two concentric circles. An inner circle indicating the origin of search (Rin = 2.5 mm, 10 mm for bees) and the outer circle indicating the minimum distance (Rout = 4 mm for flies, 16 mm for bees) that the animal had to move away from the origin. A return was defined as a movement out of the outer circle (Rout) and then coming back into the inner circle (Rout). To characterise path integration-based search and obtain the proportion of animals initiating search, we used a criterion of at least one return to the origin of the search and meander>0.85 for flies, and meander>0.8 for bees (Fig. S1).

In displacement experiments, we observed that flies and bees sometimes made small sudden and zig-zag movements where they were set down before searching. These movements were included in the latency of the start of the search as ‘pre-search walk’. We used the same criterion for speed as in control experiments for dividing the trajectories of transfer experiments into pre-search walk and walking that occurs during the search. The time between being transferred and initiation of search was termed ‘latency in walking’. If any distance was covered during the latency in walking, it was called ‘distance before walking’.

2.3 Results

2.3.1 Flies and bees initiate a local search after sugar intake

Hungry flies (N=52) and motivated honey bee foragers (N=35) when briefly fed on sugar start a local search in the vicinity of the sugar drop. The search trajectory consists of walking in a meandering path with loops and frequent returns to the location of the sugar drop (Fig. 2.2). The trajectories are normalized to the location of the food and plotted after removing the feeding duration. Therefore, the tracks depict the walking path of the flies and bees during the search.

Hungry flies (N=11) and bees (N=12) when positioned in the arena without a sugar drop did not initiate a search (Fig. 2.3,4). 61.54% (32/52) of the flies and 77.14% (27/35) of the bees initiated a search after sugar intake (Fig. 2.5). This proportion was significantly higher than the control flies and bees, where zero flies or bees had initiated search. The initiation of search was quantified as the trajectory with least one return and meander above a threshold (see methods). Control flies and bees either flew almost immediately

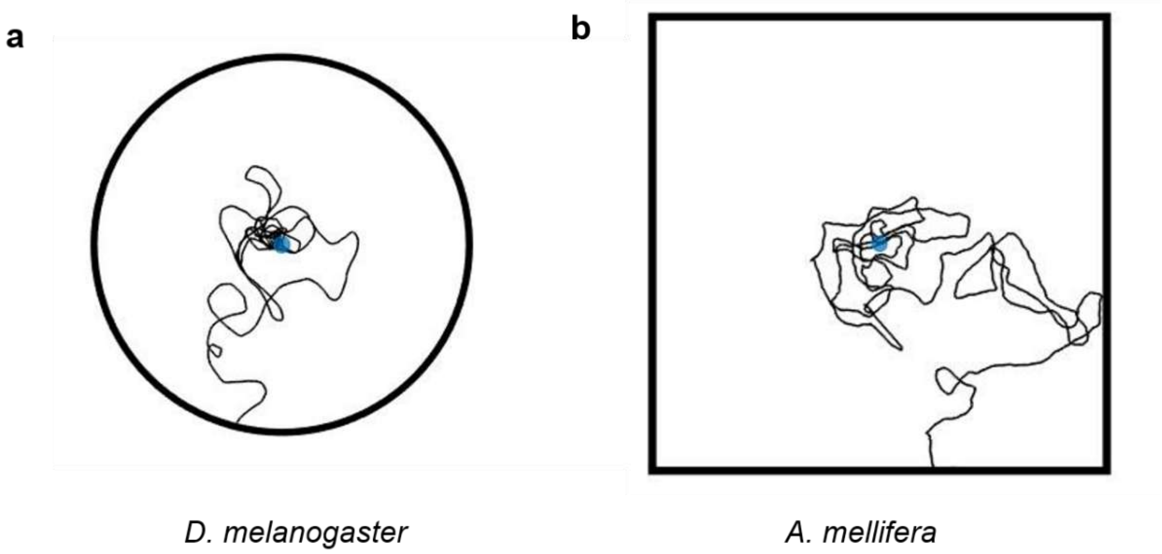


Figure 2.2: Trajectories depicting sugar-elicited search in (a) fly and (b) Honey bee.

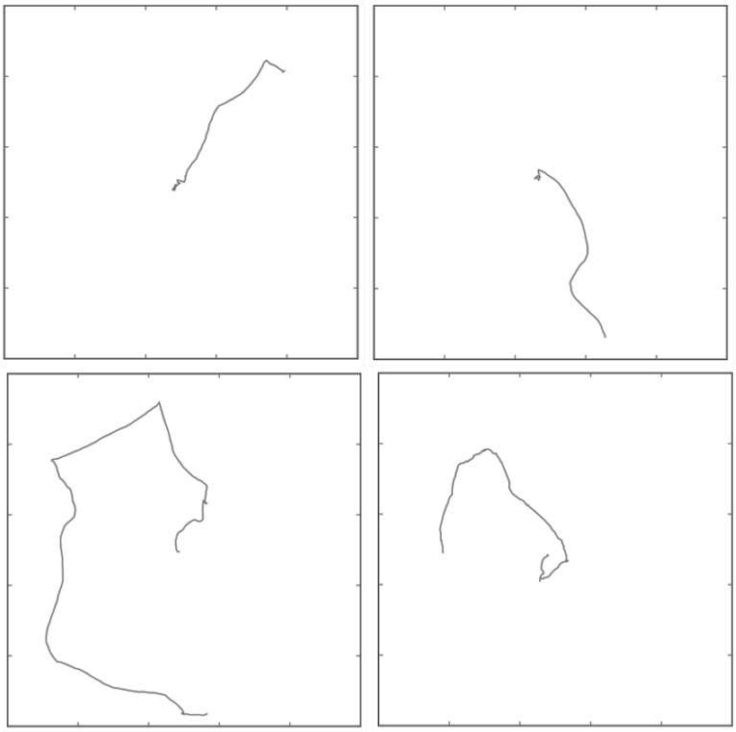


Figure 2.3: Unfed flies and bees did not initiate search. Individual tracks of four out of 11 tested starved flies that were not fed sugar.

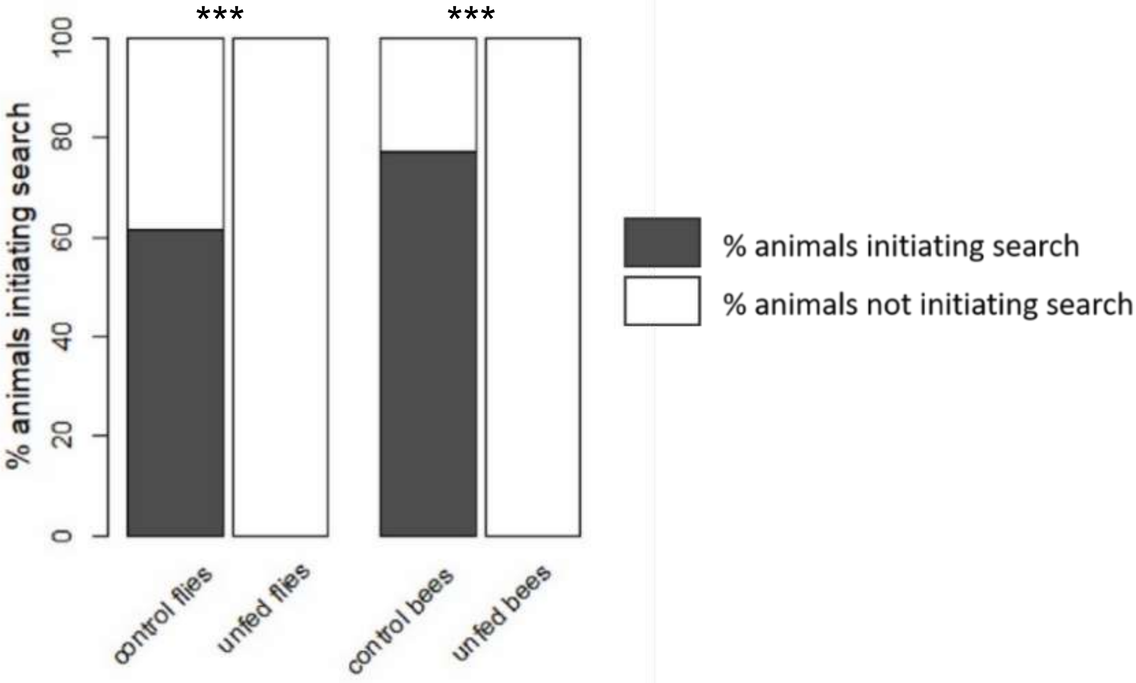


Figure 2.4: Hungry flies did not initiate search when there was no sugar stimulus. ***p<0.001, Chi-square test.

upon being introduced in the arena, walked a short distance before flying, or walked to the edge of the arena in a straight path. Additionally, there were zero returns in unstimulated flies and bees. All the analyzed parameters of the trajectories: meander, stay time, and path length, were significantly lower compared to flies and bees that were provided with the sugar stimulus (Fig. 2.6). These results demonstrate that sugar intake is necessary for the search.

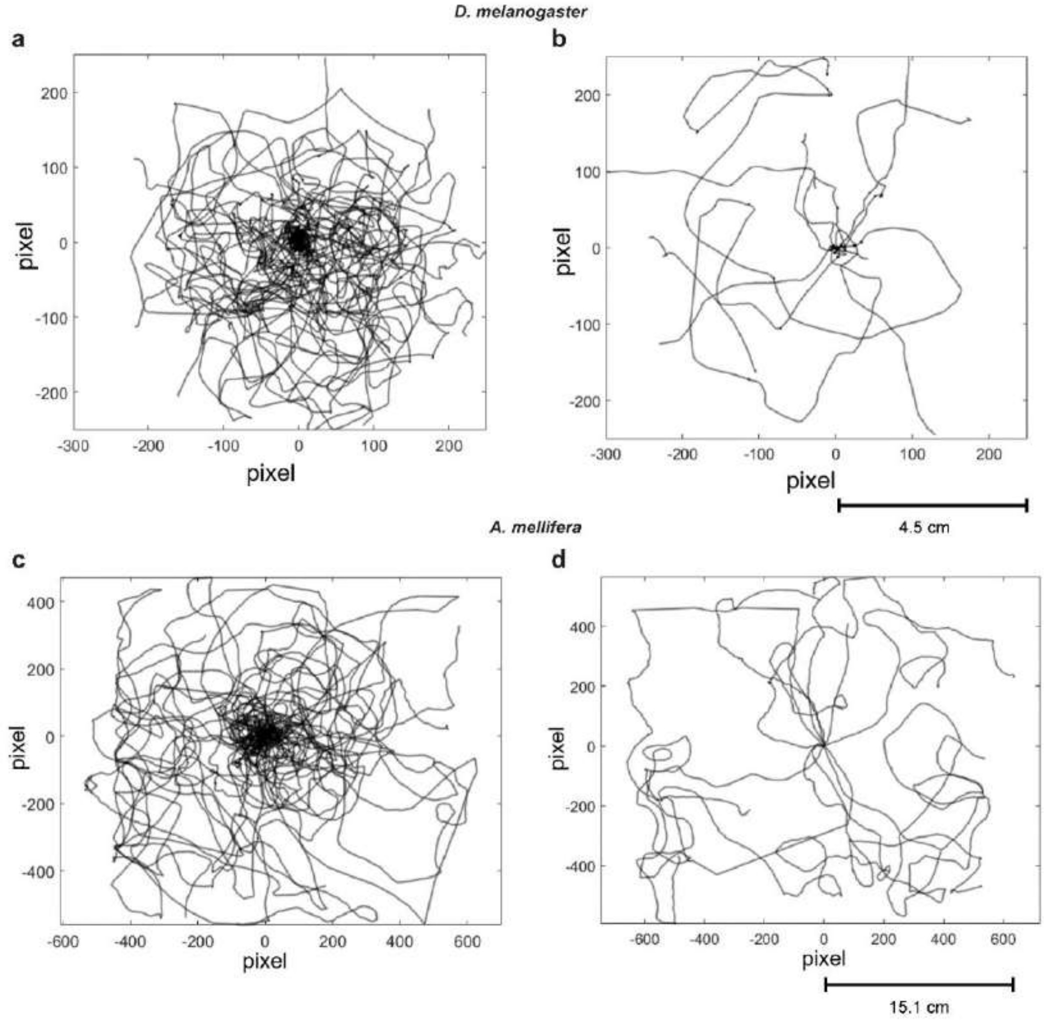


Figure 2.5: Sugar intake is necessary for initiating local search. (a) Overlay of search trajectories of 11 flies from the control group for local search stimulated with 500 mM, 0.2 μl sugar solution. (b) Overlay of the paths of the flies (N=11) which were given no sugar. (c) Overlay of search trajectories of 12 bees from control fed with 2 M, 3 μl sugar solution. (d) Overlay of path of the bees (N=12) recorded without sugar stimulation.

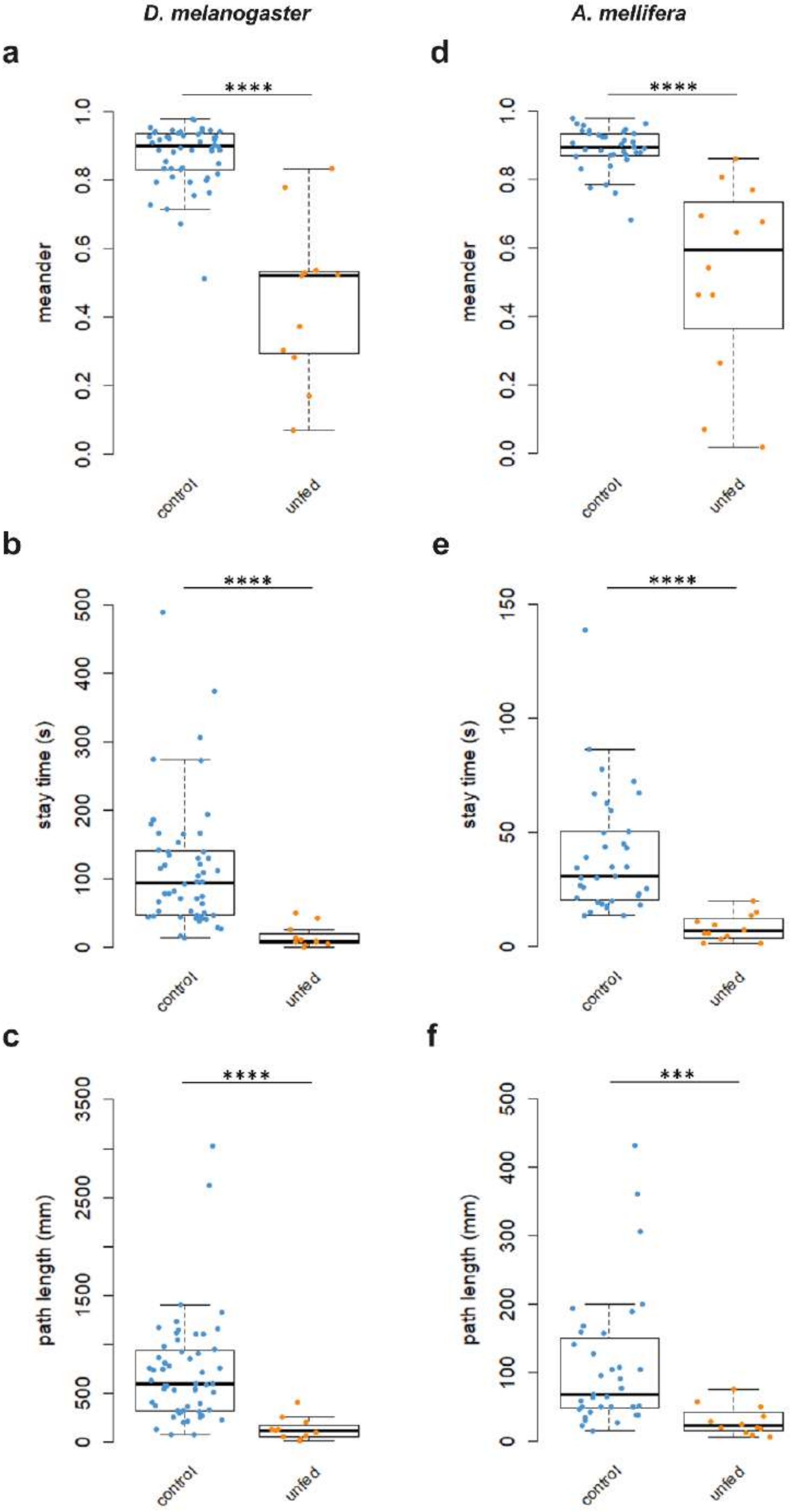

D. melanogaster
A. mellifera
a
d
****
****
meander
0.0
0.2
0.4
0.6
0.8
1.0
control
unfed
b
e
****
****
stay time (s)
0
100
200
300
400
500
0
50
100
150
c
f
****
***
path length (mm)
0
500
1500
2500
3500
0
100
200
300
400
500

Figure 2.6: Behavioural parameters were significantly lower in unfed flies and bees. (a-f) Meander, stay time and path length were smaller for hungry flies that were not given sugar reward compared to control flies and bees that were given stimulated with sugar. ***p<0.0001, ****p<0.00001, Wilcoxon Rank Sum Test.

2.3.2 Flies and bees use path integration to return to the location of the food

Flies and bees start a search around the position of the sugar drop and make frequent and precise returns to the location of the drop. 2-D heatmaps show a high occupancy near the origin indicating returns to that position (Fig. 2.7). I was interested in the question, how do flies and bees navigate to the location of the sugar drop, during the search. They could use external sensory cues or idiothetic (self-motion) cues to guide their returns. To distinguish between these two possibilities, I performed two sets of experiments. First was a series of experiments in flies where I eliminated external cues one by one, and second was a passive displacement experiments in both flies and bees.

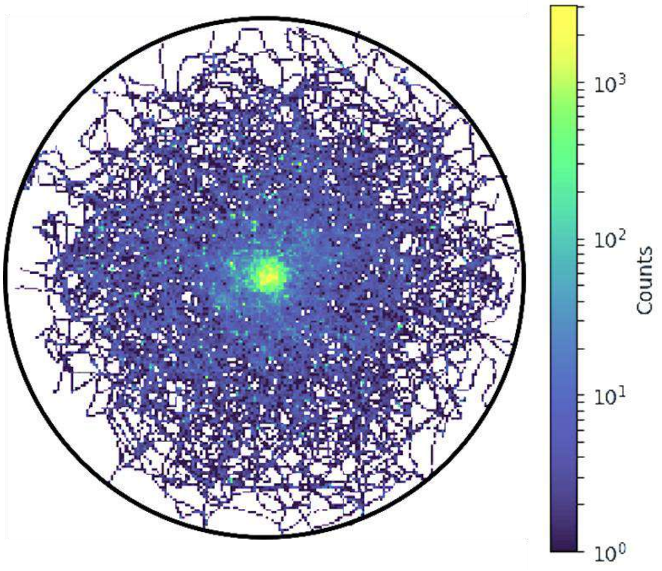


Figure 2.7: Flies make returns to the location of the sugar drop. ositional heatmap of all the flies recorded in light (N=52) showed high occupancy near the origin.

To test whether flies use visual clues to navigate their way back to the origin, I tested the flies in the dark (N=44). I then explored whether they might use chemosensory signals or cues, i.e., some emitted signal, footprint trail or dried sugar deposit. To test this, the sugar solution was provided to the flies on a retractable transparent disc placed in the arena (N=20). This was done in the dark, to eliminate visual as well as chemosensory cues. The flies initiated the search and made returns to the origin in both these conditions. Heatmaps showing positional occupancy of flies show high frequency at the centre indicating returns (Fig. 2.7). All three sets of experiments showed a similar percentage of flies initiating search (~70%). This percentage and number of returns were not statistically different across the three experiments (Fig. 2.8). These results demonstrate that flies are capable of returning to the sugar drop location without visual and chemosensory cues.

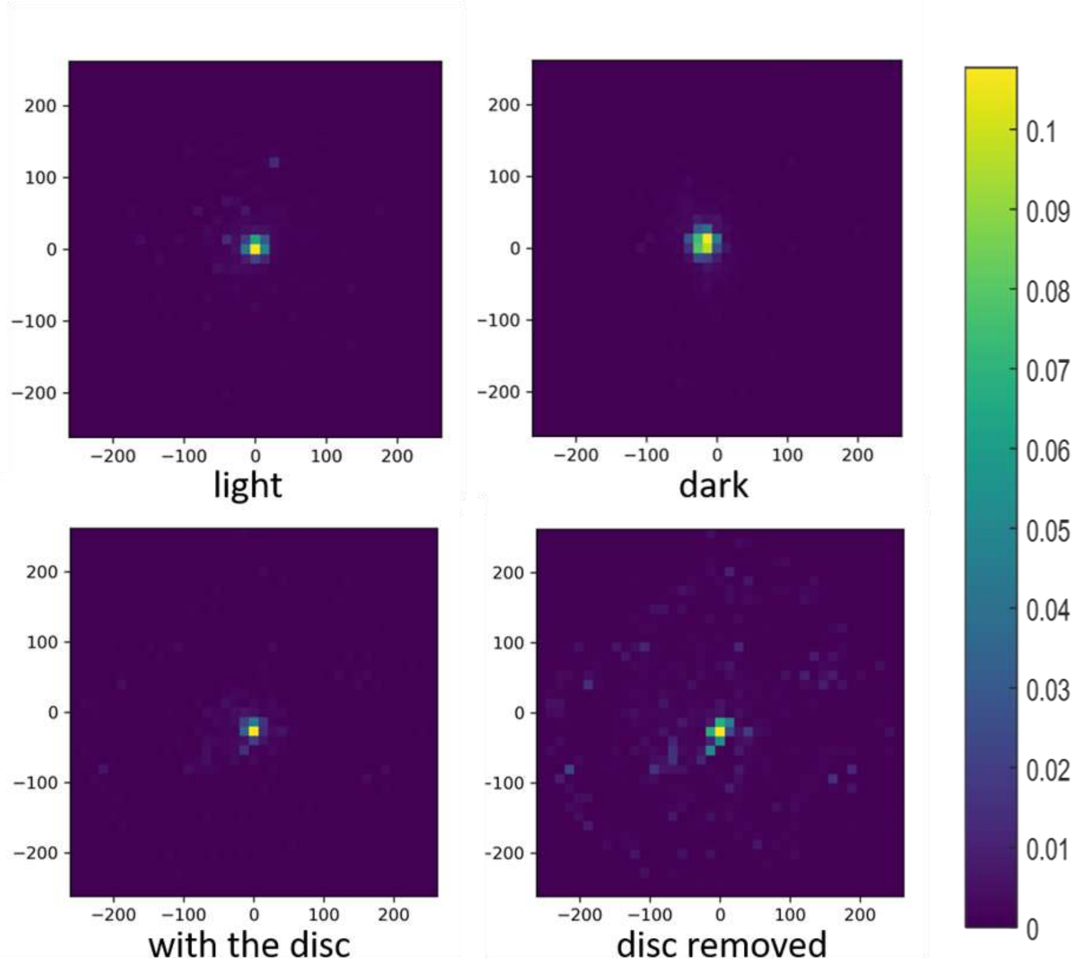


Figure 2.8: Flies made returns and navigate to the origin in the absence of external cues. Heatmaps for search in the dark and disc experiments show occupancy near the origin in the absence of visual and chemosensory cues. The bottom panel shows the trajectories with the disc present, as well as with the dosc removed, where initial part of the trajectory on the disc is removed from the analysis.

