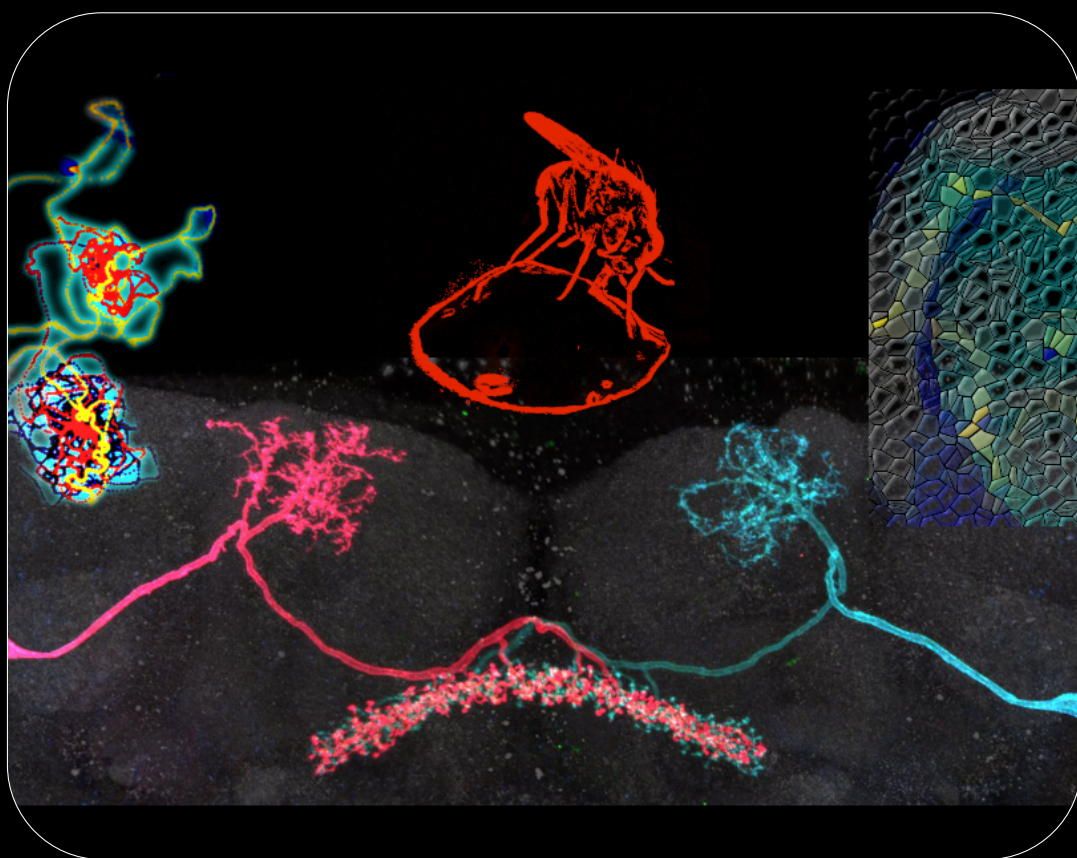


Identifying neural circuits for nutrient exploration and decision-making in *Drosophila melanogaster*

Dennis Goldschmidt



Dissertation presented to obtain the **Ph.D degree** in
International Neuroscience Doctoral Programme (INDP)

Oeiras, September, 2022

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Instituto de Tecnologia Química e Biológica António Xavier | Universidade NOVA de Lisboa

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IDENTIFYING NEURAL CIRCUITS FOR NUTRIENT
EXPLORATION AND DECISION-MAKING IN
DROSOPHILA MELANOGASTER

DENNIS GOLDSCHMIDT

A DISSERTATION
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IDENTIFYING NEURAL CIRCUITS FOR NUTRIENT EXPLORATION AND DECISION-MAKING IN *DROSOPHILA MELANOGASTER*

This doctoral thesis was written by Dennis Goldschmidt and it is the result of his own work. Any work done in collaboration is explicitly provided in each chapter. It has not been previously submitted, in part or whole, to any university or institution for any degree, diploma, or other qualification.

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Für Günter Wiese, Heiner, Oma Inge, und Mama...

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“It is in our nature to explore, to reach out into the unknown. The only true failure would be not to explore at all.” (Ernest Shackleton)

ABSTRACT

Animals adapt their foraging decisions with respect to nutrient availability and their internal needs. These decisions rely on complex computations in order to decide whether to exploit a food source or to continue exploring potentially better options. However, the neuronal circuit logic underlying dynamic computations for balancing exploration and exploitation during foraging and how they are shaped by internal states remain poorly understood.

In this thesis, I present a methodological approach to studying how the common fruit fly *Drosophila melanogaster* decides where and what to eat in order to fulfil their current and potential future nutrient needs. This method combines detailed and high-throughput, automated behavioral quantification with a large-scale neurogenetic manipulation screen to identify neuronal populations implicated in different aspects of the flies' decision-making during foraging. Specifically, we created a setup called *optoPAD* as a closed-loop optogenetics system to study neuronal circuits involved in feeding behavior. Furthermore, extending the food choice paradigm to a foraging arena with multiple yeast and sucrose patches, we study foraging decisions made by flies as they explore the arena. Using this setup, we carried out a large-scale neurogenetic behavioral screen to test the effect of silencing activity in different neuronal subsets on aspects of foraging. We discovered a neuronal population that projects to a central region of the insect brain, which is important for the decision to stop at a food patch. We showed that a single pair of neurons within this population

is required for the fly's exploratory drive, and that the activity in this neuron is modulated by the fly's protein state. Finally, I present conclusions of this study in order to form an integrative theoretical framework of the flies' decision-making during foraging with the normative goal to balance nutrient intake.

Our results reveal a neural substrate important for regulating complex, ethologically relevant foraging decisions. This provides a key step towards a mechanistic explanation of cognitive functions required to compute the exploration-exploitation trade-offs, and as such how to match foraging outcomes to physiological needs.

Keywords: Systems Neuroscience, Behavior, Foraging, Exploration, Neuronal Computation, Cognition

RESUMO

Os animais adaptam as suas decisões de procura de alimentos de acordo com a disponibilidade de nutrientes e as suas necessidades internas. Estas decisões dependem de cálculos complexos a fim de decidir se exploram uma fonte alimentar ou se continuam à procura de opções potencialmente melhores. No entanto, a lógica do circuito neuronal subjacente aos cálculos dinâmicos para equilibrar a exploração do ambiente e a utilização dos recursos alimentares durante a procura de alimentos, assim como a forma como são moldados pelos estados internos permanecem pouco compreendidos.

Nesta tese, apresento uma abordagem metodológica para estudar como a mosca da fruta comum *Drosophila melanogaster* decide onde e o que comer a fim de satisfazer as suas necessidades nutricionais actuais e futuras