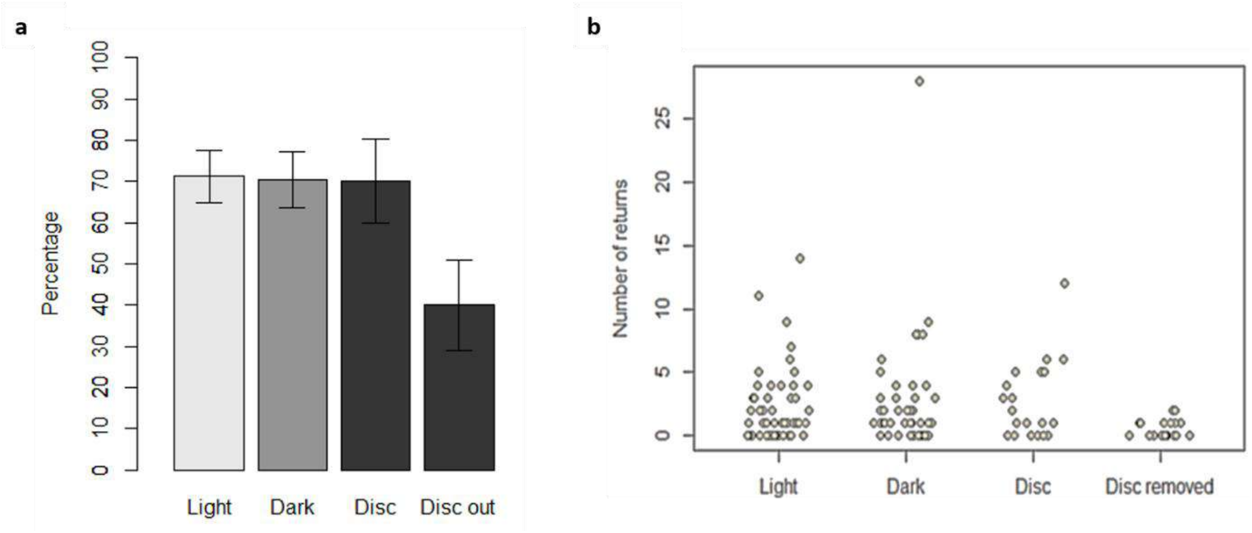
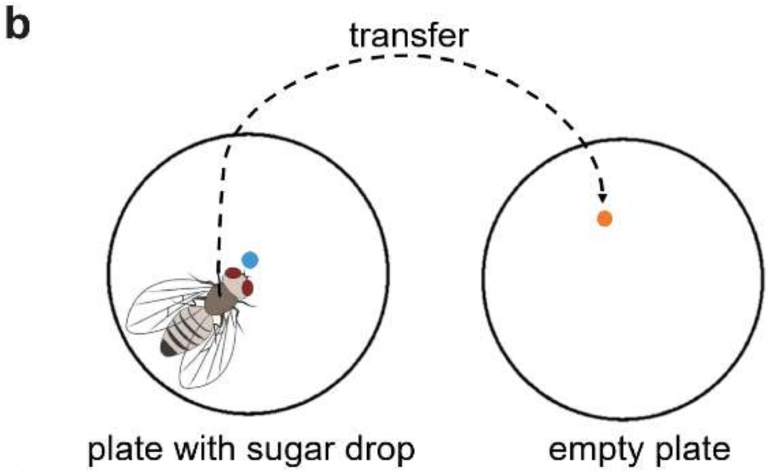


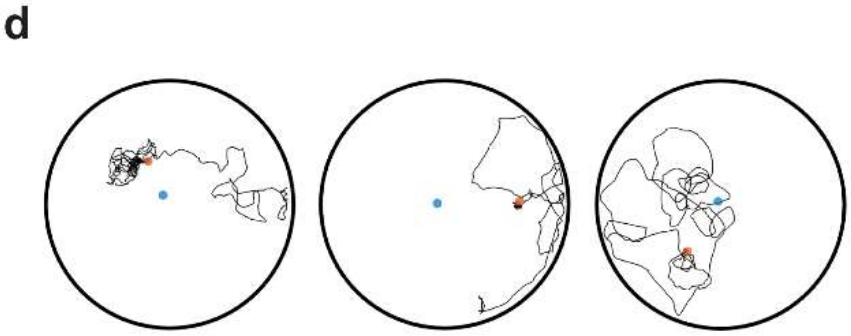
Figure 2.9: Flies return to the location of the sugar drop in the absence of external cues. (a) Percentage of flies that initiated at least one return. See materials and method for definition of returns. (b) Number of returns for each experimental condition tested.

2.3.3 Location of the sugar reward had no bearing on path integration-based local search in flies and bees

Passive displacement experiments have been successfully used to study and identify navigational mechanisms underlying food-seeking and foraging behaviours. The flies and bees were released post-feeding at arbitrary points in a fresh arena plate which had no food (Fig. 2.10 b, c).

Individual trajectories post-displacement showed that flies and bees started a search around the position where they are transferred (orange circle) and not at the location of the sugar drop (blue circle) (Fig. 2.10 d,e). In the displacement experiments, 55% flies (22/40) and 56.25% bees (9/16) initiated a search involving path integration. This


b
transfer
plate with sugar drop
empty plate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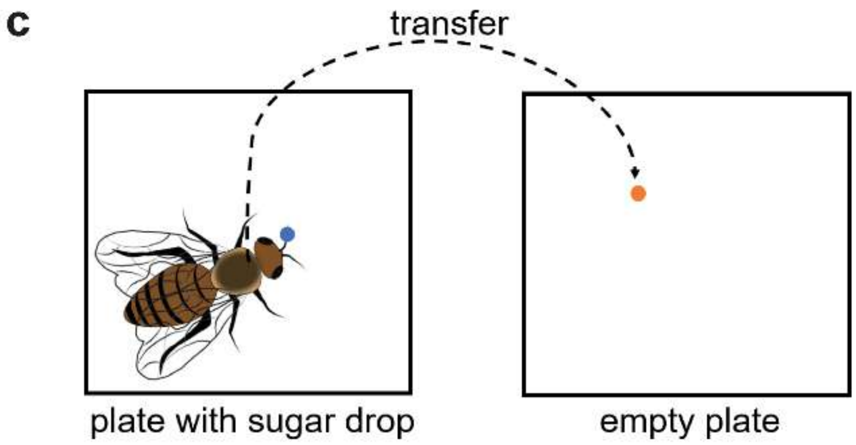

c
transfer
plate with sugar drop
empty plate

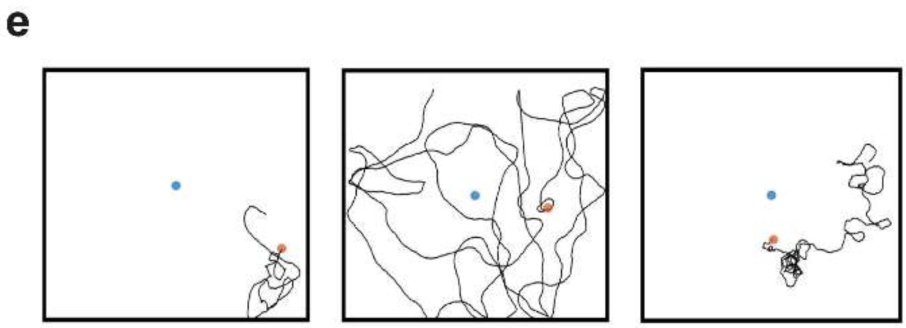

e

Figure 2.10: Flies and bees started a search involving path integration when displaced to a new location after feeding. (b,c) Schematic of the displacement experiment in flies and bees. (d,e) Individual trajectories of flies and honey bees with the location of sugar drop (in blue) and position where they are introduced in the new arena (in orange).

proportion was not different from the undisplaced controls described earlier (p=0.5299 for flies; p=0.1325 for bees, Chi-square test). These results were confirmed when the trajectories are normalized to the point where each fly and bee was transferred in the new arena. The density of positional coordinates became centred near the origin after normalization (Fig. 2.11).

However, it should be noted that there are differences in the behaviour of the two species during the behaviour. For instance, the values for meander, stay time, and path length were significantly higher in flies compared to bees in the displacement experiment (Fig. 2.12).

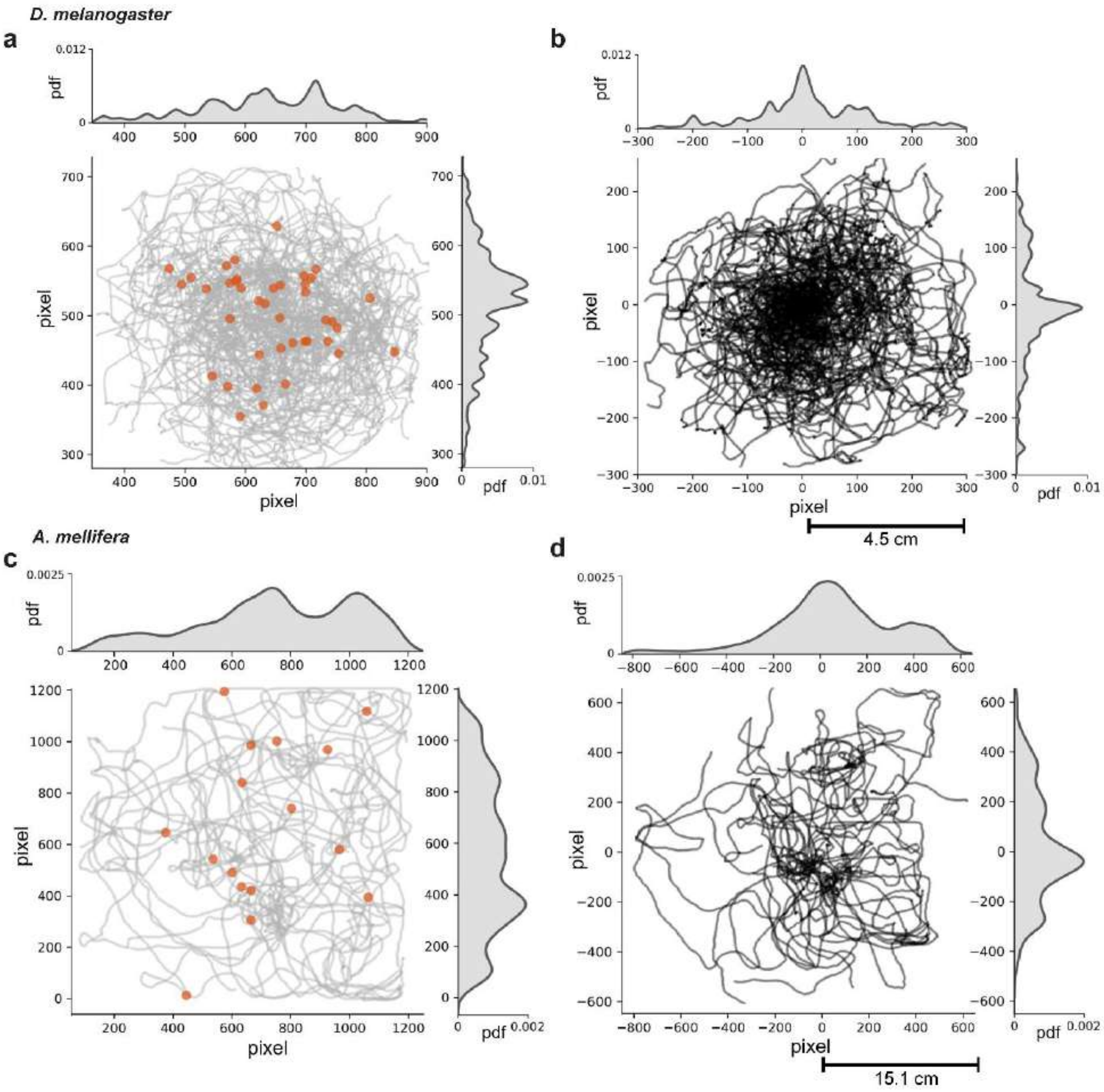


Figure 2.11: Upon passive displacement post-feeding, flies and bees started a search involving path integration around the position in the arena where they are transferred. (a) Overlay of all trajectories with the pdf (probability density function) of tested flies (N=40). The position where each fly was set down is represented with orange circles. (b) Trajectories plotted using a new coordinate system where the origin is the position where flies were introduced in the new arena. (c) Tracks and pdf of all the tested bees (N=16) with the location of sugar in orange. (d) Overlay of the trajectories of the bees after normalising to the point where they were transferred.

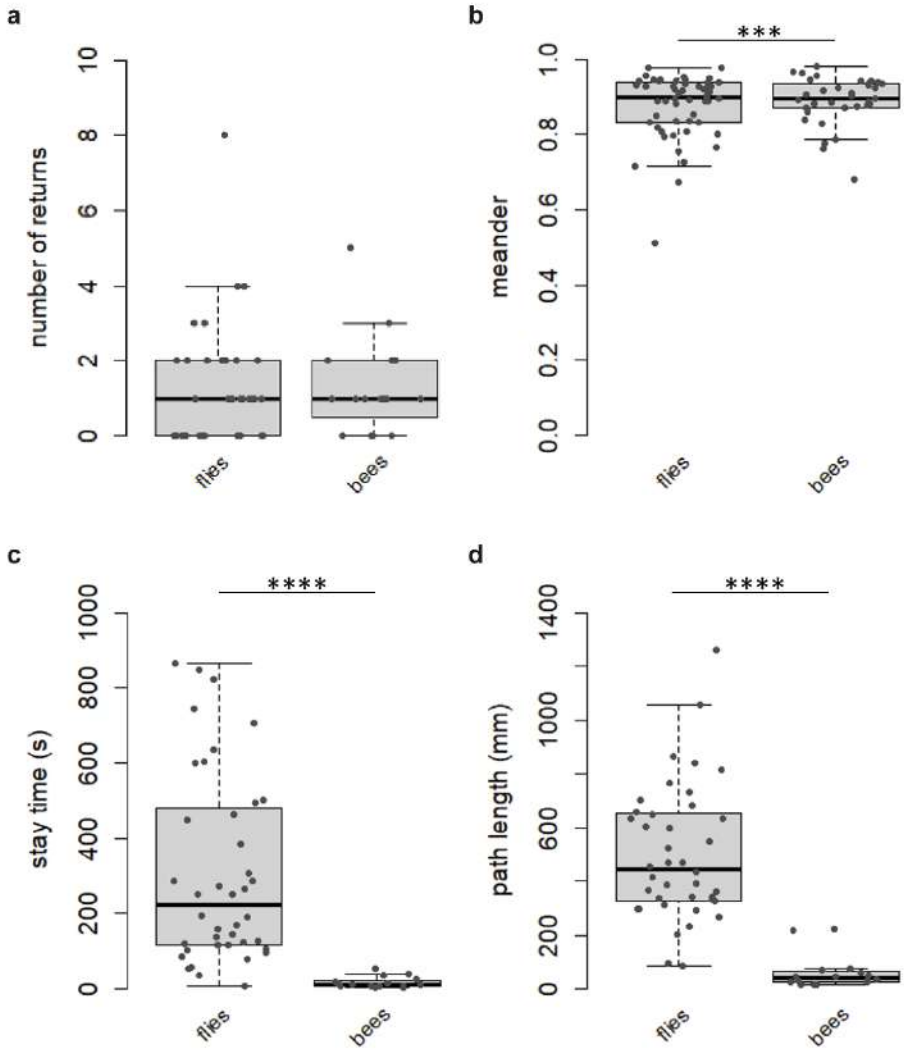


Figure 2.12: **Comparison of behavioural parameters between flies and bees for displacement experiments.** (a) Number of returns do not show a difference between the two species. (b-d) Meander, stay time and path length are lower in bees than flies. ***p<0.0001, ****p<0.00001, Wilcoxon Rank

2.3.4 Onset of walking after sugar intake starts the path integration system in flies and bees

Path integration requires active locomotion by the animal and an interesting question was whether flies used the exact location where they were released or a point near it. I noticed that after flies and bees were introduced in the new arena, sometimes they made small movements before starting to walk. Based on the analysis of the pre-search walk in the transfer experiments (see methods), I detected that for a significant majority of flies (90%) and bees (81.25%), the latency in walking and distance before walking was lower than the average (Fig. 13). The pre-search phase for these animals was extremely short and the search started almost immediately after displacement.

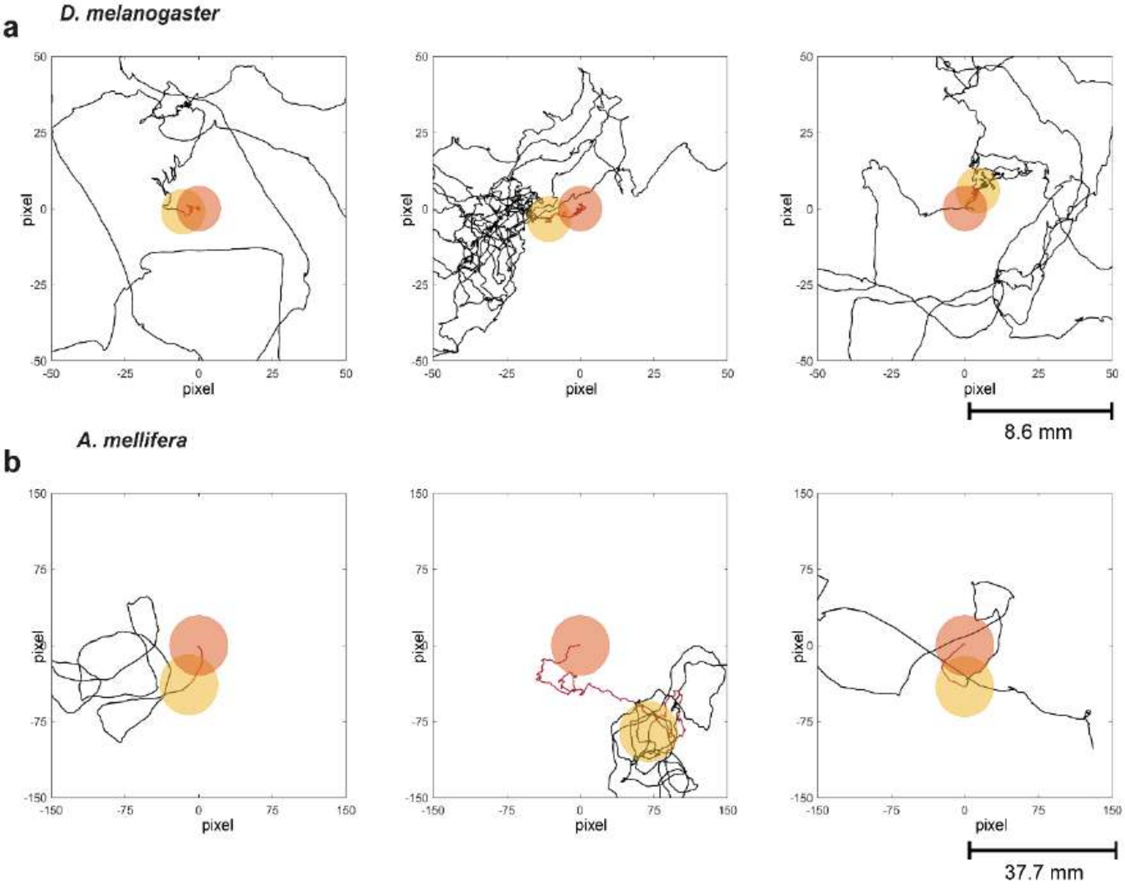


Figure 2.13: Displaced flies and bees use the end of the pre-search walk as the reference point for path integration-based search. (a) Zoomed in trajectories of the 3 (out of 4) flies with higher than average (mean±SE) latency in walking (time: 7.71±3.25 s, distance: 4.40±1.6 mm). (b) Trajectories of all the 3 bees with higher than average (mean±SE) latency in walking (time: 0.95±0.83, 7.39±6.08). Orange circle is drawn around the position where the animal was displaced as the centre and the pre-search trajectory is marked in maroon. The search trajectory is denoted in black and the yellow circle is drawn around the point of onset of walking.

For the remaining flies (4 out of 40) and bees (3 out of 16), we could mathematically distinguish the positions where the animals were displaced and where they began the search (Fig. 13). These instances allowed us to explore whether the reference point for path integration-based search is based on the position where the animal was set down, or the position after its pre-search walk, where it started walking. We observed that in these cases, the returns appeared directed towards the origin of the walking more than where they were set down. These results indicate that the position where flies and bees start walking is used as a reference point for returns.

2.4 Discussion

These experiments showed that social honey bees initiated a search behaviour after ingesting a drop of sugar which is quite similar to that of solitary flies. More importantly, our analyses indicated that sugar-elicited search behaviour is not just a simple turning behaviour but involves a set of complementary responses: change in turning frequency, path integration and initiation of learning processes. My findings suggest that this small-scale spatial orientation behaviour involves behavioural capabilities and strategies present in large-scale navigation (Frisch 1967; Wehner and Srinivasan 2003; Collett et al. 2013; Wehner et al. 2016). Thus, sugar-elicited search promises to be a fruitful behavioural paradigm to study general neural and molecular mechanisms navigation.

The significant finding of our experiments is that sugar-elicited search behaviour, first demonstrated by Dethier, is more intelligent than previously understood and comprises a set of complementary behavioural responses including an increase in turning frequency and path integration (Fig. 2.14).

Previous studies have reported the initiation of local search through the activation of multiple sugar-sensing neurons in the brain and external sensory structures in flies (Corfas et al. 2019; Behbahani et al. 2021; Haberkern et al. 2019; Titova et al. 2023). Currently it is unclear how similar or different these local searches are from sugar-elicited search behaviour as the latter is dependent on the intake of food whereas the others can also be released by the stimulation of different exteroreceptors. Regarding the sugar-elicited search behaviour, described by Dethier, we propose that sugar intake, stimulating the pharyngeal sugar sensing neurons, is not an innate releasing mechanism in the strict sense but modulates the probability to start a search involving path integration (Dethier 1957; Heiligenberg 1977; Murata et al. 2017; Ronacher 2019).

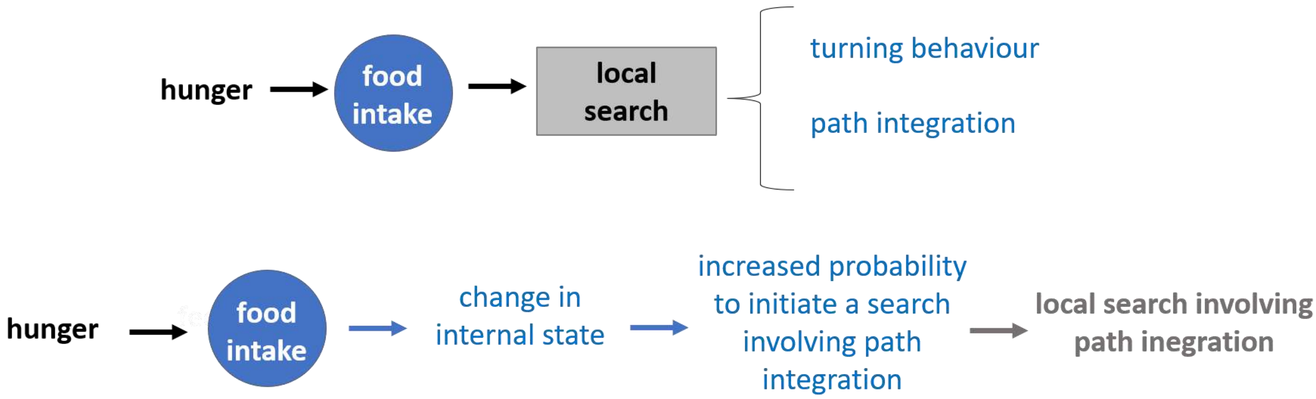


Figure 2.14: Scheme of behavioural responses involved in sugar-elicited search.

Thus, this local search behaviour involves behavioural responses and strategies that play major roles in large-scale insect navigation (Frisch 1967; Jeffery 2003; Wehner and Srinivasan 2003; Collett et al. 2013). The basic organization of the sugar-elicited search behaviour appeared to be conserved, but flies and honey bees also showed differences, which, for example, might be due to differences in general walking patterns and sensory response thresholds.

Bells's group was the first to suggest that suggested that an internal sense of spatial position may assist the fly (*Musca domestica*) in returning to the sucrose drop location (White et al. 1984). More recently, Kim and Dickinson (2017) showed that amino-acid starved mated *Drosophila* females after feeding on yeast, started a search behaviour similar to the Dethier's sugar-elicited search behaviour that involves path integration. In addition, Zeil et al. (2013) provided some evidence that the Banded Sugar Ant (*Camponotus consobrinus*) likely uses path integration during local search behaviour supporting our finding that path integration can be used during small-scale search behaviours.

My results show that that search behaviour involves idiothetic orientation; that is the fly is able to estimate its current position with respect to the position of the drop, the starting position of the search, as a reference and perform path integration. Reasons to return to the original location might be the probability that the food source is not depleted or has been replenished. Flies and bees likely use several different sensory systems, e.g., vision, olfaction, and gustation, to find their way back. However, our removable disc experiments under dark conditions demonstrate that they are capable of repeatedly returning to the start of the search in the absence of visual, olfactory, and gustatory cues. Furthermore, probabilistic analyses using transformed trajectories showed that returning to the sugar drop location cannot be explained solely by an increased turning frequency.

Passive displacement experiments have been successfully used to identify navigational mechanisms underlying food-seeking and foraging behaviours (Müller and Wehner 1988, 1994; Dacke et al. 2020; Patel et al. 2022). My passive displacement showed that feeding position is not necessarily the origin of the search. Feeding increases the probability to initiate the search involving path integration. These experiments provide clear evidence that the onset of walking and not the cessation of food intake starts the path integrator in both species. The location where the flies and bees started walking was used as the origin of search instead of the location where they found the food. This was most evident in a few of our experiments, in which the flies (4/40) and bees (3/16) made small movements away from the location where they were displaced, before starting the search. In those cases, the returns were distinctly oriented to the position where they started their walking trajectory. Evidently, the path integration system should be functionally coupled to the initiation of (self-) locomotion (Wehner and Srinivasan 2003; Seidl 2006; Beetz et al. 2022).

Thus, we conclude that flies and likely also bees are capable of using self-motion (idiothetic) cues, e.g., proprioceptive input, to navigate back to the sugar location. The most basic definition of path integration is keeping track of one’s own movement using self-generated (idiothetic) motion signals to be able to return back to the starting point of that movement irrespective of the distances traveled (Seyfarth et al. 1982; Mittelstaedt and Mittelstaedt 2001).

To summarize, the sugar-elicited search behaviour in flies and honey bees involves more complex navigational procedures than previously assumed. Given our results, Dethier (1957) original suggestion that sugar-elicited search behaviour and honey bee dance communication are closely related might not be so wrong (Brockmann and

Robinson 2007; Barron and Plath 2017). In this case, sugar-elicited search assay provides the opportunity to use *Drosophila* and its neurogenetic toolkit to study the neural circuits and genetic mechanisms underlying food search behaviour, navigation, and path integration (Neuser et al. 2008; Sitaraman et al. 2008; Ofstad et al. 2011; Murata et al. 2017). Parallel experiments in honey bees will allow to determine the behavioural differences between flies and a master of insect navigation, as well as verify whether the behavioural responses in the lab assay correspond to those used in large scale navigation in nature (Wehner 1999; Riley et al. 2003; Jacobs and Menzel 2014). Additionally, we propose that food-intake during foraging has the capability to activate the path integration system in flies and honey bees, and this interaction might have been elaborated during evolution to guide the walking pattern of the honey bee dance. Therefore, sugar-elicited search behaviour in flies can be used to gain insights into the organization of honey bee dance.

3. Solitary sugar-elicited behaviour and social dance communication

3.1 Introduction

Workers of eusocial bees have evolved elaborate communication behaviours to recruit nest-mates to leave the hive and forage food for the colony (Lindauer 1971; Michener 1974). Honey bees are unique in communicating the location of profitable food sources to nest-mates via the waggle dance. The dance indicates the direct flight path to the food source (Frisch 1967; Riley et al. 2005, Alves et al. 2023). The waggle run duration and direction correlate with the flight distance and direction to the food source and are presumably under the control of the brain’s path integration system when navigating while foraging (Riley et al. 2005; Menzel et al. 2011; Chatterjee et al. 2019). The initiation of dance behaviour, the number of waggle runs, and the intensity of the movements depend on the food quality, the arousal state of the forager and her interactions with receiver bees in the hive (Frisch 1967; Dyer 2002; Seeley 1995). Recruitment behaviours of stingless bees and bumblebees have similar relations between the initiation and intensity of the locomotor displays and the food reward but lack communication of navigational information (Lindauer and Kerr 1958; 1960; Dornhaus and Chittka 2001; Hrncir et al. 2004; 2011).

Dethier was the first to suggest that the dance is a highly evolved form of innate local search behaviour observed in solitary flies. Hungry flies when given a small amount of sugar start a local search around the food reward. He described this behaviour in blowflies and called it ‘dance’ while referring to honey bee dance as a delayed response to sugar intake (Dethier 1957). He made the intriguing observation that the flies initiated their search after they had been displaced from the location of the sugar drop. Recent studies have provided evidence that this search behaviour involves path integration, a mechanism by which the insects monitor their walking trajectory to calculate the distance and direction necessary to navigate back to the original location where they found and consumed the food (Kim and Dickinson 2017; Murata et al. 2017; Brockmann et al. 2018). Additionally, it has been demonstrated that local searches performed in different behavioural contexts can be triggered by activating various olfactory and gustatory sensory neurons in flies (Corfas et al. 2019; Haberkern et al. 2019; Behbahani et al. 2021; Titova et al. 2023).

I have demonstrated that sugar intake leads to local search in hungry *Drosophila melanogaste*r adults and motivated *Apis mellifera* nectar foragers. Passive displacement experiments show that the stimulus (sugar intake) and the response (search) can be spatially separated. Here, I was interested in understanding the initiation and regulation of the behaviour. This study addresses two key questions regarding the initiation of sugar-elicited search behaviour in *Drosophila melanogaster* and *Apis mellifera*. First, if sugar intake and the initiation of search behaviour can be spatially dissociated, how long do the motivational effects of the food reward persist? Second, what is the temporal dynamics of the honey bee waggle dance when a delay is introduced between sugar intake and the performance of the dance?

By impeding locomotor behaviour after feeding, we demonstrated that the heightened motivation to initiate the search lasted for a minimum of 3 min in both species. These results suggest that sugar elicits two independent behavioural responses: path integration and increased turning, with the initiation and duration of path integration system being temporally restricted. In a comparable delay experiment with foraging honey bees, we observed a significant reduction in the probability to initiate dance behaviour only after 15 min, while the number of circuits significantly declined at 3 min.

My findings show that sugar intake in the sugar-elicited search behaviour and honey bee dance communication initiates the behaviour but modulates the temporal dynamics of the behaviours differently, suggesting a more complex regulation of dance communication. Such a comparative study lays the groundwork to study the neural underpinnings of the honey bee dance using sugar-elicited search as a paradigm. Since neurogenetic manipulations are difficult in honey bees, one can use *Drosophila* to gain insights into brain modules that organise dance behaviour.

3.2 Materials and Methods

3.2.1 Delay experiments in flies

For delay experiments, the fly was allowed to feed for 40 s and then housed inside a tube (inner diameter 4.6 mm~1.5 fly length) to impede walking. We observed the flies to make sure that they were not moving inside the tube. The tube was cushioned on both ends, giving little to no movement for the fly, since we did not want the fly to start walking while it was inside the tube. This prohibited the fly from initiating search for specific durations. The delay durations tested were 30 s (N=18), 1 min (N=19), 2 min (N=16) and 3 min (N=21). After the experimental delay duration elapsed, the fly was introduced to a fresh Petri dish at an arbitrary position and its behaviour was recorded. Each experimental day consisted of tests with several time delays with the starved flies from the same batch in order to reduce the effect of day and observer bias. The dataset from displacement experiments (reported in chapter 2) was used as a control time-point and referred to as instant transfer.

3.2.2 Delay experiments in honey bees

For delay experiments, we had to modify the protocol of the fly experiments, because bees became agitated when we used the same method. Instead of housing the bees inside a tube to impede locomotion, an inverted tube (inner diameter: 14.9 mm, ~1 bee length) was placed over the bee to immobilise it. The inverted tube with the bee was gently moved away from the sugar drop and closer to the edge of the Petri dish for the elapsed delay duration. The Petri dish was also moved in some cases to an arbitrary location in the square arena. The tube was cushioned, and there was no space for the housed bee to walk.

The delay durations tested were 30 s (N=11), 1 min (N=13), 2 min (N=11) and 3 min (N=10). At the end of the period, we gently slid the Petri dish from underneath the bee and simultaneously removed the tube from the top. Thus, the bee was introduced to the fresh arena and we recorded its behaviour. We tested several time delays on the same day in an arbitrary fashion to reduce the effect of day and observer bias. The dataset from passive displacement experiments (reported in chapter 2) was used as first time-point in the series and referred to as instant transfer.

3.2.3 Honey Bee Dance experiments

Apis mellifera colonies (N=3) were obtained from a local beekeeper. The colonies were housed in an outdoor flight cage (16 m × 4 m × 4 m) on the campus of the National Centre for Biological Sciences, Bangalore, India. A pollen feeder was advertised outside of experimental hours. The day-night length as well as the temperature conditions inside the flight cage were the same as the natural conditions in Bangalore. The colony was transferred to an observation hive for recording the dances. We trained the foragers to an unscented sucrose solution (1M) from 11-12:30 PM using a gravity feeder kept on a channel plate (Fig. 3.1) 11 m away from the hive entrance. 15-20 bees were individually marked (Posca 5M, ‘Uni’ Mitsubishi Pencil, India) to form a foraging group.

Each replicate of the experiment was performed over four days and three observers were present at the feeder throughout the experiment. One person recorded the number and timing of the foraging trips. The other caught all recruits (non-marked foragers) coming to the feeder to keep the foragers motivated to dance throughout the experiment (von Frisch 1967). These bees were released back to the hive after the observation time. The third person performed the time delay procedure after the foragers collected sugar water.

On day 1, we recorded the dance activity of the marked foragers. The video was then analysed to identify the most active dancers (4-6) from the group (George and Brockmann, 2019). This was done based on ranking the bees based on the dance intensity (the ratio of number of circuits to the number of dances) and dance probability (the ratio of the number of dances to the number of foraging trips). These active dancers were randomly assigned to either the experimental or control group. On days 2-4, the experimental group underwent treatments while the control bees foraged freely. The foraging trips of the experimental group consisted of treated trips (where the delay was performed) and untreated trips (where no delay procedure was performed). We used one foraging group from colonies 1 and 3, while colony 2 was used for two foraging groups.

To introduce time delay post-feeding, we took advantage of the natural feeding behaviour of bees. After the foragers collect sugar water, they retreat their proboscis and walk a couple of steps away from the channel containing sugar solution before flying (Fig. 3.1). Just as the bees were about to take flight, a tube was gently inverted over the bee, similar to how we restrained the bees post-feeding in search experiments. This tube was painted black from outside and cushioned so that there was no space for the housed bee to walk inside (14.9 mm, ~1 bee length). The treatment was started after the bees in the experimental group completed five foraging trips.

Time delays that we tested in this experiment were: 30 s, 1 min, 2 min, 3 min, 5 min, 10 min and 15 min. Since our camera allowed real-time monitoring, we decided to test longer time delays because we did not see an extinction of dance for up to 3 min. An

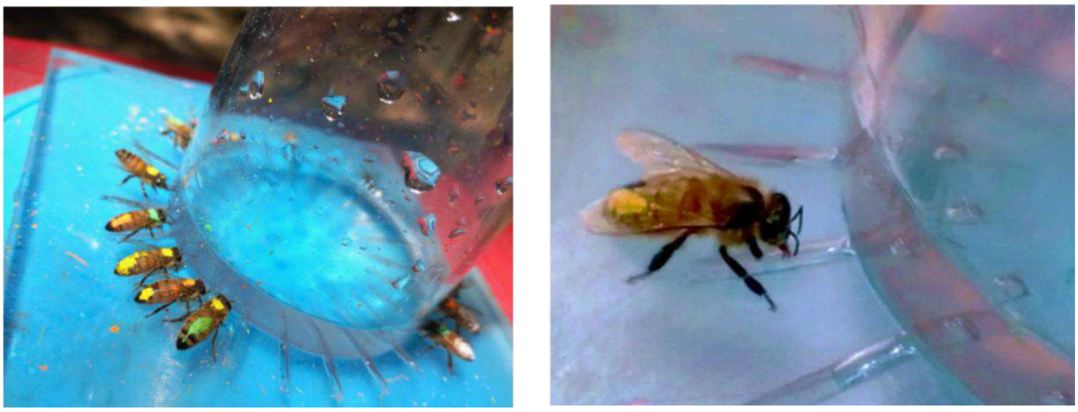


Figure 3.1: Honey bees at the feeder. Individually marked honey bee foragers feeding at an artificial feeder. The sugar is presented via a gravity feeder placed on top of a radial channel plate. The right panel shows a single bee feeding. The delay was introduced after the bee stopped drinking and retracted its proboscis from the channel.

instant treatment was also done, where the tube was inverted over the bee post-feeding for 3-4 s (same duration as instant transfer in Honey bee search experiments).

After the delay duration elapsed, the tube was lifted and the forager flew back to the hive. We tested each bee in the treatment group for all the time delays on each day to eliminate the effect of the day. Trips where a forager started feeding again after we performed the delay treatment were removed from the analysis. In total, 148 treatments over 11 individuals were tested (instant: N=26, 30 s: N=21, 1 min: N=24, 2 min: N=19, 3 min: N=20, 5 min: N=19, 10 min: N=9, 15 min: N=10).

3.2.4 Dance recording and analyses

The dances were recorded at 60 fps with a mounted video camera (GoPro Hero 8 Black CHDHX-801) and the videos monitored in real time. A temporary barrier at the nest entrance between the two faces of the observation hive directed the forager traffic to the side facing the camera. Since the food source was very close to the hive (11 m) the waggle run duration for the dances was very short (Gardner et al. 2008; Seeley 1995). Videos were analysed manually using Virtual Dub 1.10.4 (http://www.virtualdub.org/). Change-point analysis, which is a statistical method for identifying abrupt changes in time series data, was done using BEAST (https://github.com/zhaokg/Rbeast, Zhao et al. 2019).

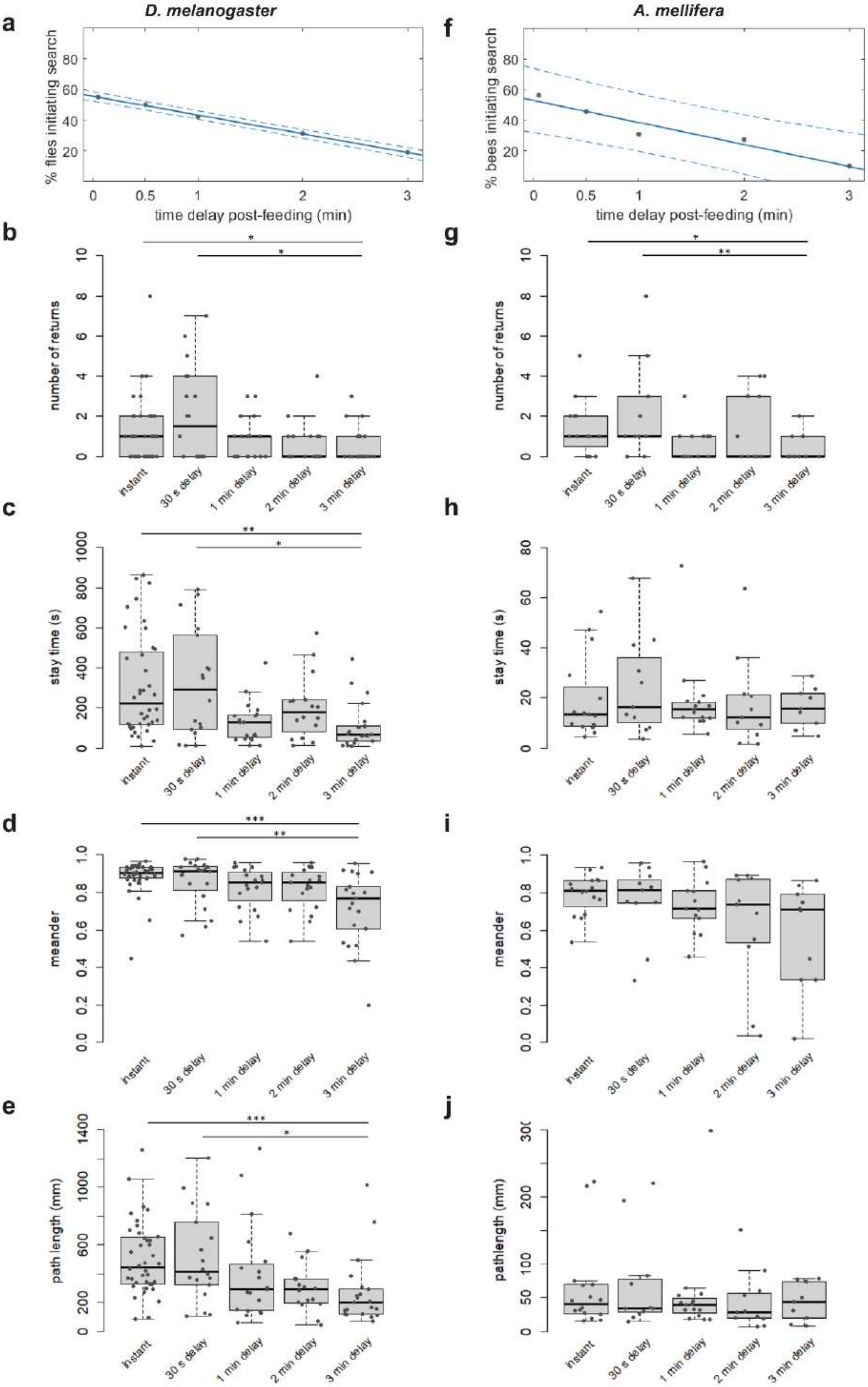
3.3 Results

3.3.1 The heightened motivation to start a path integration-based local search lasted at least for 3 min post-feeding

Passive displacement experiments suggested that sugar intake increases the probability that a fly or bee would start a path integration-based search after feeding. If the sugar intake is not an innate releasing stimulus for search, what happens if an interval is interposed between sugar intake and the opportunity to search? This question was approached by prohibiting flies and bees from walking in the arena for different durations after sugar intake. To understand the time dynamics of initiating a local search using path integration, I did a set of delay experiments (30 s, 1 min, 2 min and 3 min) to increase the time gap between feeding (the stimulus) and walking (the behaviour). Flies: N=40 for instant, N=18 for 30 s, N=19 for 1 min, N=16 for 2 min, N=21 for 3 min; Bees: N=16, for instant, N=11 for 30 s, N=13 for 1 min, N=11 for 2 min, N=10 for 3 min.

Percentage of flies and bees that initiated a path integration-based search at each tested delay duration declined linearly and was significantly reduced after 3 min (Fig. 3.2 a, f). Only 19.04% flies (4/21) and 10% bees (1/10) initiated a search when delayed for this duration (Fig. 3.3). This proportion was significantly lower than instant transfer ($p<0.001$ for flies; $p<0.05$ for bees, Chi-square test). The negative slope of this decline (units: min^{-1}) was similar in both species (dm: -12.18; am: -14.37, $p=0.98$, t-test for linear regression).

Delay duration also influenced the intensity and duration of the behaviour. The number of returns was significantly reduced for both flies and bees for the 3 min delay condition (Fig. 3.2b, g). Meander, stay time and path length declined significantly for the 3 min delay period in flies, but not in bees (Fig. 3.2c-e, h-j). 2D histograms depicted that as the delay period after feeding increased, the spatial position of the trajectories became less centred towards the origin of walking for both flies and bees (Fig. 3.4, 3.5). The probability density function for the total tested flies (21) after 3 min delay appears concentrated near the origin. However, it is the result of the trajectory of one fly out of


a
D. melanogaster
% flies initiating search
80
60
40
20
0
0.5
1
2
3
time delay post-feeding (min)
f
A. mellifera
% bees initiating search
80
60
40
20
0
0.5
1
2
3
time delay post-feeding (min)
b
number of returns
*
*
instant
30 s delay
1 min delay
2 min delay
3 min delay
g
number of returns
*
**
instant
30 s delay
1 min delay
2 min delay
3 min delay
c
stay time (s)
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*
instant
30 s delay
1 min delay
2 min delay
3 min delay
h
stay time (s)
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Figure 3.2 Effect of delay post-feeding on initiation and parameters of path integration-based local search in flies and honey bees. (a) Percentage of flies that initiate the search decrease with the increased delay post-feeding and fits a linear model f(x) = m*x + c where m=-12.18 (-13.23, -11.14), c= 55.44 (53.67, 57.21), adjusted R-squared: 0.9971 with 95% confidence bounds, $p<0.00001$, t-test for linear regression. (b) Number of returns, (c) meander, (d) stay time and (e) path length of flies for 3 min delay is reduced compared to instant transfer and a short delay (30 s). (f) Proportion of bees that initiate search decreases with increased delay durations post-feeding. The linear equation is f(x) = m*x + b2 where m=-14.37 (-21.55, -7.196), b= 52.77 (40.66, 64.89), adjusted R-squared: 0.9083 with 95% confidence bounds, $p<0.001$, t-test for linear regression. (g) Number of returns in bees reduced for 3 min as compared to instant transfer and a short delay (30 s). (h-j) meander, stay time and path length, did not change with delay duration in honey bees. *$p<0.05$, **$p<0.001$, ***$p<0.0001$, Kruskal-Wallis test with Dunn correction, p-values adjusted with the Holm method.

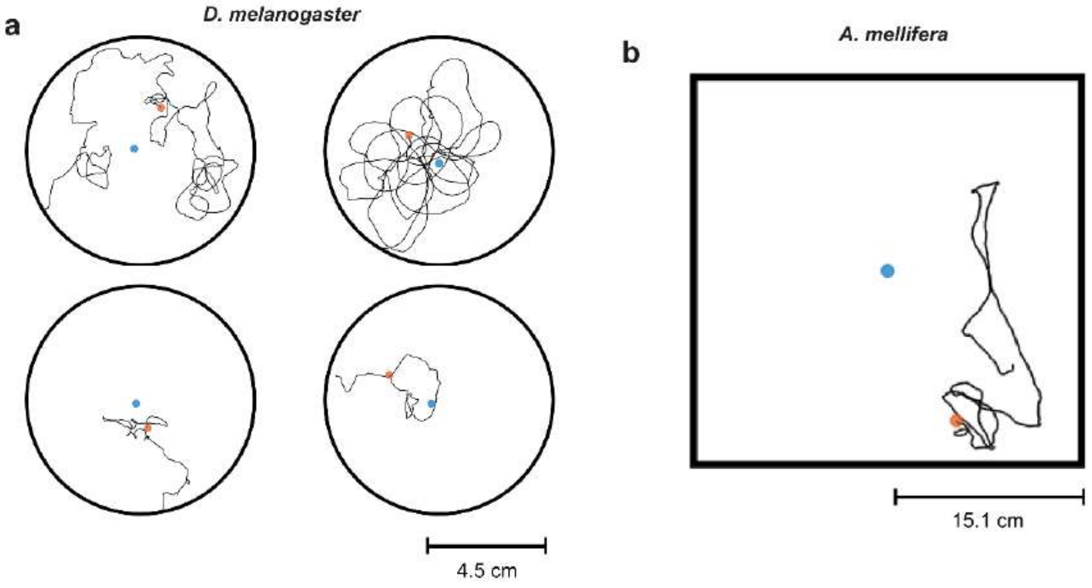


Figure 3.3: Less than 20% of tested flies and bees initiate local search for a delay of 3 min. (a) Trajectories of the four flies out of 21 tested flies for 3 min delay that initiated a path integration-based search. (b) Trajectory of the only bee (out of 10) which initiated a path integration-based search.

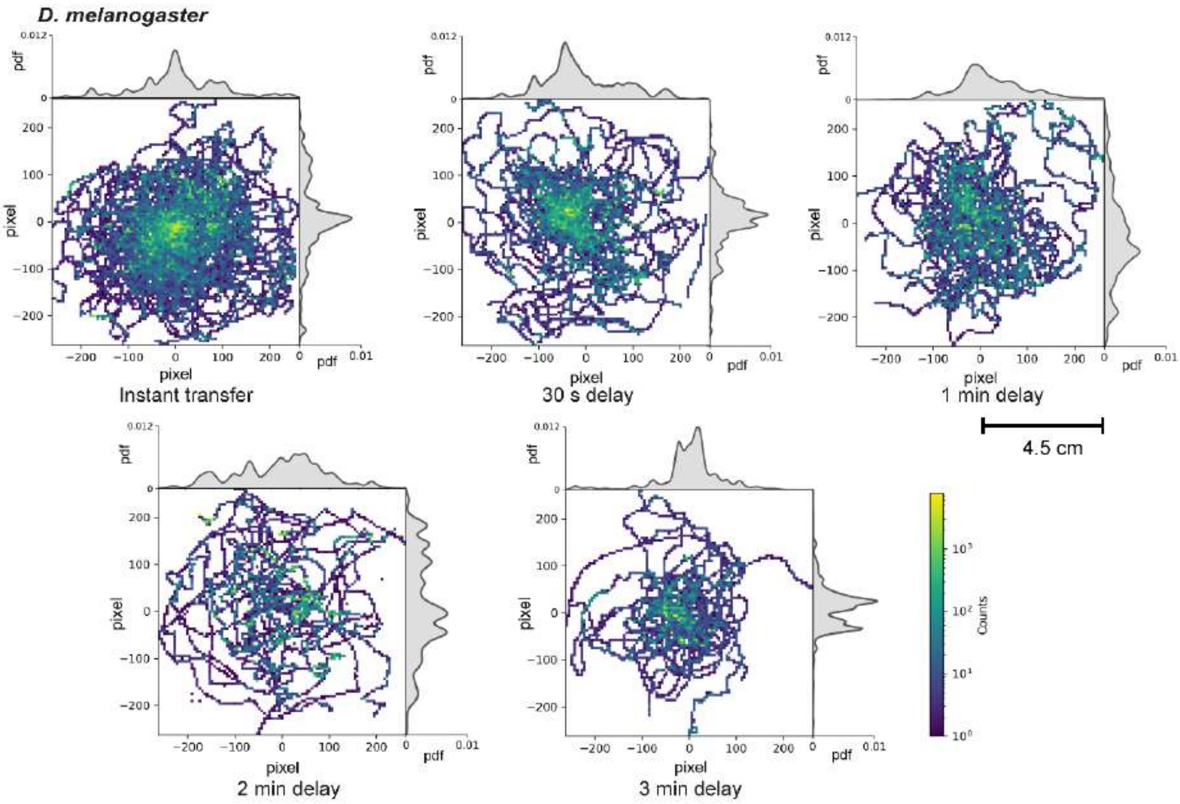


Figure 3.4: Search in flies became less centred towards the origin with increased delay duration. Heatmaps with pdf (probability density function) of walking trajectories of all tested flies for tested durations show that as the time delay increases, the trajectories spread out from the origin and flies spend less time near the origin.

the four that had initiated a path integration-based search for this delay duration (Fig. 3.3).

Interestingly, while the delay inhibited the initiation and intensity of the search for 3 min in flies, their walking trajectories showed higher values for meander, stay time, and path length but not for the number of returns compared to unfed controls (Fig. 3.6). Notably, the delay duration had no effect on the latency to start walking after transfer (Fig. S2). Regression analysis revealed that the decline in these parameters was not strongly correlated with delay durations (Table S2).

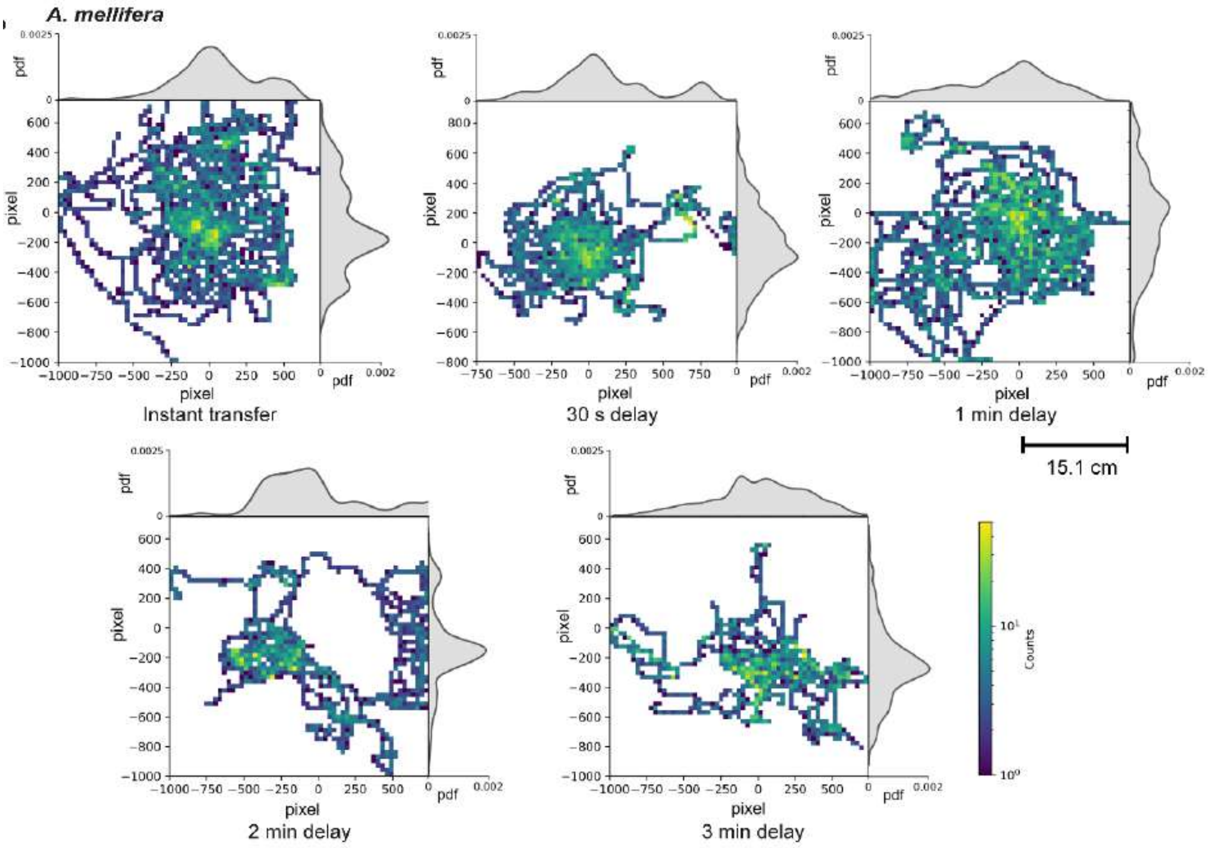


Figure 3.5: The search in bees was less centred towards the origin of walking as the delay duration increases. Trajectory heatmaps with pdf (probability density function) of bees for tested delay durations showed that as the time delay increases, the trajectories became more spread out from the origin and bees show less occupancy near the origin.

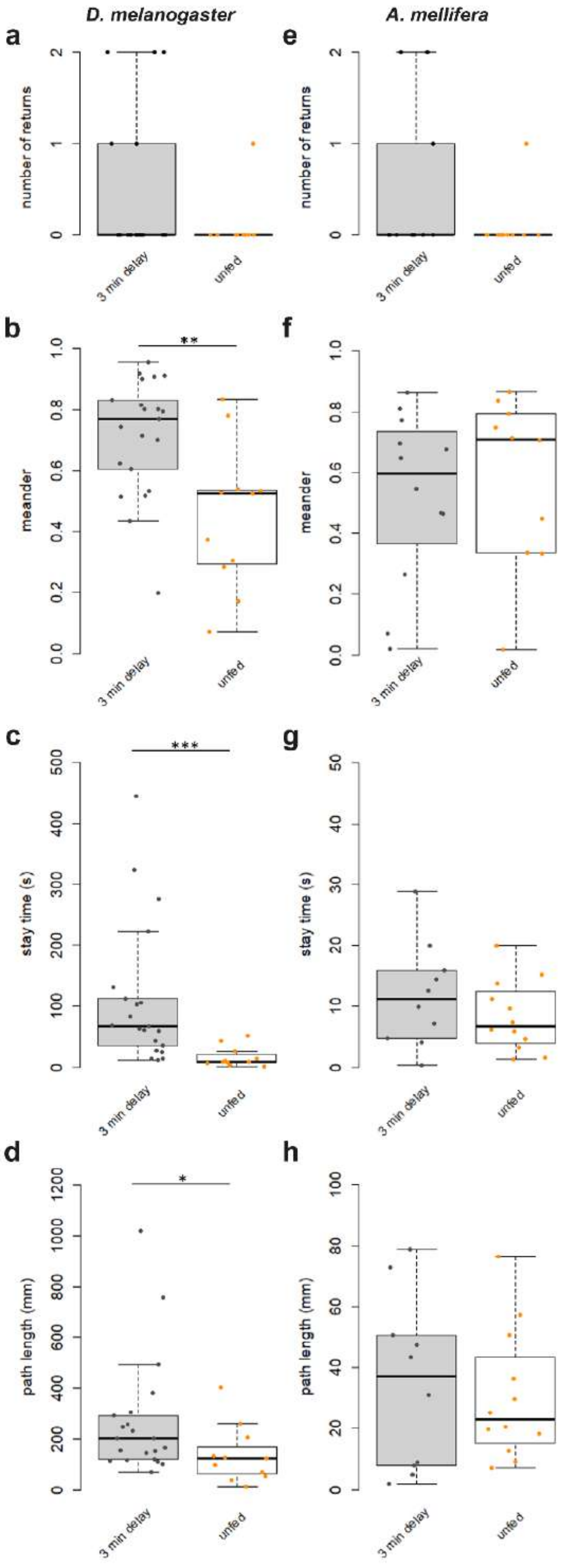

D. melanogaster
A. mellifera
a
e
b
f
c
g
d
h
number of returns
meander
stay time (s)
path length (mm)
3 min delay
unfed
**
***
*
0
1
2
0.0
0.2
0.4
0.6
0.8
1.0
0
100
200
300
400
500
0
10
20
30
40
50
0
200
400
600
800
1000
1200
0
20
40
60
80
100

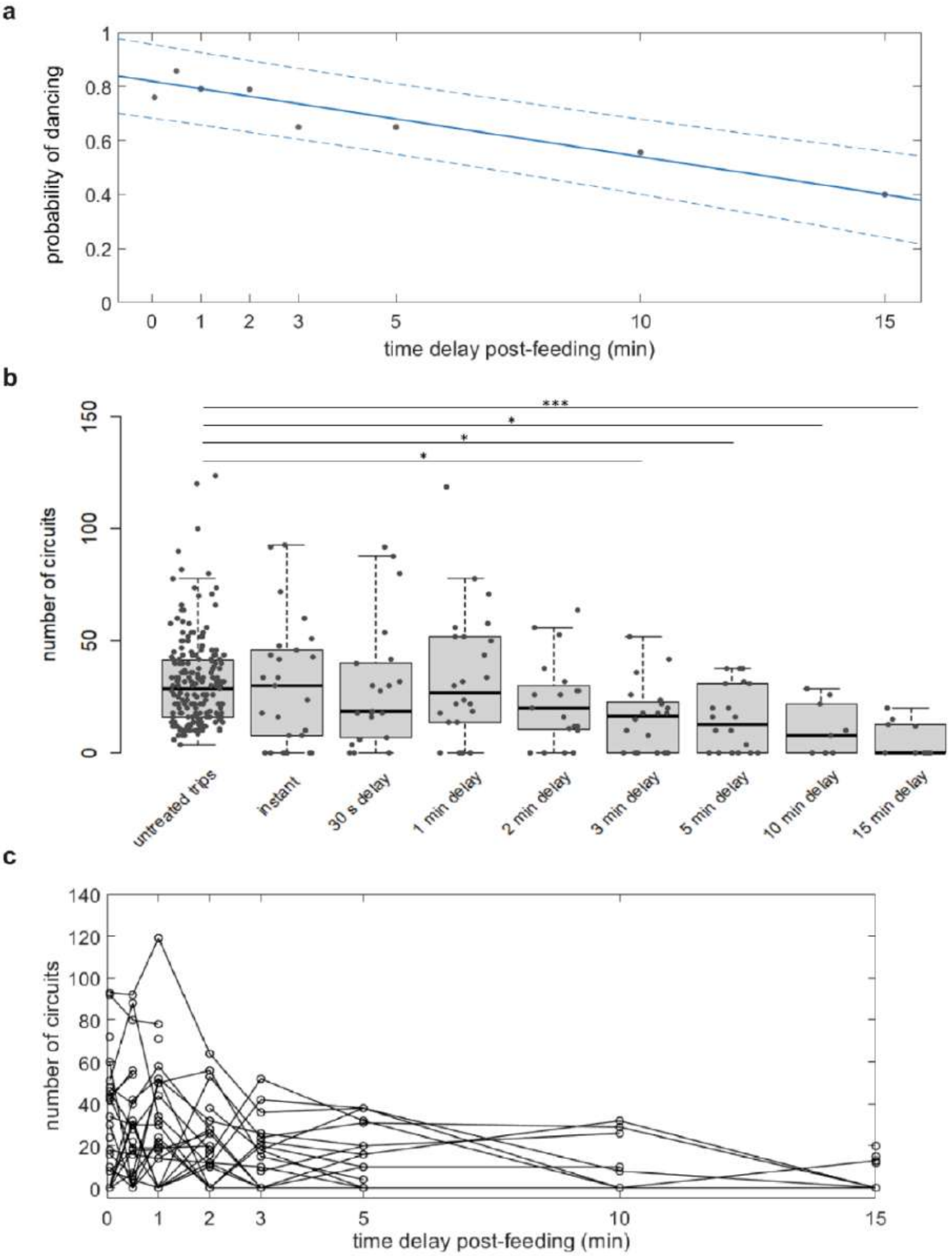
Figure 3.6: Behavioural parameters of unfed flies and bees compared to those with 3 min delay. (a,e) Number of returns are not different between flies and bees after 3 min delay compared to the unfed control. (b-d) Other parameters associated with the behaviour: meander, stay time and path length were significantly higher for delay of 3 min than unfed controls in flies. (f-h) These parameters did not show a difference between 3 min delay and delay and unfed bees. *p<0.05, **p<0.001, ***p<0.0001, Wilcoxon Rank Sum Test.

3.3.2 Delaying departure of foragers after sugar intake at the feeder reduced the probability of dancing

Based on Dethier's argument that there are temporal and spatial similarities between a fly's search behaviour for food and initiation of the bee dance, we tested the effect of delay durations between sugar collection at the feeder and return to the hive to initiate dance. Foragers were assigned into two groups: treatment and control, based on their dance activity. We calculated the probability of dancing post-delay as the ratio of the number of foraging trips with dances post-treatment to the number of total trips for the respective delay treatment.

The probability of dancing reduced linearly with delay duration (Fig. 3.7a). This decline but was not significant at 3 min (p=0.9656), while we observed a significant reduction in the probability after a delay of 15 min (dance probability=0.4, p<0.05 instant-15 min, p<0.001 30 s-15 min, Chi-square test).

There was no significant difference in the number of circuits between the dances of the control bee and the dances of untreated trips of the experimental bees across all foraging groups (Fig. S8). Therefore, to assess the effect of delay, we compared the number of circuits in treated foraging trips with the number of circuits in untreated trips. There was a significant decline in the number of circuits for the delay period of 3 min (Fig. 3.7b). Moreover, longer delay durations of 5 min, 10 min and 15 min resulted in a further decrease in circuits compared to untreated trips, with the most significant reduction occurring after 15 min (Fig. 3.7b, Table S3).


a
probability of dancing
0
0.2
0.4
0.6
0.8
1
0
1
2
3
5
10
15
time delay post-feeding (min)
b
number of circuits
0
50
100
150
***
*
*
*
untreated trips
instant
30 s delay
1 min delay
2 min delay
3 min delay
5 min delay
10 min delay
15 min delay
c
number of circuits
0
20
40
60
80
100
120
140
0
1
2
3
5
10
15
time delay post-feeding (min)

.

Figure 3.7: Initiation of dance and number of circuits reduce with delay duration in honey bees. (a) The probability of dancing for bees decreased with delay duration with a linear model: f(x) = m*x + b where m= -0.1485 (-19.5, -10.2), b=0.6918 (64.83, 73.54), adjusted R-squared: 0.8878 with 95% confidence bounds; $p<0.00001$, t-test for linear regression. (b) Number of circuits significantly reduced when the delay increased to 3 min and reduced further as the delay was increased to 15 min. *$p<0.05$, ***$p<0.0001$, Kruskal-Wallis test with Dunn correction, p-values adjusted with the Holm method. (c) Individual bees have a general decline in the number of circuits with increased delay duration. Each line represents a single bee that underwent different delay treatments after sugar collection.

At the individual level too, bees exhibited a decline in the number of circuits as the delay duration increased (Fig. 3.7c). Regression analysis showed a strong correlation between delay duration and decline in the number of circuits (Fig. 3.8a). Change-point analysis detected a pronounced reduction in the number of circuits for delay durations of 1 min and 3 min (Fig. 3.8b).

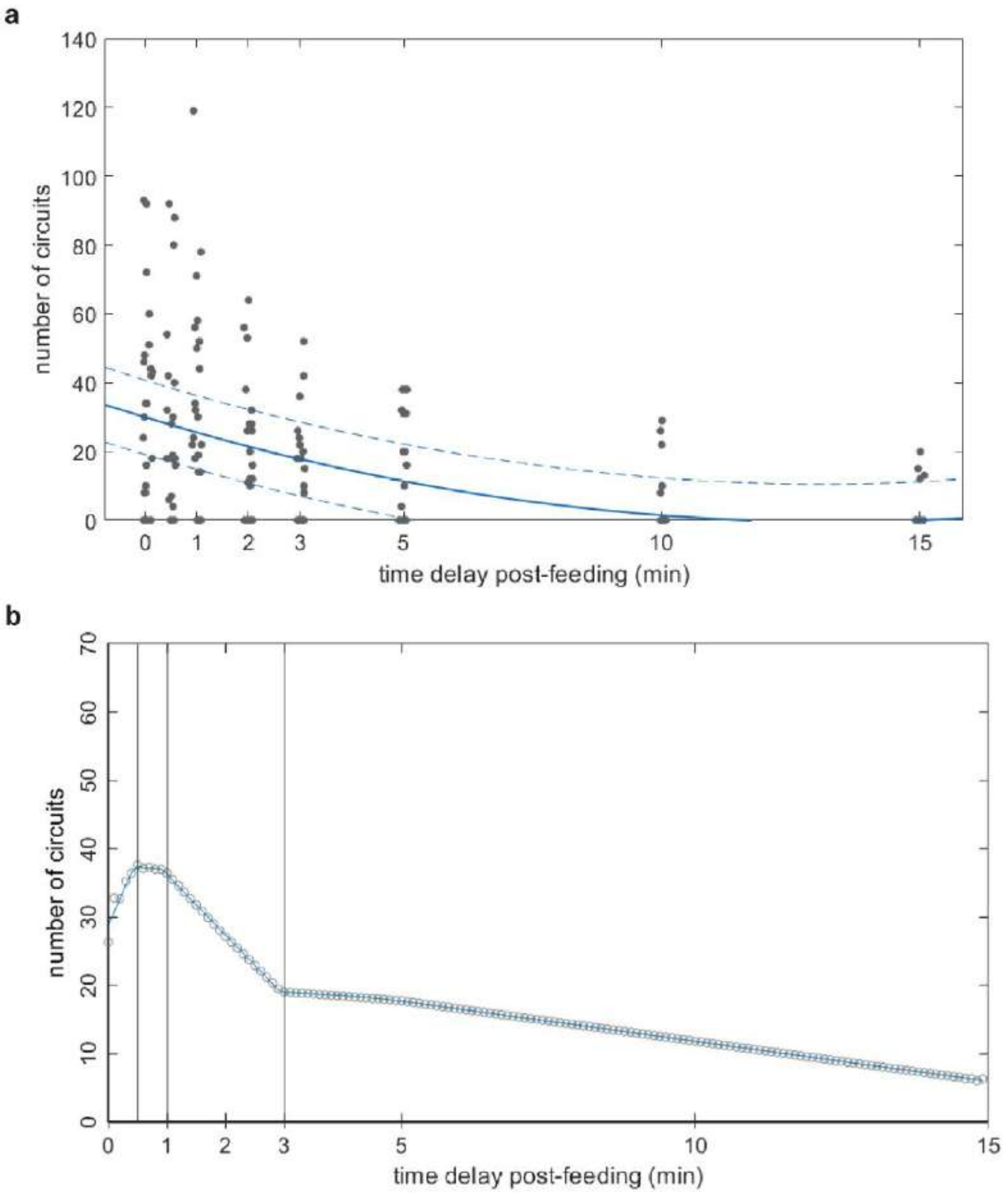


Figure 3.8: Regression and change-point analysis for number of circuits in honey bee dance experiments. (a) Decline in the number of circuits decreases with time delay post-feeding. Linear model Poly2: f(x) = a1*x^2 + c2*x + c3 where a=2.855 (2.02, 3.69), b=-14.16 (-15.9, -12.41), c=17.09 (15.87, 18.3), R-squared: 0.9491 with 95% confidence bounds. (b) 3 min is detected as one of the change-points with a p=0.98. Two other change points were identified as 30 s and 1 min (p=0.99).

3.1 Discussion

Earlier studies on the sugar-elicited search behaviour in blow flies and house flies highlighted that the intensity and duration of the search depends on the "central excitatory state", starvation period, sugar concentration and the volume taken in, supporting the idea that the sugar intake modulates the behaviour (McGuire and Tully 1986; Mayor et al. 1987). In our delay experiments, the modulatory effect of the sugar stimulus (D.m.: 0.2 µl, 500 mM; A.m.: 3 µl, 2 M) to initiate the search lasted for 3 min in flies and honey bees. After this duration, the decrease in the percentage of animals starting a path integration-based search was significant.

In addition, we observed a significant reduction in the number of returns for both species, while only flies showed a pronounced decline in meander, stay time and path length for this duration. Stay time and path lengths for honey bee trajectories were considerably shorter than those for fly trajectories, even in control experiments. There is a possibility that the parameters we used were not suitable to detect an effect of delay duration on behaviour in honey bees.

Although our analyses indicated that for the majority of flies, the 3 min delay reduced the initiation of path integration-based search, the meander, stay time and path length remained higher compared to controls not fed on sugar. However, the unfed flies and bees did not make any returns and the values were not statistically different from the trajectories for 3 min. This finding may suggest that after 3 min, the path integration system is not active after this duration. High turning behaviour is a common strategy for finding additional food in a restricted area (Bell 1990). Since increasing turning frequency without monitoring the path trajectory is less energy-intensive, its initiation and duration might be less temporally restricted than path integration-based search (Bell 1990; Sterling and Laughlin 2015). Therefore, path integration guided walking and increased turning frequency in sugar-elicited search could be independent behavioural modules of food search, and regulated by different modulatory dynamics.

The honey bee dance experiments in which we experimentally delayed the departure of foragers from a sugar feeder showed that the motivation to initiate a dance in the hive lasted at least for 15 min. The differences in the effective delay durations between search and dance are certainly a consequence of the different behavioural contexts. The motivation to recruit nestmates after food collection should last longer than searching for food around a site where they found a small amount of sugar. Although the experimental conditions were different, the slopes of decline for initiation of search (-14.04 min-1) and dance (-0.1485 min-1) in honey bees was comparable (p=0.8730, t-test for linear regression), which might suggest that search and dance are regulated by the same neuromodulatory system. Moreover, studies have elucidated the involvement of neurotransmitters, such as octopamine, in modulating honey bee foraging behaviour.

Several scenarios have been proposed on how dance communication might have evolved (Dornhaus and Chittka 1999; Price and Grüter 2015; Barron and Plath 2017; Alves et al. 2023). Successful foragers of the closely related stingless bees and bumblebees perform excited sugar-dependent locomotor displays or runs over the nest area that alert their nestmates (Lindauer and Kerr 1958; 1960; Dornhaus and Chittka 2001; Menzel 2019). However, none of these behaviours display any features that are correlated to the flight direction towards the food source.

The finding that sugar-elicited search behaviour activates the path integration system at least suggests an ancestral connection between neural pathways of food reward and the path integration system which was recently shown to involve the central complex (Dyer et al. 2002; Brockmann and Robinson 2007; Heinze and Homberg 2007; Heinze et al. 2018; Collett 2019). Flies and bees might exhibit similar (ancestral) modulatory circuits regulating the activity of the navigational system (Barron et al. 2007; Schröter et al. 2007; Busch et al. 2009; Menzel 1999; 2009; Yang et al. 2015).

As neuro-genetic tools to study the mechanisms of behaviour in honey bees are still in their infancy, *Drosophila* might provide interesting experimental opportunities to identify candidate neural pathways involved in honey bee dance behaviour (Chen et al. 2021; Carcaud et al. 2023). The significance of such experiments depends on designing behavioural experiments that reflect honey bee behaviour, something that hasn’t been explored to great extent, yet. Even if the neural circuitry is more complex, findings in *Drosophila* can provide sufficient information to help the research efforts in honey bees.

4. Exploring the mechanistic role of walking in path integration in local Search using flies

4.1 Introduction

Idiothetic path integration, a mechanism used by diverse species, including humans, allows animals to keep track of changes in direction and distance from a starting point, enabling effective navigation even without external references (Mittelstaedt and Mittelstaedt 1980; 2001). Use of path integration in navigation and homing has been demonstrated in desert ants, spider and bumble bee navigation. (Muller and Wehner 1988, Seyfarth et al. 1982, Chittka et al. 1999). Previous chapters detail the finding that both flies and honey bees use path integration in sugar-elicited local search.

This chapter delves into the role of path integration in the context of local search behaviour in flies, with a particular focus on the mechanistic aspects related to walking. The proposed source of path integration in this behaviour is traced back to proprioceptive inputs generated through the self-motion of the fly. Proprioceptive information in flies is mediated by chordotonal organs, such as Johnston's organs in the antennae and femoral chordotonal organs (FCO) (Kavlie and Albert 2013; Shanbhag et al. 1992).

The sensory neurons on the legs report the load and joint angles from the legs back to the central nervous system, and thereby influence the firing of the motor neuron during walking. Several mechanosensors have been reported in flies, including Nanchung, Inactive, NompC, Pickpocket, Painless, and Piezo, facilitating the integration of sensory information with motor commands (Tuthill and Wilson, 2016). Nanchung, a member of the *Drosophila* Transmembrane Receptor Potential channel (TRP) family, localizes to the cilia of tarsal, tibial and femoral chordotonal organs in the legs (Kim et al. 2003). It is also expressed in the antenna and AMMC antennal mechanosensory and motor centre in the brain.

The literature extensively documents the role of TRP channels in various aspects of *Drosophila* behaviour, from sensory perception of sound, gravity sensing, hygrosensation and thermotaxis (Kim et al. 2003; Liu et al. 2007; Rosenzweig et al. 2008; Kamikouchi et al. 2009). Notably, Nanchung protein has been implicated in adult walking behaviour and posture (Akitake et al. 2016). Nanchung has also been shown to be important in maintaining step precision during walking (Mendes et al. 2013).

Therefore, I explored the role of Nanchung by using Nan36a mutants, with their impaired proprioceptive capabilities, exhibit differences in local search behaviour compared to wild-type flies. Additionally, Nanchung was inactivated using thermogenetics in a Nan expressing fly line using UAS-Gal4 system (Nan>Shi^{TS}). Shibire^{TS1} is a temperature sensitive allele of dynamin which blocks neurotransmitter release at the synaptic junction when temperature is changed from permissive (19° C) to restrictive (32° C) (Kitamoto 2001).

There is evidence of counting steps in desert ants (Wittlinger et al. 2006; 2007). Ants use their stride length as a pedometer and animals with elongated ("stilts") or shortened legs ("stumps") take larger or shorter strides, respectively and misgauge travel distance. Therefore, sugar-elicited search can be used to test whether flies use stride information for path integration. Drawing parallels with studies on desert ants and their stride-integrator system, the chapter also proposes experiments using sugar-elicited search behaviour to assess whether flies utilize stride information in path integration. I did a manipulation experiment where the legs of the flies were clipped to impair feedback. Since path integration does not rely on external cues, I performed all the experiments in this chapter, in both light and dark.

4.2 Methods

4.2.1 Testing Nanchung mutants to test the role of proprioception

Adult males of Nan^{36a} mutants were used for experiments. 2-day old flies were starved for a 29± 2hrs. The starvation duration was calculated by using the duration for 90% survival of the food-starved population. Canton-S flies were tested as control. The behaviour was recorded under light and dark conditions. CS in light, N=31; Nan in light N= 32; CS in dark N=32; Nan in dark N=32

4.2.2 Inactivating Nan neurons thermogenetically

The behavioural set-up was made amenable to thermal manipulations using a heater and PID (Proportional–integral–derivative) controller to set and maintain the temperature. UAS-Shi^{TS} and the modified set-up was tested using OK371 (neuromuscular junction) gal4, where activation Shibire^{TS} caused reversible paralysis.

Nan>Shi^{TS} (32° C) is the test group wherein neurons expressing nanchung are synaptically blocked by activating UAS- Shi^{TS} at restrictive temperature (32° C). Room temperature (24^{o} C) was used as permissive temperature. Appropriate genetic (Nan gal4 flies are crossed with wild type w1118) and temperature controls were used (N=25, for each group).

4.2.3 Visualizing Nan expression

Virgin females from Nan-Gal4 and males from UASmCD::GFP were crossed to visualize nan protein expression (Nan-gal4 X UASmCD::GFP). F1 generation adults were collected. Brains, legs and antennae were dissected in 1 X PBS (Phosphate Buffer Saline) and fixed in 4% paraformaldehyde for 20 minutes. Tissues were then washed with PBS containing 0.3% Triton X-100 (PTX) for 15 minutes. The tissue was then mounted in Vectashield. The slides were viewed and imaged using a confocal microscope Olympus FV1000.

4.2.4 Testing the behaviour on large arena

Flies were individually tested on 160 mm diameter plate for large arena. To contain the visual field of the flies, the arena was surrounded by a white cylindrical tube (72.7 mm height, 184 mm inner diameter) made of polyvinylchloride resin. The arena was illuminated from the bottom by a large panel of cool white SMD LEDS. The light intensity was 240 lux at the centre of the arena, measured using TENMARS TM-203 Data Logging Light Meter1214 mm lens, Azure). Flies were filmed for twenty minutes or until they reached the periphery of the arena and limbed over. Rest of the protocol was the same as small arena (2.2.2). These experiments were recorded in light as well as dark. Small arena in light N=50; large arena in light N-52; small arena in light N=40; large arena in dark N=44.

4.2.5 Leg clipping experiments

Male flies of Canton-S (CS) strain were used in all the experiments. 2-day old male flies were anesthetized on ice before the clipping. All three pairs of legs were clipped at the tibia (Fig. 4.1). Flies were then allowed to recover overnight and starved the next day. Flies that survived the surgery as well as the starvation, and were able to walk after the treatment were used for the behavioural experiments. The flies were starved of food (with access to water) before the behavioural experiments. The behaviour was recorded on a 90 mm Petri dish whose surface was made rough using a 320-grit sandpaper to provide traction for the flies to walk. Sham flies (treated same as tested flies except for leg clipping), were used as a control. All the experiments were done in both, light and dark (N=30, for each group).

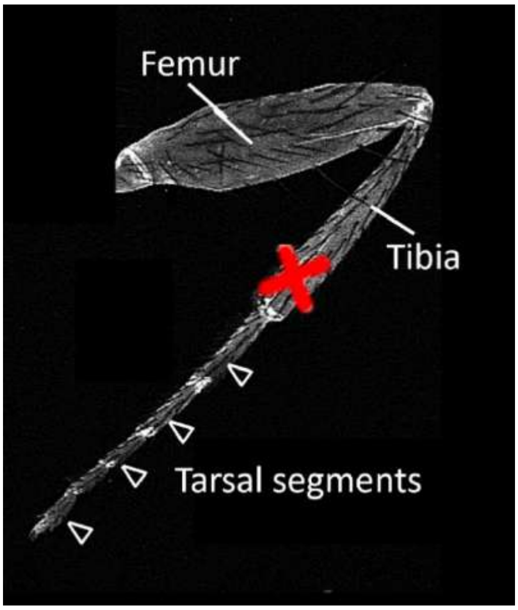


Figure 4.1: Leg clipping experiment. All six legs were clipped near the distal end of tibia shown in red.

4.2.6 Quantifying additional homing clusters

I developed a pipeline where in each fly trajectory is tested for whether there are points on the arena, besides the origin where the flies home. A histogram was plotted and the points that show a signal above the threshold (decided for each dataset, based on return probability) are selected as potential points. The points had to be a minimum of the 26.5 pixels (3 fly lengths) away from the origin to be taken up further. The trajectories were then normalized to those X and Y coordinate and curve-fitted for a power equation model or sum of sines. An R-square value above 0.75 was considered a good fit. Fig. 4.2 shows the flowchart of the developed pipeline.

4.2.7 Testing scale-free walk

The analysis for scale-free index and Lévy walk was done based on a previous study done (Tsai and Chou, 2019 for scale-free index. The walking distance was calculated over the entire trajectory for different time windows. The average of walking distance to the power of *q* was calculated in different windows. This distance was raised to q in values of 0 to 5, with increments of 0.2 and plotted with the time on a log-log plot. The

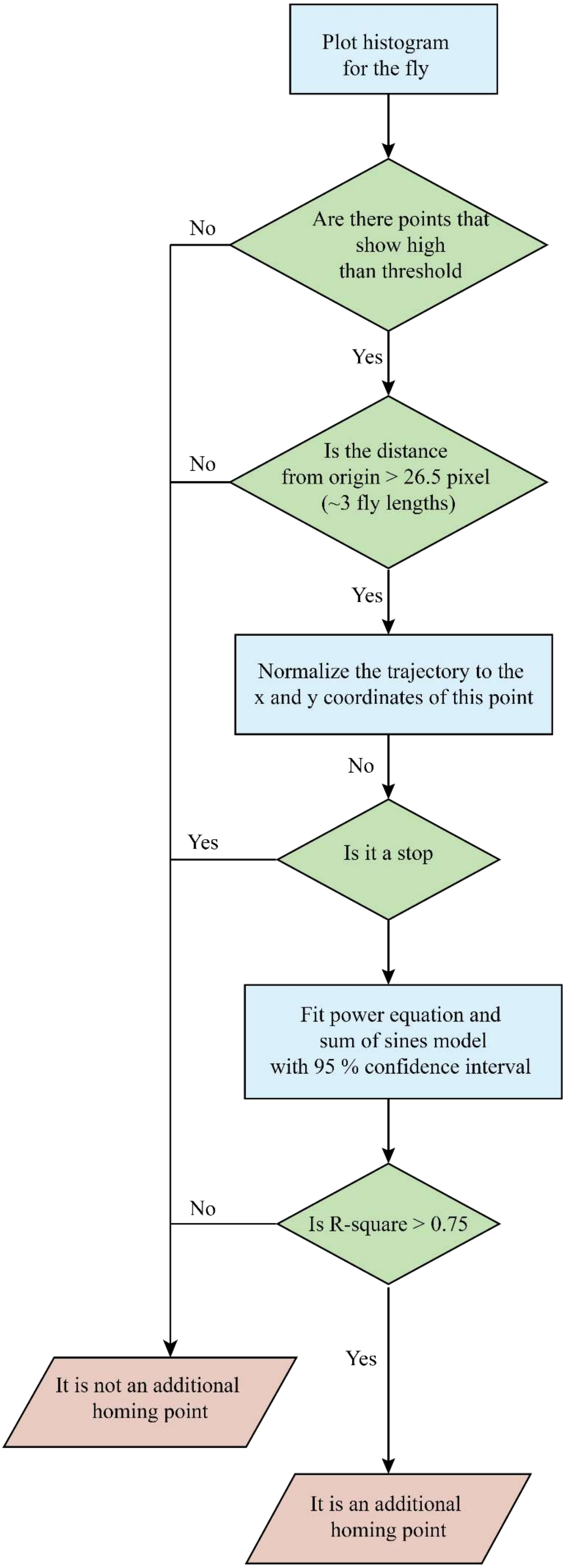

Plot histogram for the fly
Are there points that show high than threshold
No
Yes
Is the distance from origin > 26.5 pixel (~3 fly lengths)
No
Yes
Normalize the trajectory to the x and y coordinates of this point
No
Is it a stop
Yes
Fit power equation and sum of sines model with 95 % confidence interval
Is R-square > 0.75
No
Yes
It is not an additional homing point
It is an additional homing point

Figure 4.2: Flowchart for quantifying additional homing clusters.

slope was represented by ζ ($\zeta=\alpha q$) where when the power-law scaling $\alpha<0.5$, the movement of subjects was considered to be odd. When α approximated 0.5, the subject movement was considered to be a random walk. When $\alpha>0.5$, the walking patterns were considered to be scale-free, as they followed a power-law function. Trajectories of leg clipping experiment and large arena were tested.

4.3 Results

4.3.1 Nanchung mutants have impaired returns during search

The expression of Nanchung protein was observed in the Antennal mechanosensory and motor centre (AMMC) in the brain, chordotonal organ in the leg, and antennae, and was consistent with the literature (Fig. 4.3). I wanted to study the role of Nanchung in path integration using both mutants and inactivating the sensory neurons expressing Nan with thermogenetics.

The Nan^{36a} mutants showed that occupancy of flies near the origin appeared defective. Positional heatmaps indicated that proprioceptive deficit flies attempt returning to the starting position during search but the returns seem imprecise (Fig. 4.4 top panel). This became more apparent when visualized closely near the origin. Nan^{36a} flies indicate a shift in homing near the origin in both light and dark conditions (Fig. 4.4 bottom panel). The number of returns were significantly reduced for Nan^{36a} mutants in the dark compared to light (Fig. 4.5a). The percentage of flies initiating path integration-based search was significantly lower in Nan^{36a} mutants in dark from CS flies in both light and dark, but not in comparison to Nan^{36a} flies in light (Fig. 4.5b). Search time was significantly lower in the mutants as well as CS flies in dark, than CS flies in light. Meander was also lower in Nan^{36a} mutants in dark compared to CSflies in light but path length was not significantly different across groups (Fig. S4).

The set up was modified to block Nanchung by activating UAS-Shi^{TS} at restrictive temperature (32^{o} C) to study the effect on search behaviour. The thermogenetic inactivation was tested using OK371>UAS-Shi^{TS}, and was successful. Nan>Shi^{TS} (32^{o} C) denoted the group where Nan was blocked when temperature is raised from 24^{o} C

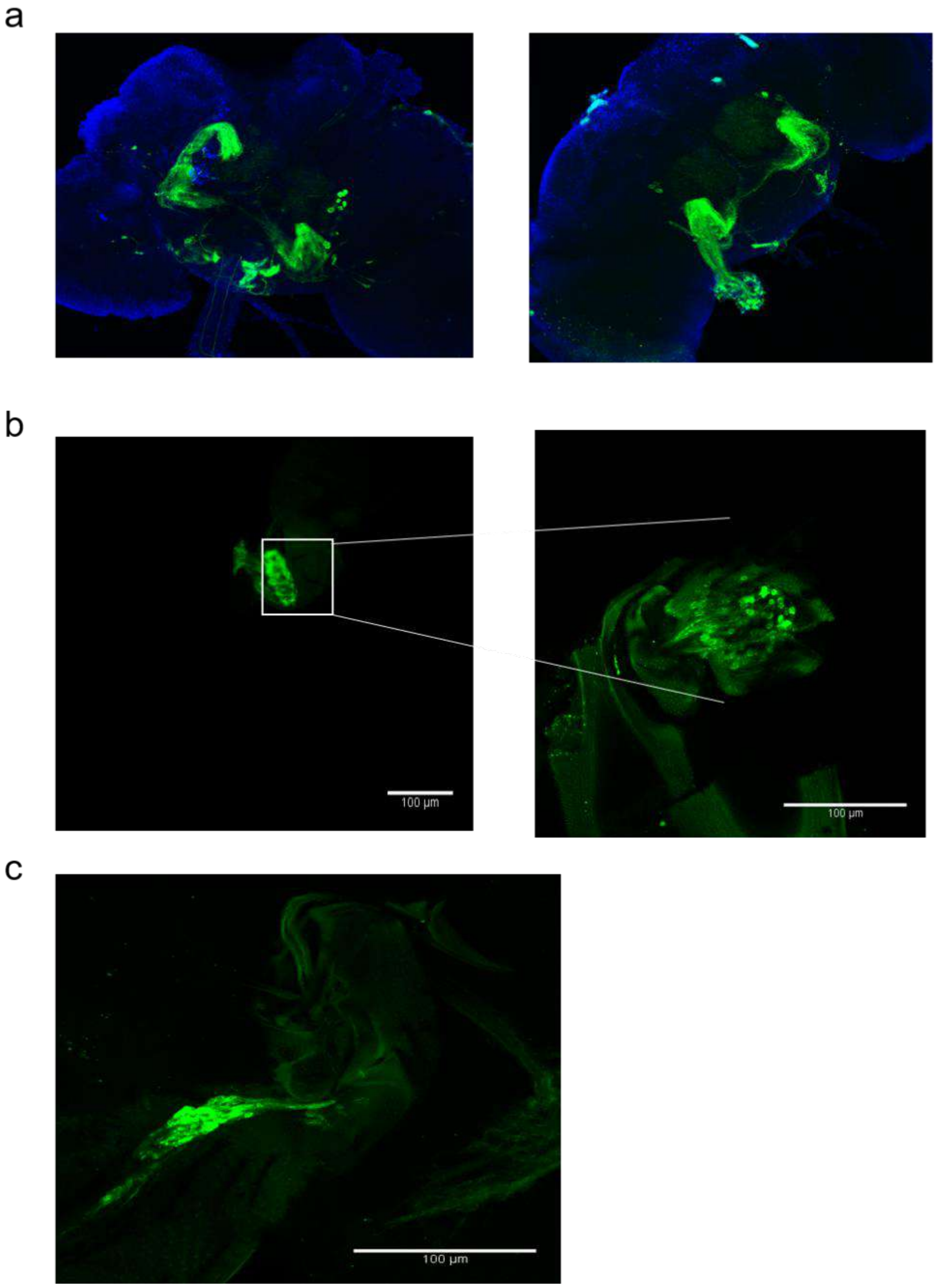


Figure 4.3: Nanchung protein has characteristic expression. Expression of nan-Gal4 in (a), (b) antennae, (c) legs and (d) brain (AMMC) visualized by UAS-GFP, which labels the cytoplasm. (Scale bar, 100µm). Expression of nanchung protein in the (b) Johnston's organs, present on the second-segment of the antennal lobe. (c) Leg chordodontal organs, located at the femur of the leg. (scale bar,100µm).

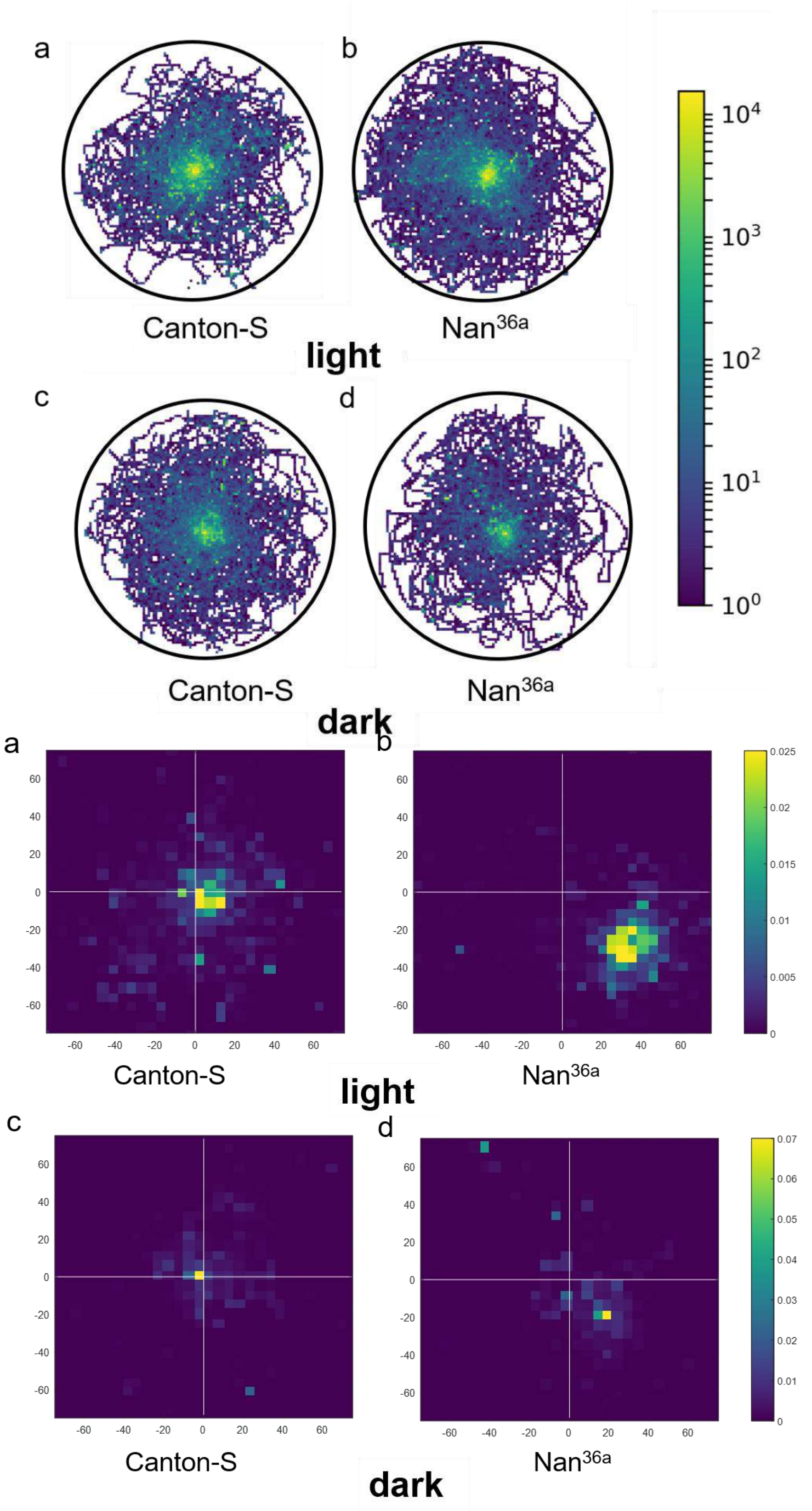


Figure 4.4: Positional heatmaps for Nan^{36a} indicated impaired returns. Top panel (a-d): The heatmaps showed a deviation from the origin for Nan mutants. Bottom panel (a-d): The heatmaps plotted near the origin (±80 pixels on x and y axes) showed a shift in homing near the origin in Nan^{36a} mutants in both light and dark.

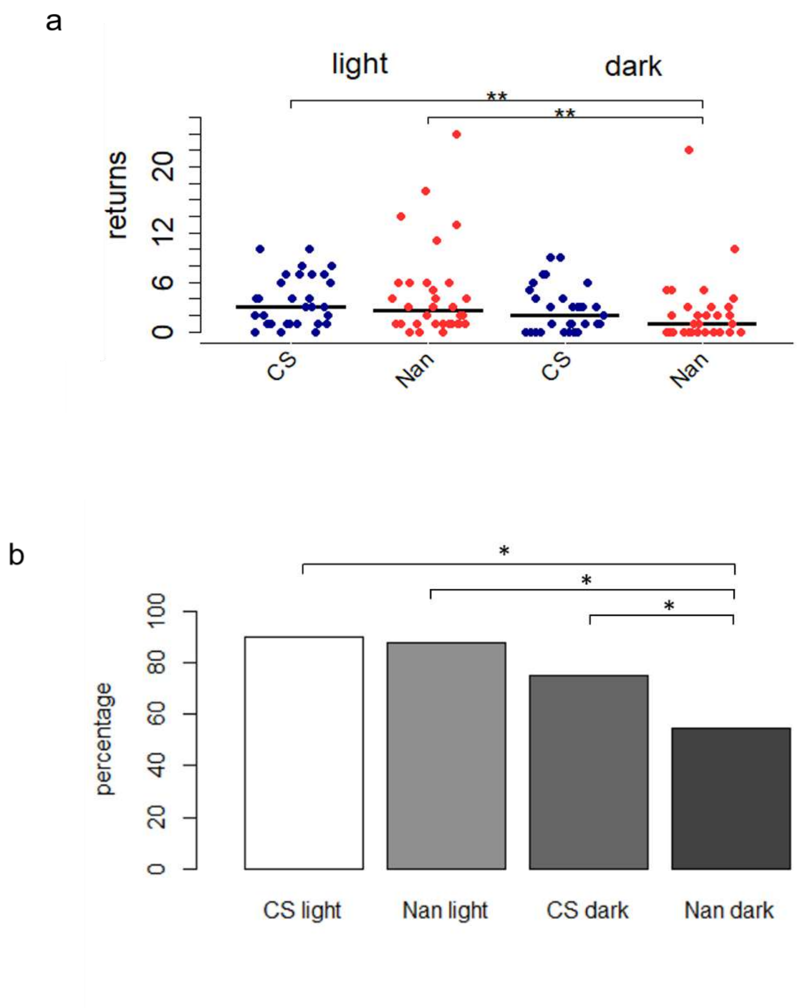


Figure 4.5: Path integration-based search was disrupted in Nan^{36a} mutants. (a) Nan mutants in dark had significantly lower returns compared to Nan mutants in light ($p=0.005$), and CS in light ($p=0.007$). (b) Percentage of flies with path integration-based search was reduced in Nan mutants in dark compared to light ($p=0.0041$), and CS in light ($p=0.0018$). $^{*}p<0.05$, $^{**}p<0.01$ Kruskal-Wallis test with Dunn correction, p-values adjusted with the Holm method.

to 32° C. Nan gal4 flies are crossed with wild type flies (w1118) as a genetic control (Nan>+) and tested at 32° C as a temperature control. The number of returns did not vary across test and control flies (Fig. 4.6). Since Nan^{36a} mutants had a defect in returns, two additional parameters as a measure of imprecision in error: error in returns and maximum displacement between subsequent returns was quantified, but no significant differences were found between test and control flies (Fig. S5, S6). Additionally, the returning behaviour was compromised 32° C, even in the CS flies, which made it difficult to ascertain the data of these experiments (Fig. S7). Thus, the data for Nan>Shi^{TS} was not included further in the study.

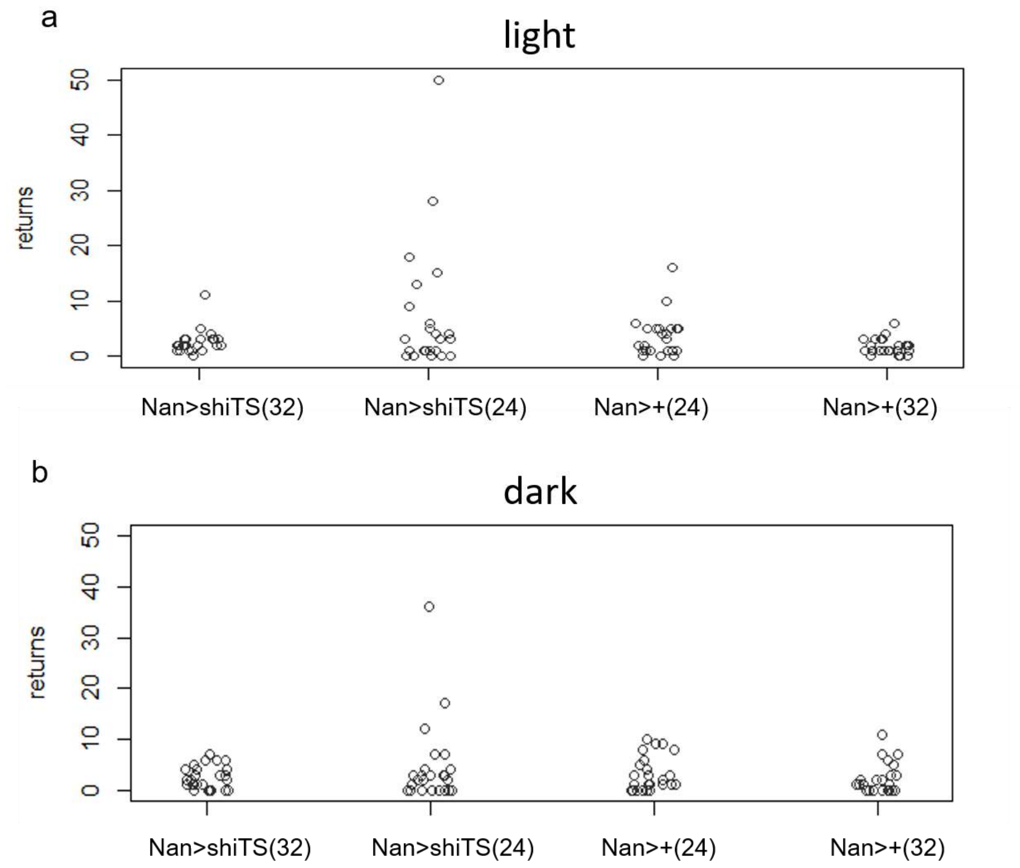


Figure 4.6: The returns were not affected when Nanchung was thermogenetically inactivated using Shibire. Kruskal-Wallis test with Dunn correction, p-values adjusted with the Holm method.

4.3.2 Shortening the leg length had no effect on path integration at the origin of the search

The leg clipping experiment was done to test how perturbing feedback from leg could affect the behaviour. The feeding time and walking speed of legs clipped flies were quantified to see the effect of the treatment and were not statistically different from the control (Sham) flies (Fig. 4.7). The heatmaps of the flies and homing near the origin with legs appeared to be the same between flies with clipped legs and controls (Fig. 4.8). The number of returns and percentage of flies initiating path integration-based search were not different either (Fig. 4.9).

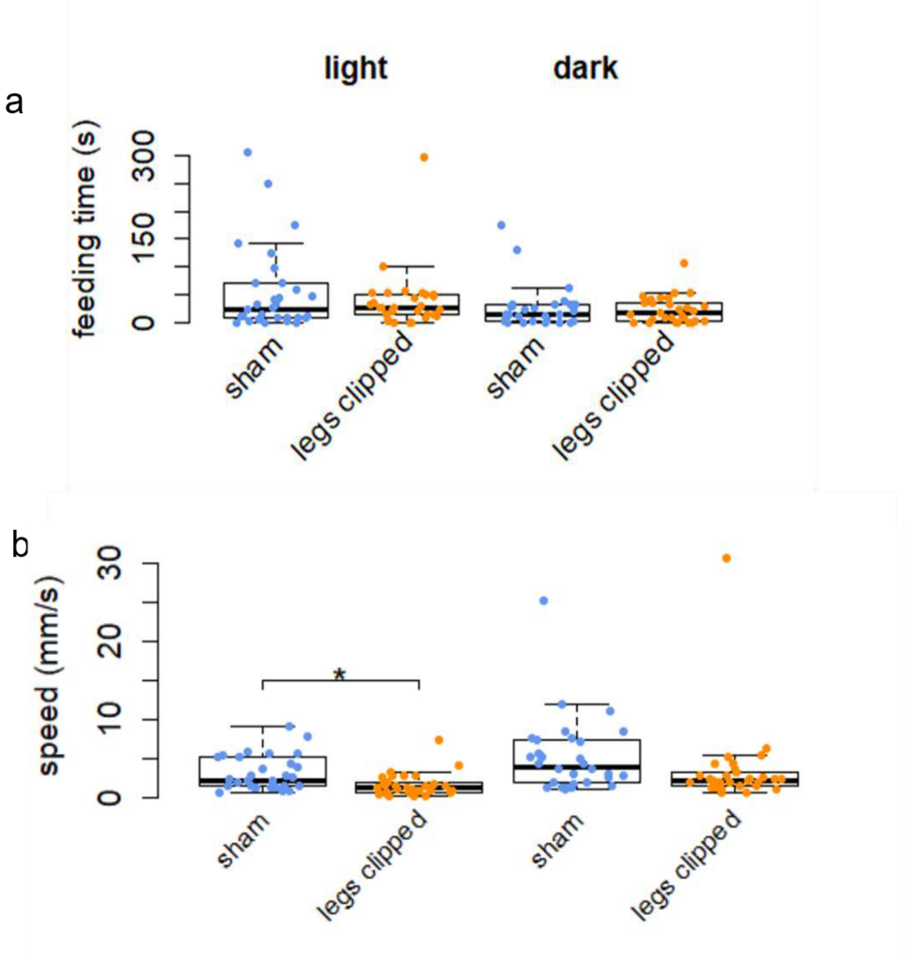


Figure 4.7 The feeding time did not change for legs with clipped flies, however the speed was lower for flies in light as compared to sham.

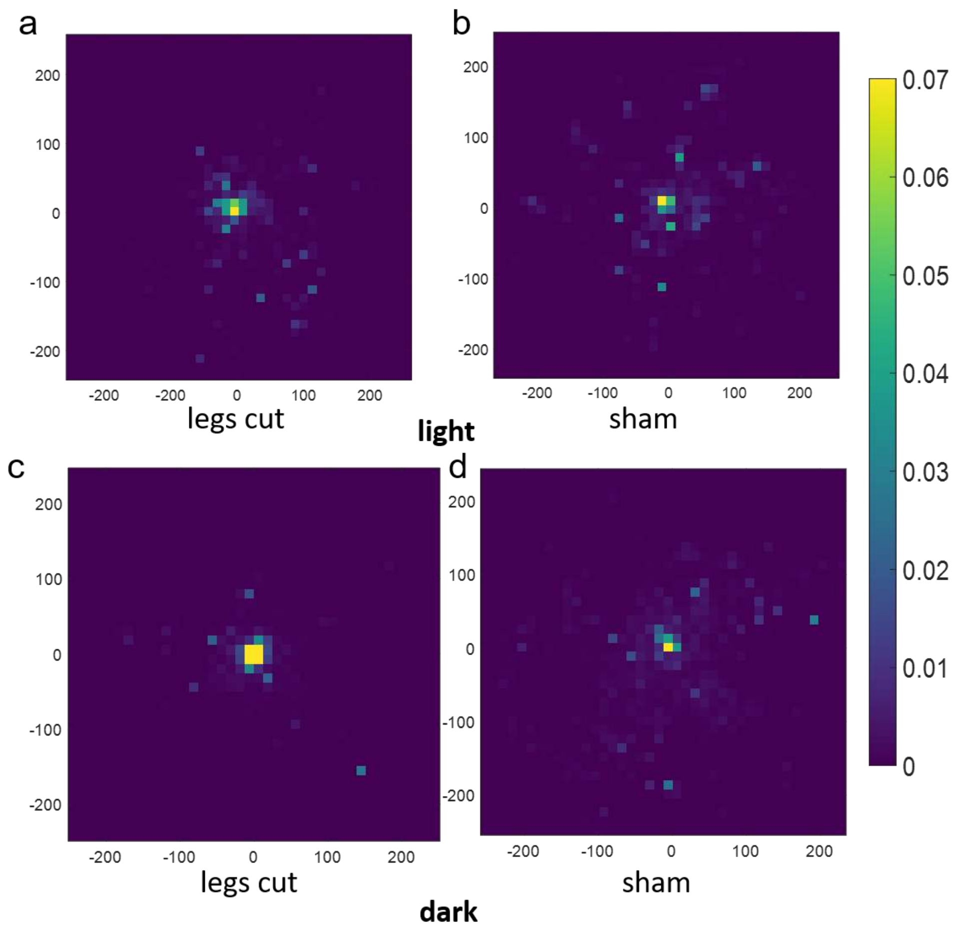


Figure 4.8: The heatmaps did not show a difference when the leg length of CS flies was shortened.

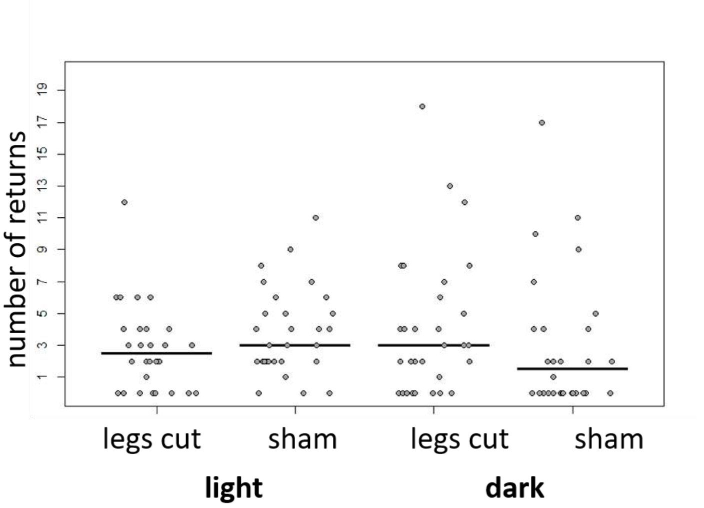
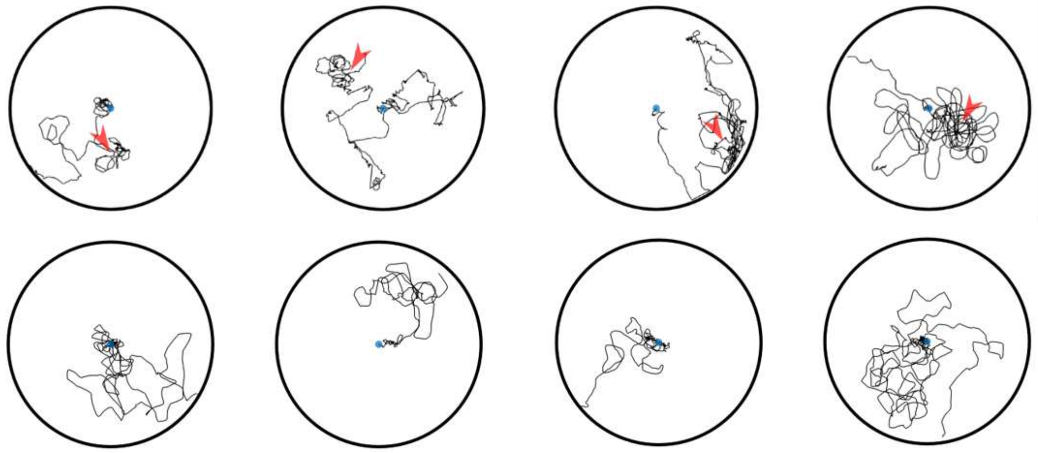


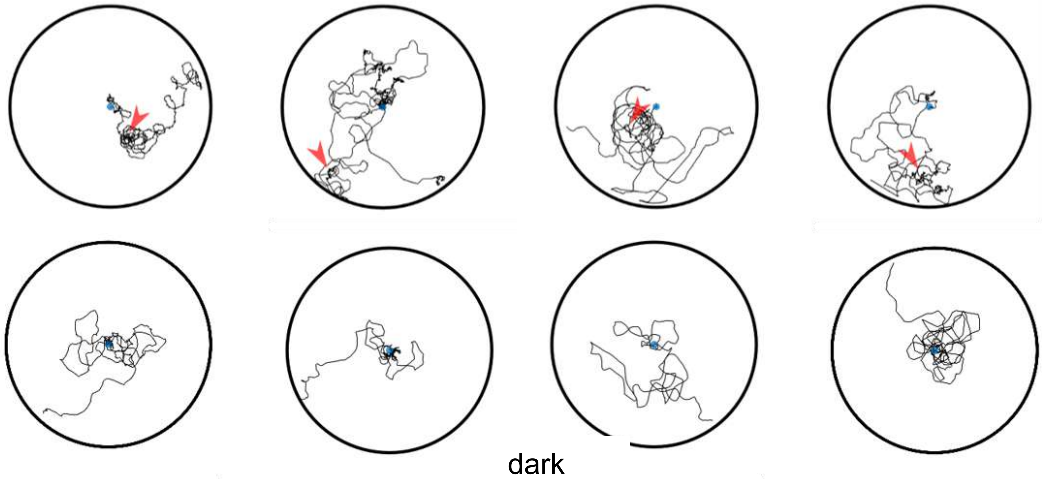
Figure 4.9: The number of returns to the origin did not show a difference when the leg length of CS flies was shortened.

4.3.3 Local search consisted of smaller clusters of homing at points in addition to the origin, in flies with shorter legs

The leg clipping experiments were done with the aim to test how impairing proprioceptive feedback would affect path integration. Therefore, I had first studied the homing near the origin of the search, which was not affected in flies with clipped legs. However, the individual trajectories of the flies indicated homing to arbitrary points in the arena, in addition to the origin (Fig. 4.10). Interestingly, these clusters were also observed for the flies tested on a large arena (Fig. 4.11).

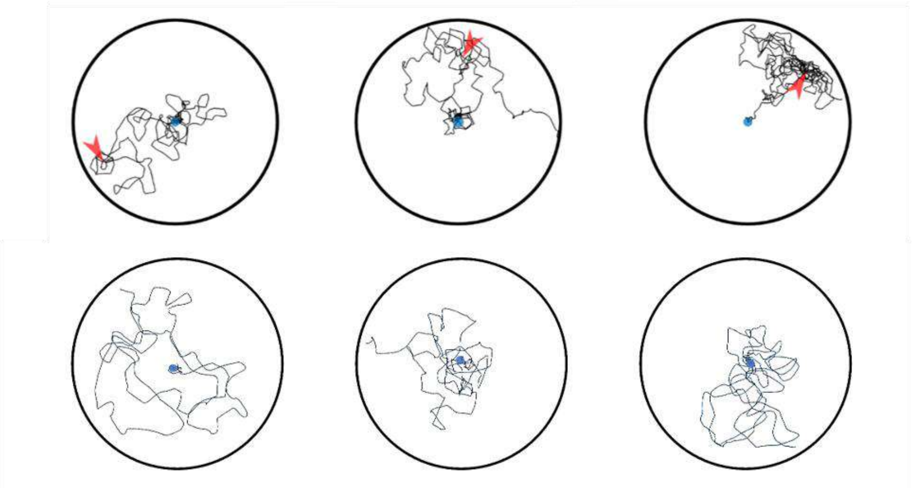


light

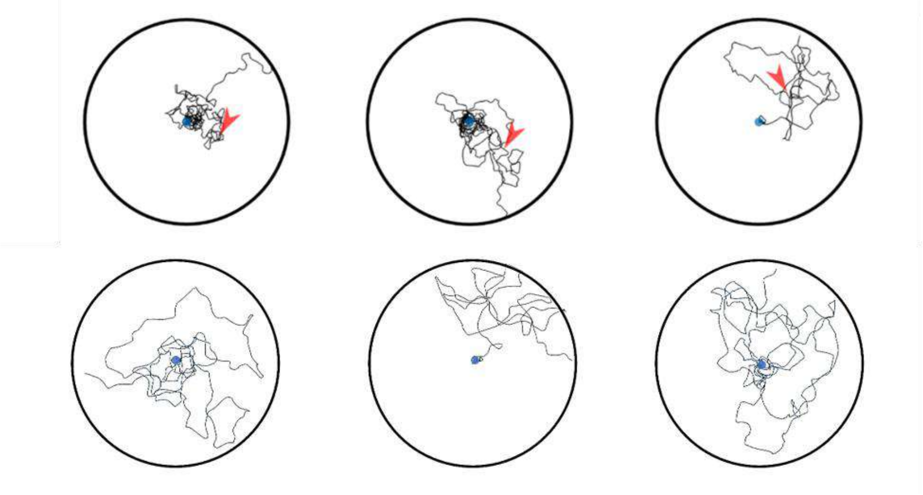


dark

Figure 4.10: Presence of small localized clusters of homing in flies with shorter legs. Examples of trajectories of flies with clipped legs (top panel in both light and dark) are trajectories and sham flies (bottom panel in both light and dark) Blue circle shows the origin of the search, and the red arrow marks these additional clusters in flies with clipped legs.



light



dark

Figure 4.11: Presence of small localized clusters in flies tested in large arena. Trajectories from large arena where the origin of the search for each fly is represented by the blue circle and additional clusters are shown by the red arrow. For both light nd dark conditions, bottom panel indicates flies in small arena.

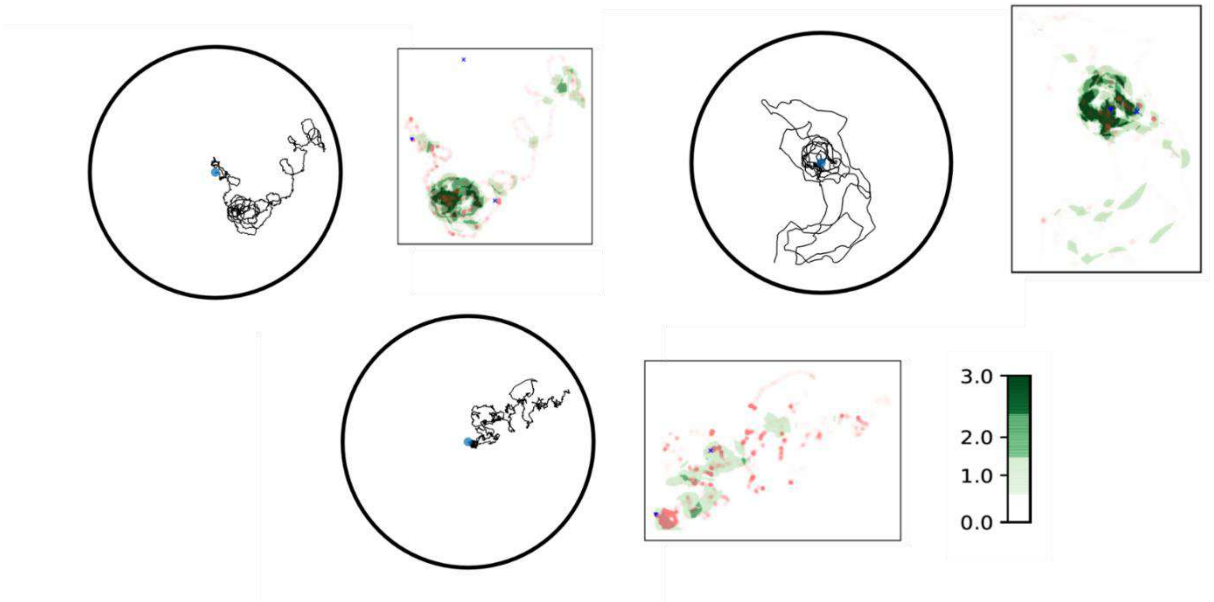


Figure 4.12: The additional clusters show high density around the origin of the clusters.

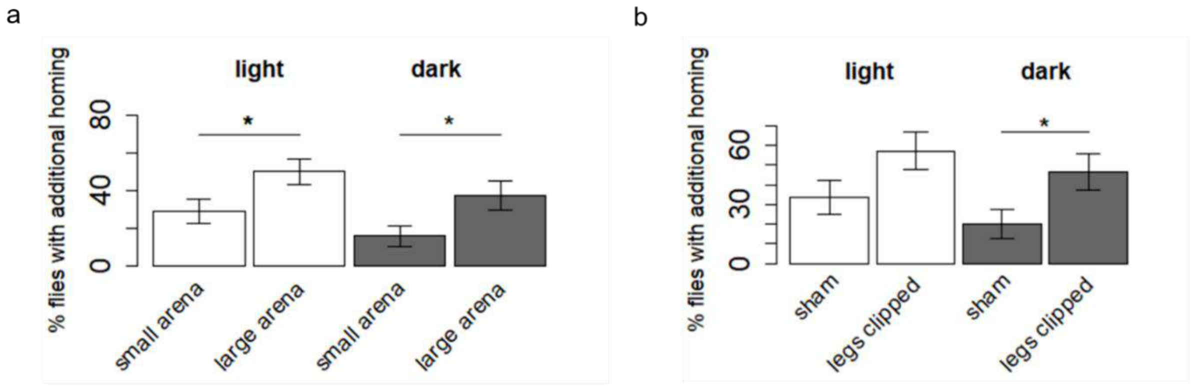


Figure 4.13: Quantifying the presence of additional clusters of homing. (a) The flies in large arena had a significantly higher frequency of the additional clusters in large arena compared to small arena, in both light (p=0.0287) and dark (p=0.0246). (b) Higher percentage of flies with shorter legs showed these homing clusters than control flies, in the dark (p-value=0.0685, in light; p-value=0.0285, in dark). *p<0.05, Chi-square test.

The probability density function was high around these clusters, which suggested that the flies were either spending a lot of time and/or were looping around a centre (Fig. 4.12). To test whether presence of these clusters was by chance or statistically significant, I quantified the frequency of observing in both the sets of experiments using an analysis pipeline (Fig. 4.2). The percentage of flies that had additional clusters was significantly higher in large arena in both light and dark, and in flies with shorter legs only in the dark (Fig. 4.13).

These trajectories exhibited clustering which looked similar to scale-free walking (Fig. S9). Hence, I tested whether the behaviour fits a scale-free model by testing the trajectories of these two sets of experiments for Lévy walks: legs clipped flies and large arena (see methods). All tested groups had power-law scaling $\alpha>0.5$, indicating scale-free walking pattern (Table S4). The value of α was higher in flies with clipped legs compared to controls, in light as well as dark (Fig. 4.14a). However, there was no significant difference in α between small arena and large arena (Fig. 4.14b, Table S5).

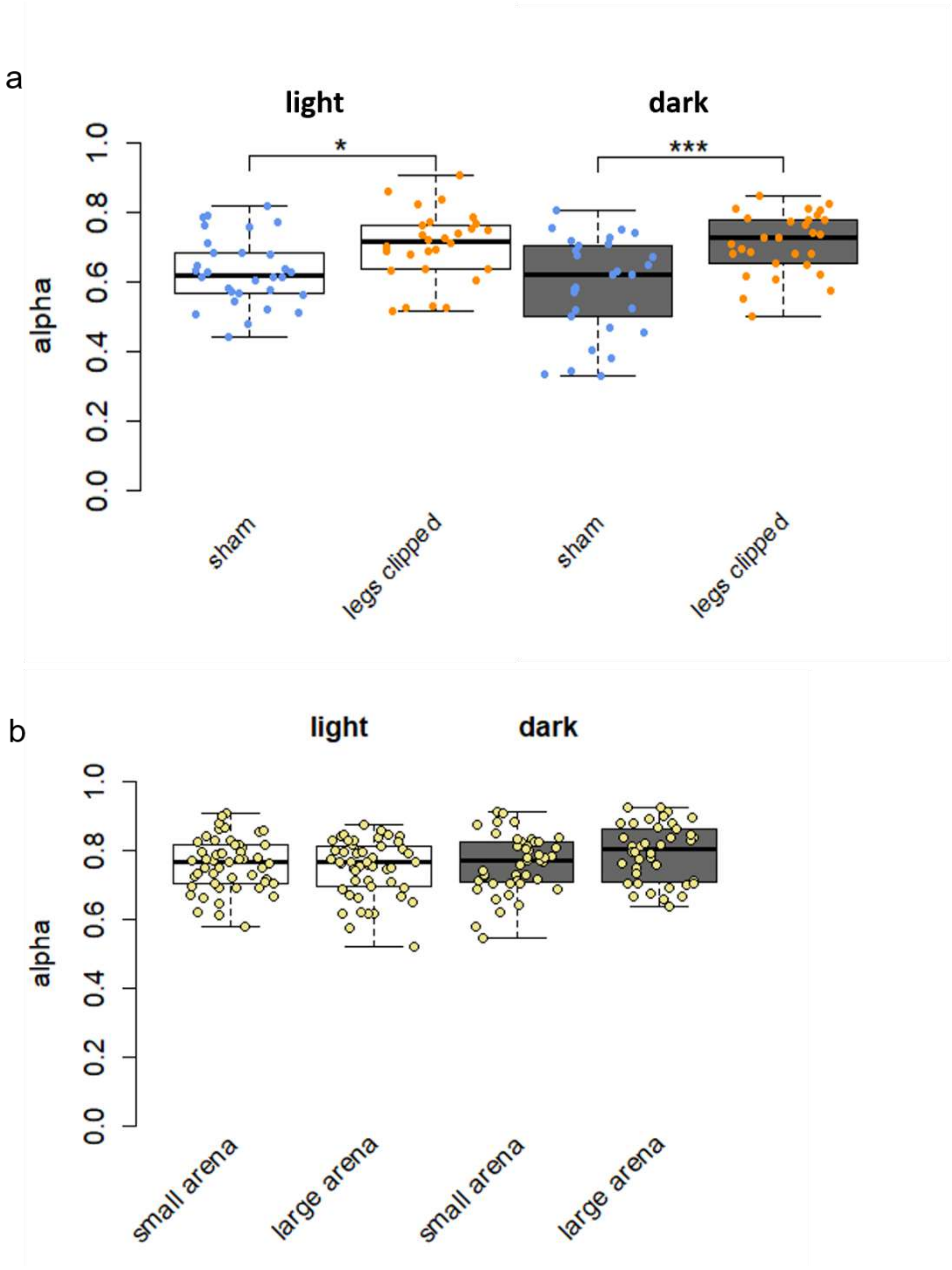


Figure 4.14: Testing scale-free walk (a) Flies with clipped legs had higher values of α than control flies, in light as well as dark (p=0.031 in light; p=0.00063 in dark). *p<0.05, ***p<0.0001, Wilcoxon Rank Sum Test. (b) The values of α were not significantly different between large arena and small arena, but were higher than 0.5 indicating scale-free walk. Kruskal-Wallis test with Dunn correction, p-values adjusted with the Holm method.

4.4 Discussion

In order to generate idiothetic information for path integration, the animal needs to estimate its own position as a function of body movements in space i.e. distance and direction. This is achieved through proprioceptive inputs which rely on mechanoensory neurons. Out of the many mechanosensors, mostly TRP channel proteins together contribute to proprioceptive cues in *Drosophila*. To this end, two kinds of experiments were performed: with Nanchung deficient mutants (Nan^{36a}) and attempting to synaptically block Nanchung. Nan^{36a} showed a defect in returns to the origin. Positional heatmaps showed a left shift away from the origin while homing in both light and dark conditions. Number of returns and percentage of flies initiating path integration-based search were significantly lower for the mutants in the dark. It should be noted that the returns of mutant flies in light were not different from control flies.

While path integration does not require any visual cues, it is prone to accumulating errors, and is thus complemented by visual cues provided by both the sky and the terrestrial environment errors (Benhamou 1990; Michel and Antonsen 1996; Merkle and Wehner, 2010). It is possible that Nan^{36a} flies relied on some visual inputs in light and were able to be return to the origin more accurately. This could explain why the phenotype was more pronounced in dark. It is possible that the returns in mutants were early in the search and flies were not able to navigate to the origin later in the trajectory.

Additionally, other mechanosensory channels like NompC and inactive could be compensating for the effect of inactivating Nan. Nan itself has been implicated in a wide-spectrum of physical and chemical stimuli like hearing, gravity sensing, nociception and thermotaxis (Kim et al. 2003; Gong 2004; Sun et al. 2009, Fowler and Montell 2013). Even though extensive literature has been published on invertebrate proprioception, exact mechanism and pathways involved in the activation of these mechanosensors remain unclear. It is also not well understood as to what extent each channel contributes to the sensory-feedback in the chordotonal organs.

The legs clipping experiment was done to test how proprioceptive inputs from the legs feed into the behaviour. Among the walking insect that use path integration, only desert ants (*Cataglyphis fortis*) have been shown to have a pedometer and count steps (Wittlinger et al. 2006; 2007). However, the legs of the ants were clipped after they had found food. Thus, their onward journey to food was with the legs intact and the homebound journey was with shorter legs. However, due to the nature of the behaviour, the flies were allowed to recover for a day after amputation in my experiments. It is possible that the flies adapted to the new leg length and altered proprioceptive inputs over time.

However, an interesting phenotype was observed in the flies with shorter legs. I found that in leg clipped flies, there were additional clusters with path integration and high turning. Same observation was made when the arena used was much larger, allowing more space for flies to search. The large arena was 3.16 times the small arena in area. This finding suggested that sugar intake provides the motivation to start a search, which may include smaller localized clusters. These clusters can be further tested to see if the behaviour can be explained by random chance, turning alone or a combination of turning and path integration. This would lead to a deeper understanding of the involvement of path integration in the behaviour.

5. Conclusion and future directions

5.1 Conclusions

Vincent Dethier, in his seminal paper "Communication by Insects: Physiology of Dancing" highlighted similarities between the search behaviour in flies and the dance communication of honey bees suggesting an ancestral connection between the solitary behaviour giving rise to the social communication (Dethier 1957; 1964). This thesis aimed to dig deeper into these connections, exploring the potential evolutionary and functional implications of the observed similarities between fly search behaviour and honey bee dance communication. We developed a lab set up to study sugar-elicited search behaviour on flies and honey bee foragers (Shakeel et al. 2023). Here, I summarize the major finding of this thesis.

5.1.1 Connection between solitary sugar-elicited behaviour and honey bee dance

Dethier made a distinction between solitary search in flies and dance, that the sugar ingestion and dance are separated in space and time. The sugar is ingested by the forager at the flower and the dance occurs inside ethe hive after the bee flies back home. Sugar-elicited search is initiated after the intake of sugar, indicating a connection between sensory input (sugar stimulus) and navigational behaviour. The honey bee dance is triggered by the successful foraging experience of an individual, linking food intake to the subsequent communication of the location to nest mates.

I was interested in exploring the relation between the stimulus and behaviour. Passive displacement experiments showed that stimulus (sugar) and behavioural response (local search) can be spatially and temporally dissociated in flies and bees (Shakeel and Brockmann 2023). Sugar intake increased the probability of initiating a search but the actual onset of walking triggers the path integration system to guide the search. To determine the duration of the motivational effect that ensues after sugar intake, I studied the temporal dynamics of the initiation of behaviour after sugar ingestion using a comparative approach (Shakeel and Brockmann 2023).

A series of delay experiments was performed after sugar ingestion, in flies and honey bees for search, and in bees for dance. When prevented from walking after feeding, flies and bees maintained their motivation for a path integration-based search for a duration of 3 min. In flies, turning and associated characters were significantly reduced during this period but remained higher than in flies without sugar stimulus. These results suggest that sugar elicits two independent behavioural responses: path integration and increased turning, with the initiation and duration of path integration system being temporally restricted. Honey bee dance experiments demonstrated that the motivation of foragers to initiate dance persisted for 15 min, while the number of circuits declined after 3 min following sugar ingestion.

5.1.2 Mechanistic basis of sugar-elicited search

Dethier had drawn attention to similarities in the highly tortuous walking patterns, and the stimulus (intake of sugar) which leads to both the behaviours. Local search behaviour in flies involves a walk with a high turning rate and frequent returns to the location of a sugar drop. However, the question of how they navigate to make returns remained unexplored. Additionally, I wanted to understand the mechanistic basis of the behaviour. It has been demonstrated that the search behaviour involves path integration (Kim and Dickinson 2017; Brockmann et al. 2018). The role of self-motion cues, potentially involving mechanosensory inputs from the legs, contribute to the search behaviour. I studied this using proprioceptive deficient mutant flies which showed an impairment in returns which was stronger in dark. I also explored how the inputs from legs affect walking and path integration during local search. Flies with shorter legs showed additional clusters of searches and Lévy walks. Furthermore, displacement experiments showed that the reference point for the path integration control of the search is the starting point of walking.

5.2 Summary

We propose that food intake during foraging increases the probability to initiate locomotor behaviours involving the path integration system in both flies and honey bees, and this ancestral connection might have been co-opted and elaborated during the evolution of dance communication by honey bees. Additionally, this study supports the idea that we can use local search as a paradigm to study initiation and control of path integration system using flies. Furthermore, the sugar-elicited search behaviour in flies can be used to study neural pathways involved in initiating and regulating the dance communication in honey bees.

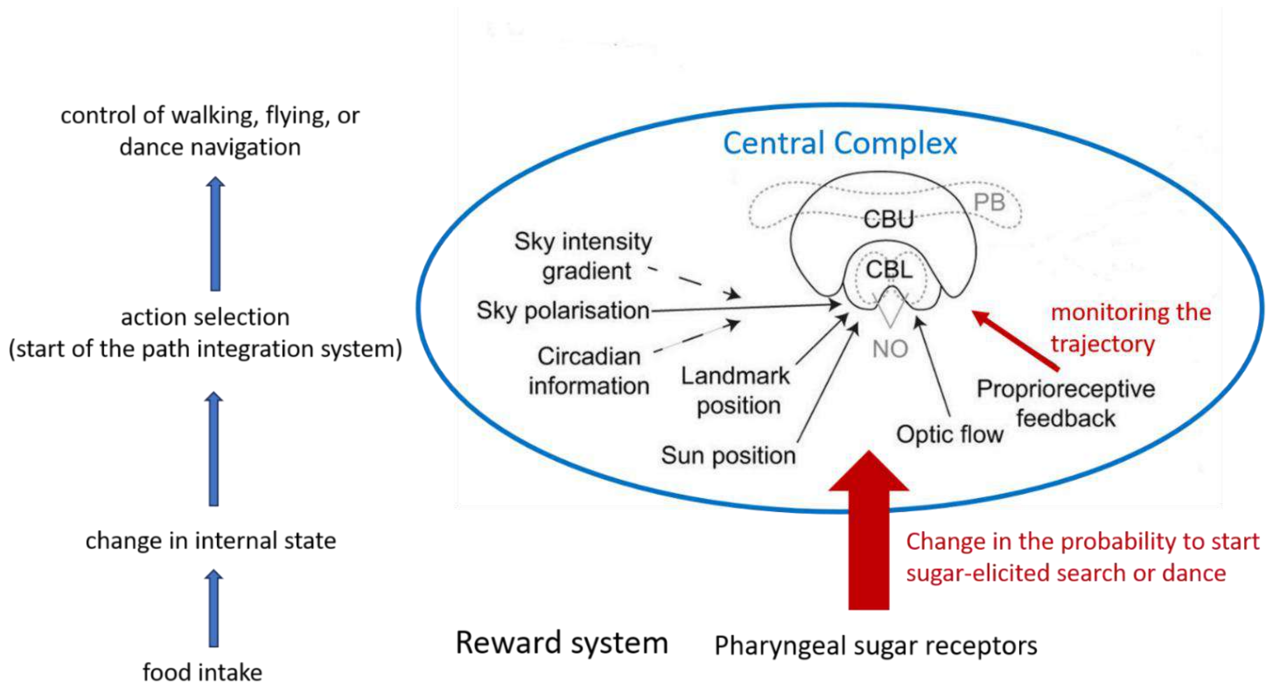


Figure 5.1: Model summarizing major conclusions of the study. Inputs to the central complex from Fig. 1.1 are supplemented based on the results (Modified from Barron and Plath 2017).

5.3 Future directions

5.3.1 Identifying neurotransmitter and neuromodulator system involved in initiating and regulating search and dance

To explore which neurotransmitter/neuromodulator system might be involved in both behaviors, we can measure changes in neurotransmitter/neuromodulator like dopamine and octopamine titres using mass spectrometry (MS). Our collaborators have developed MS method to quantify different important biogenic amines from honey bee and *Drosophila* brains (Ramesh and Brockmann 2018; Parkhitko et al. 2020: Chatterjee et al. 2021). The hypothesis is that in both behaviours, search and dance, sugar-intake induces comparable changes in the same neurotransmitter/neuromodulators and that these are then likely involved in regulating both behaviors. Flies and bees can be collected at different time points after sugar ingestion, in the same manner as the delay experiments. Flies and honey bee for time points post-feeding and impeded from initiating behaviour (10 s, 30 s, 1 min, 2 min, 3 min and 5 min) will be collected for the search experiments. Similarly, foragers in the dance experiment will be collected at the feeder at different time points (10s, 30s, 1 min, 2 min, 3 min, 5 min, 10 min, 15 min) along. Unfed controls should be collected as well. Although, this study will only result in correlative data, it will give interesting insights into the dynamics of brain changes after sugar ingestion.

5.3.2 Studying neural and mechanistic basis of path integration

Due to challenges of doing manipulation studies in honey bees, understanding the neural basis of memory and navigation has been elusive. This work shows that sugar intake activates the path integration system in flies and honey bees. Sugar-induced search behaviour in flies combined with the powerful neurogenetic toolkit in flies can be used as a paradigm study the neural pathways involved in dance communication in honey bees. Additionally, the sensory input pathways that activate path integration can be studied using Drosophila. This thesis investigated the role of mechanosensory neurons, while chemosensory pathways that are involved in activating path integration following food intake have not yet been studied.

In two of my experiments: large arena and legs clipped, there were additional clusters of homing observed. The observation of these clusters in large arena, without any manipulation indicates that this is a feature of the behaviour and should be analysed further. The nature of these clusters can be examined to test and whether they involve path integration. This can be done by running simulations on the randomized trajectories to see if the behaviour can be explained by random chance, turning alone or a combination of turning and path integration.

5.3.3 Studying neural basis of sugar-elicited search

5.3.3.1 Modification to the behavioural set up to achieve neuronal silencing

Proprioceptive inputs impaired flies using Nan>Shi^{TS} did not work because of the tools used to achieve neuronal silencing i.e. the temperature of 32°C had an effect on the behaviour. Thus, instead of thermogenetics, the setup can be modified using optogenetics for neuronal silencing by using UAS-enphr2.0 and UAS-GTACR2. However, establishing optogenetic method in freely walking flies is difficult, there have been some recent successes reported by some research groups. Additionally, to explore the role of mechanosensory inputs specifically from the legs, Dacshund (specific to leg) can be used to drive in combination with Nan gal4 (Mendes et al. 2013). This will help in elucidating the specific role of proprioceptive inputs in self-motion cues for navigation.

5.3.3.2 Identifying neural substrates of the behaviour by neurogenetically silencing regions of the brain

Various central complex and mushroom body Gal4 lines can then be tested with neuronal inactivation to see the effect on sugar-elicited search. Neuromodulators can also be tested in a similar manner to identify the post-ingestive signal(s) involved in the behaviour.

6 References

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7. Appendix

7.1 Supplementary figures

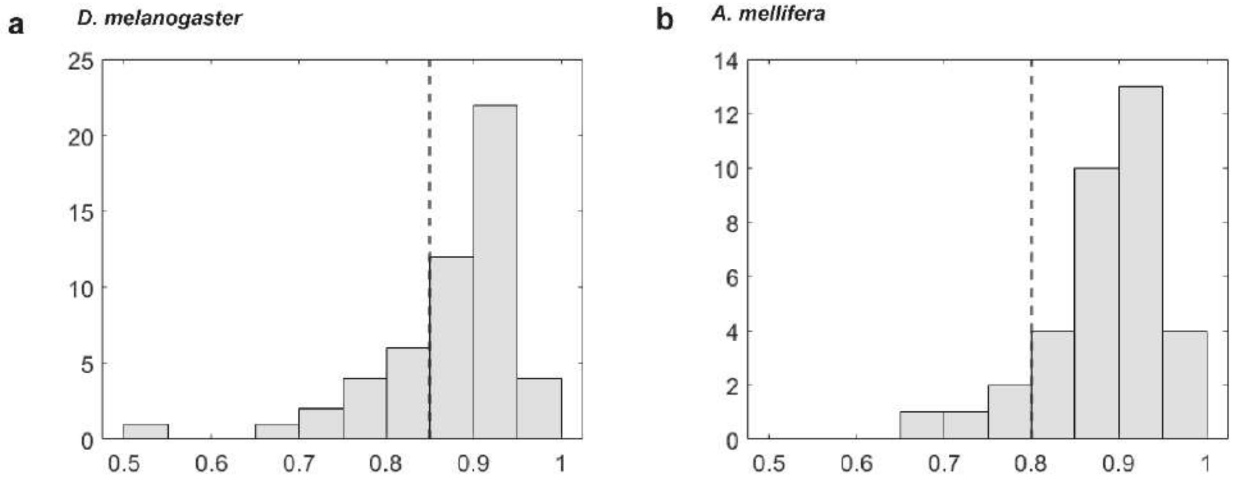


Figure S1: Histogram of meander from control experiments. (a) meander>0.85 for flies; (b) meander>0.8 for bees was used as a threshold on meander for characterizing path integration-based local search.

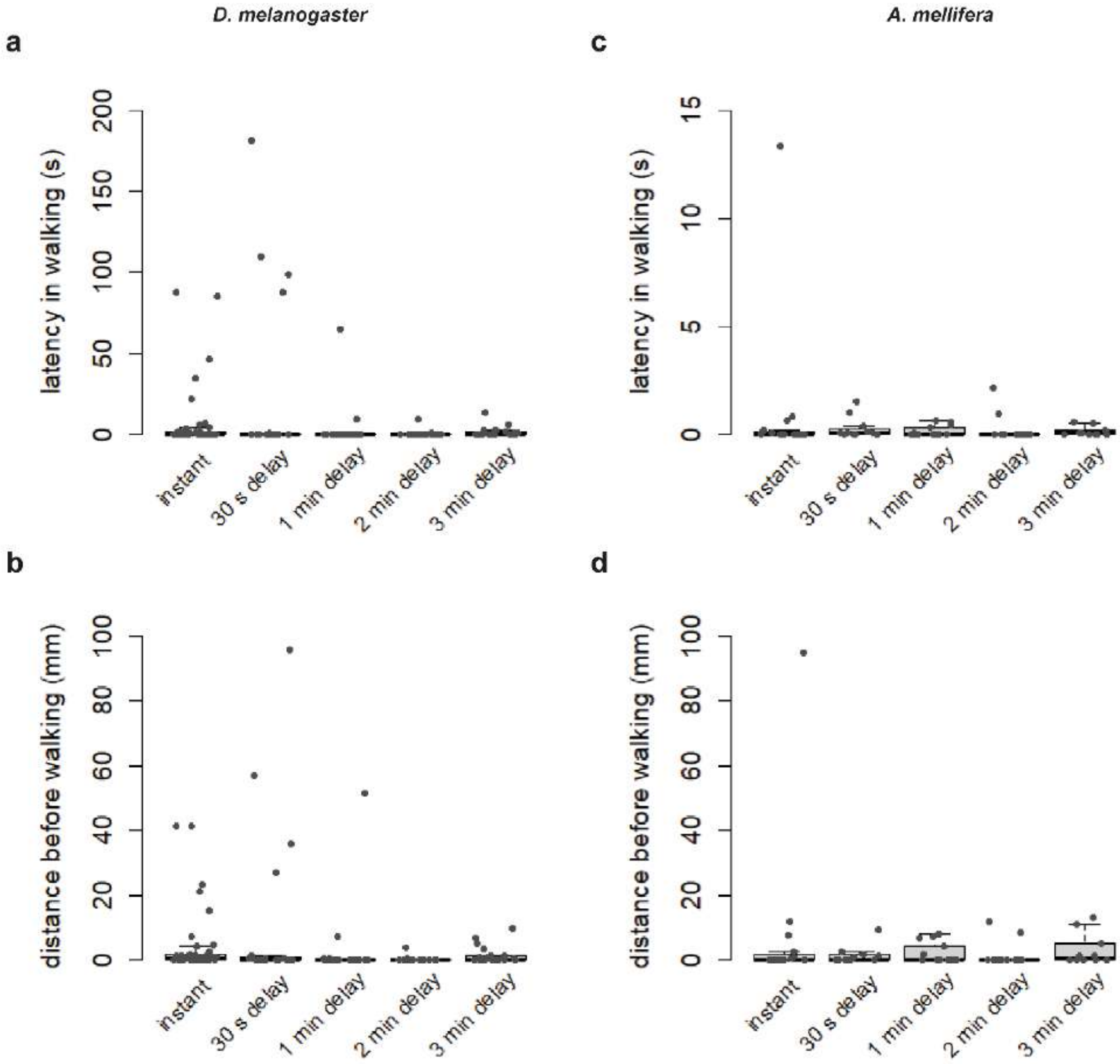


Figure S2: There is no difference in latency to walk post-transfer across delay durations. (a,b) Latency in walking and distance covered before walking does not change with increased time delay post-feeding in flies. (c,d) Bees do not show a difference in delay in walking and distance covered before walking with increased delay post-feeding. Kruskal-Wallis test with Dunn correction, p-values adjusted with the Holm method.

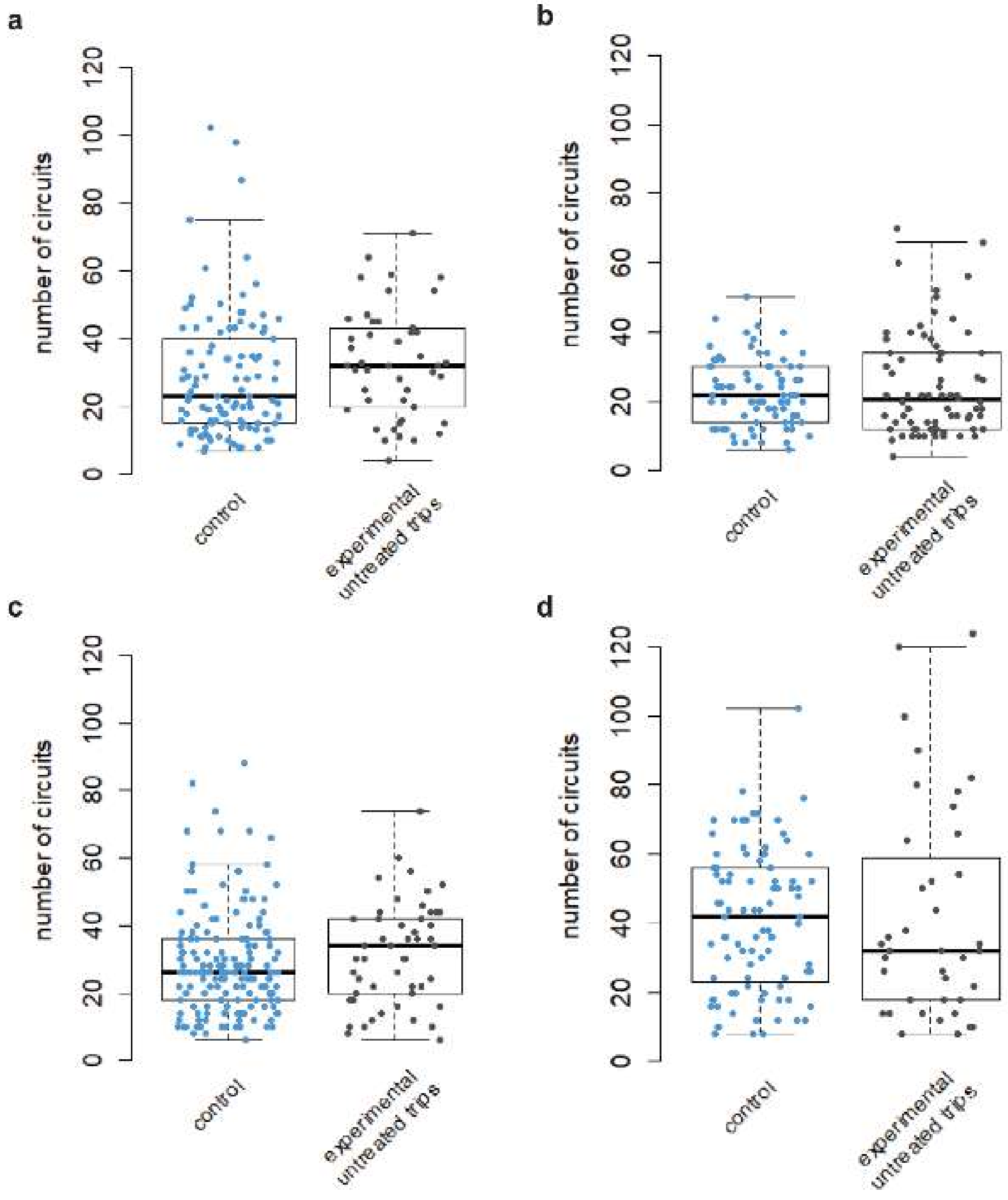


Figure S3: Number of circuits across do not get affected in untreated trips. (a-d) The number of circuits for the control group and untreated trips of experimental group (do not show a difference across all four foraging groups (group 1 p=0.07013; group 2 p=0.983; group 3 p=0.1298; group 4 p=0.7505, Wilcoxon Rank Sum Test).

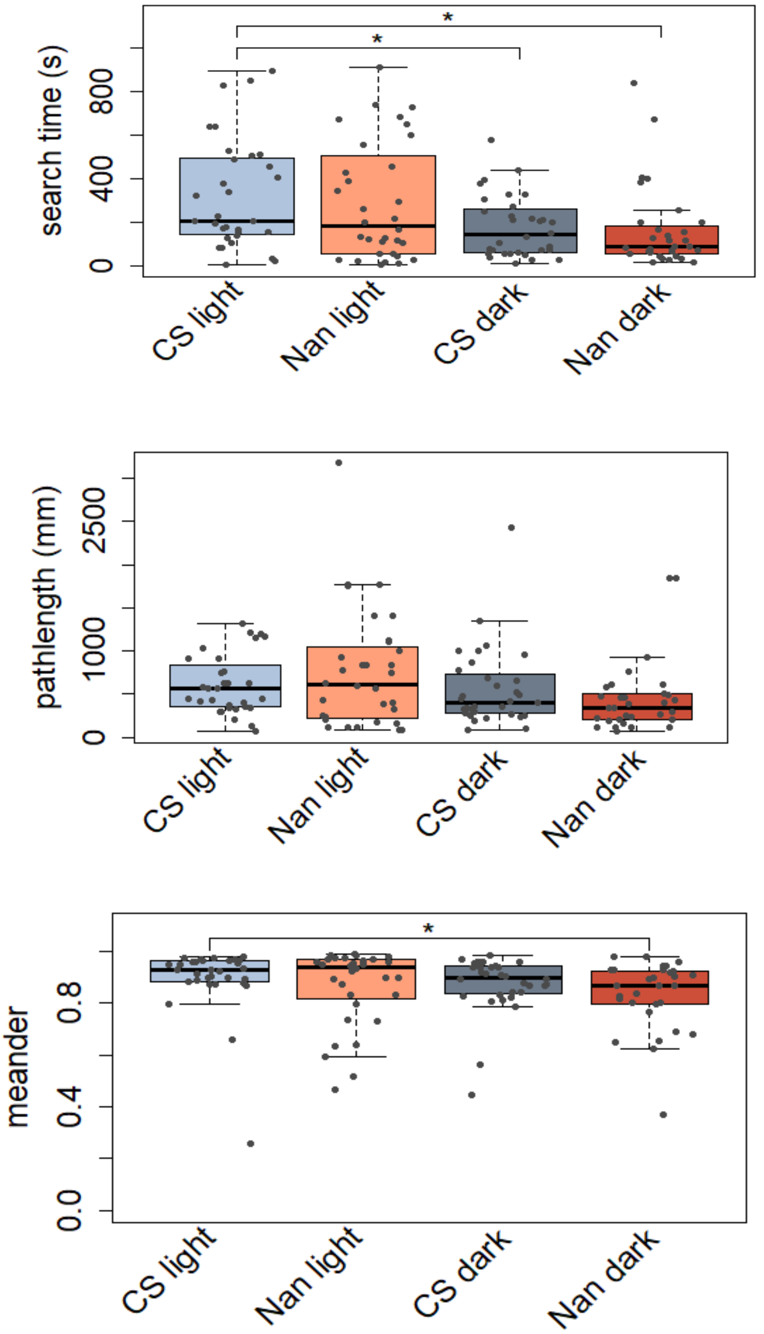


Figure S4: Comparison of parameters of search for Nan^{36a} mutants. Search time was statistically lower between CS in light and dark (p=0.027) and CS in light and Nan^{36a} in dark (P value=0.015). The was no difference in path lengths across test groups. The meander showed statistically significant difference between Canton S flies in light and nan36a in dark (p=0.044). *p<0.05 Kruskal-Wallis test with Dunn correction, p-values adjusted with the Holm method.

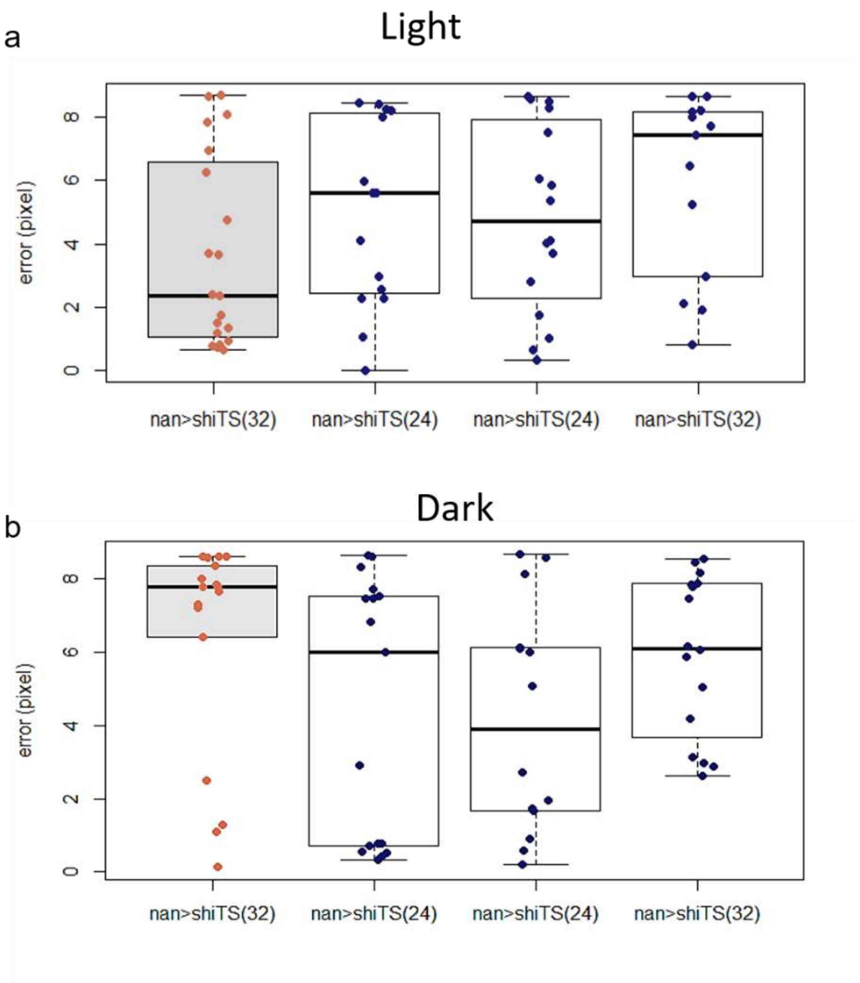
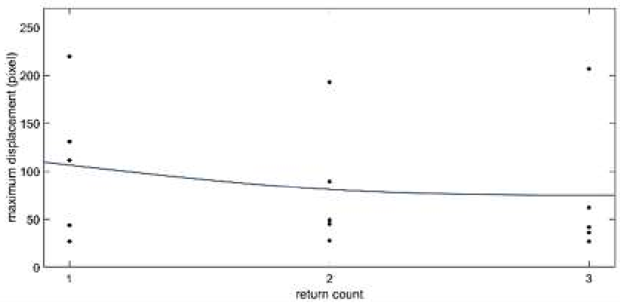
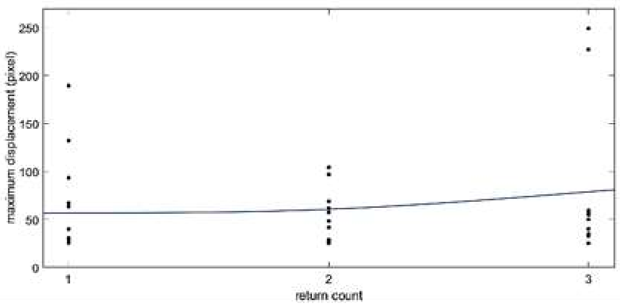


Figure S5: Quantification of error in returns for Nan mutants. First four returns were analysed for the error, defined by local minimum when the fly is making a return. There was no significant differences between test and control flies in either light or dark. Kruskal-Wallis test with Dunn correction, p-values adjusted with the Holm method.

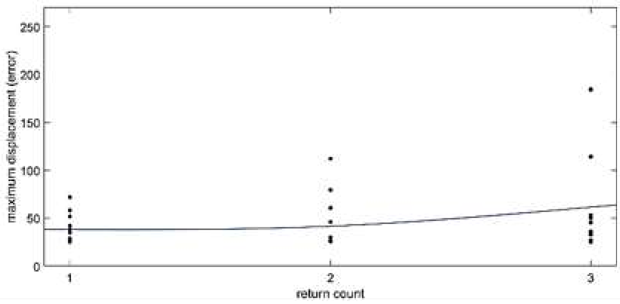
Light



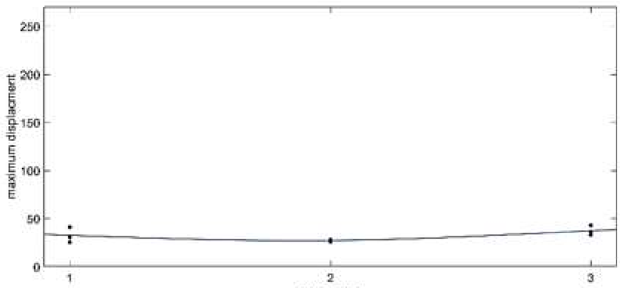
Nan> Shi^{TS} (32° C)



Nan> Shi^{TS} (24° C)

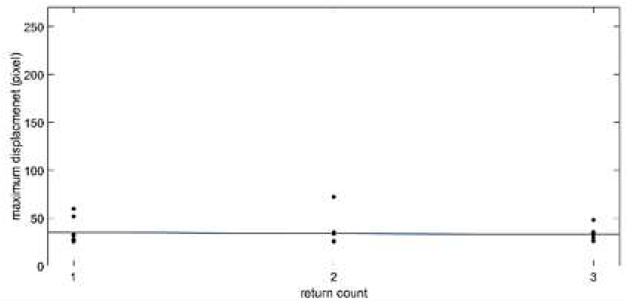


Nan> + (24° C)

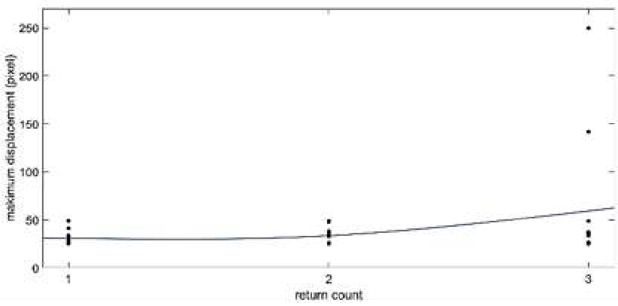


Nan> + (32° C)

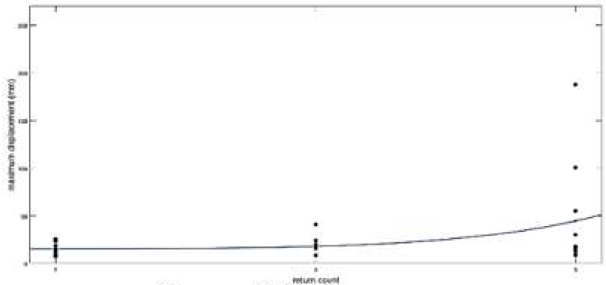
Dark



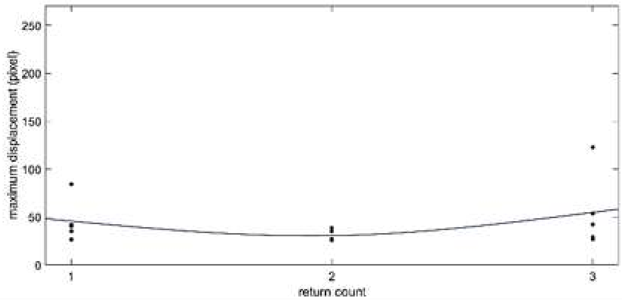
Nan> Shi^{TS} (32° C)



Nan> Shi^{TS} (24° C)



Nan> + (24° C)



Nan> + (32° C)

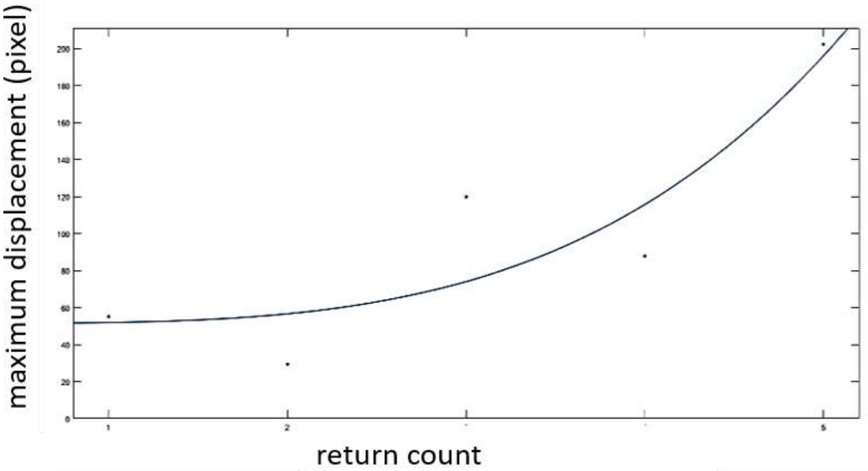
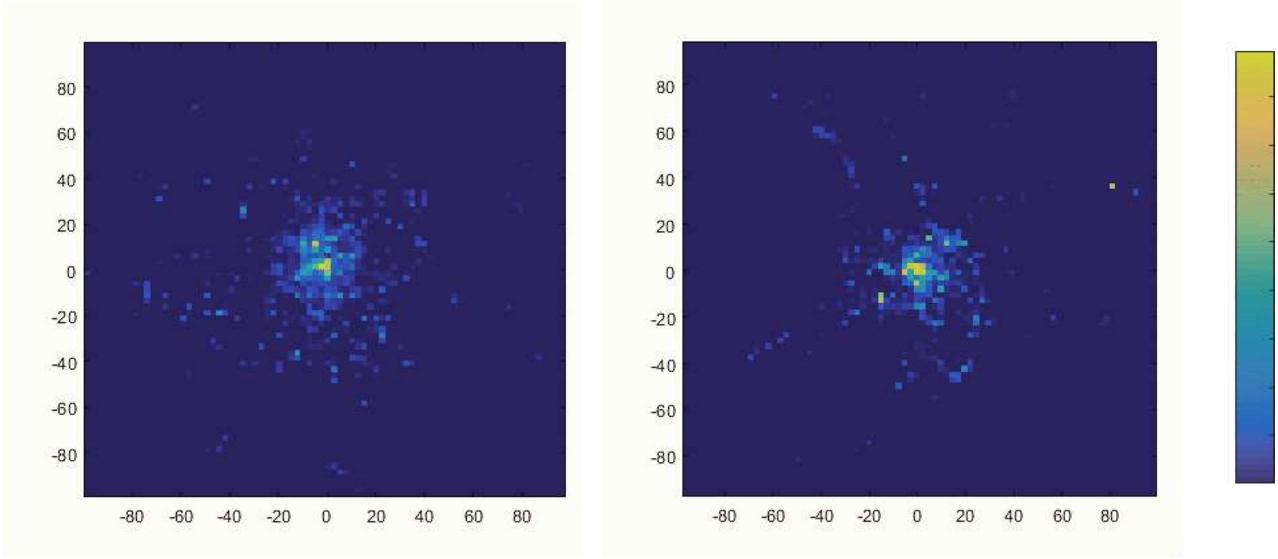


Figure S6: Quantification of maximum displacement between subsequent returns. First three returns (maximum number of returns common across all groups) were analysed to quantify the maximum displacement of the fly before it made the next return. CS flies at room temperature go further away from the origin before each return, as the search progresses (top panel). However, the slope of these plots for Nan>shiTS and Nan>+ did not follow the same curve as CS controls (shown separately).



CS in light at 32° C

CS in dark at 32° C

Figure S7: Temperature has an effect on the search behaviour of flies. CS flies tested at 32º C showed that the heatmaps were scattered near the origin ((±100 pixels on x and y axes) in both light and dark.

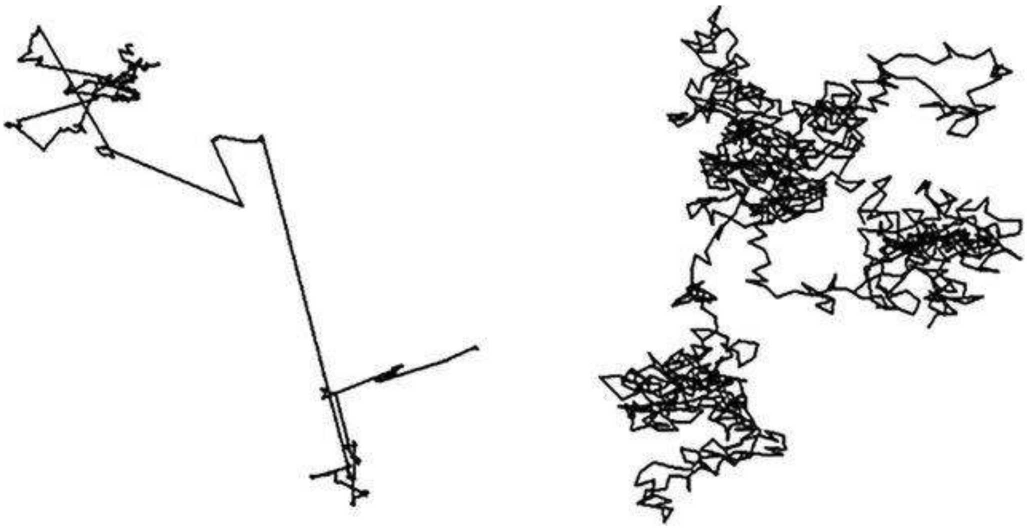


Figure S8: An example of a Lévy walk (left) and a Brownian walk (right). Adapted from Reynolds 2018

7.2 Supplementary tables

Table S1: The *Drosophila* media composition used (for 1 L of media)

corn flour	80 g
D-glucose	20 g
Sugar	40 g
Agar	8 g
Yeast powder	15 g
propionic acid	4 ml
TEGO	1.25 g in 3 mL of ethanol
Orthophosphoric acid	600 µL)

Table S2: Regression analysis on the behavioural parameters did not find a correlation with the delay durations in flies and bees

D. melanogaster					
Parameter	Slope	Intercept	Adjusted R-squared	t-statistic	p-value
number of returns	-0.32	1.42	0.043	1.1172e-10	0.0146
meander	-0.052	0.89	0.163	2.2557e-84	3.0523e-06
stay time	-66.04	300.03	0.104	1.7434e-19	2.6659e-4
path length	-87.52	511.32	0.112	8.2088e-28	1.5933e-4
A. mellifera					
Parameter	Slope	Intercept	Adjusted R-squared	t-statistic	p-value
number of returns	-0.32	1.57	0.031	2.1685e-06	0.0931
meander	-0.07	0.79	0.116	3.5176e-28	0.0042
stay time	-2.47	21.5	0.006	1.3988e-08	0.2434
path length	-11.36	72.75	0.013	8.5762e-07	0.1833

Table S3: p-values for comparison of number of circuits for dance experiments

Kruskal-Wallis test with Dunn correction, p-values adjusted with the Holm method.

Group	Comparison	p-value adjusted
1	2min-3min	1.000
2	2min-5min	1.000
3	3min-5min	0.996
4	2min-30s	1.000
5	3min-30s	1.000
6	5min-30s	1.000
7	2min-10min	1.000

8	3min-10min	1.000
9	5min-10min	1.000
10	30s-10min	0.754
11	2min-1min	1.000
12	3min-1min	0.316
13	5min-1min	0.311
14	30s-1min	1.000
15	10min-1min	0.176
16	2min-15min	0.442
17	3min-15min	1.000
18	5min-15min	1.000
19	30s-15min	0.106
20	10min-15min	1.000
21	1min-15min	0.013
22	2min-instant	1.000
23	3min-instant	1.000
24	5min-instant	1.000
25	30s-instant	1.000
26	10min-instant	0.558
27	1min-instant	1.000
28	15min-instant	0.063
29	2min-untreated	1.000
30	3min-untreated	0.020
31	5min-untreated	0.020
32	30s-untreated	1.000
33	10min-untreated	0.036
34	1min-untreated	1.000
35	15min-untreated	0.001
36	instant-untreated	1.000

Table S3: Values of power-law scaling α (mean±SD)

Group	Light	Dark
Sham	0.63±0.09	0.59±0.14
Legs clipped	0.70±0.10	0.71±0.08
Small arena	0.76±0.074	0.76±0.08
Large arena	0.75±0.08	0.79±0.08

Table S4: p-values for comparison of values for α

Kruskal-Wallis test with Dunn correction, p-values adjusted with the Holm method.

Comparison	p-value adjusted
Large arena in dark – Large arena in light	0.177
Large arena in dark - Small arena in dark	0.432
Large arena in light - Small arena in dark	1.000
Large arena in dark - Small arena in light	0.273
Large arena in light - Small arena in light	1.000
Small arena in dark - Small arena in light	0.795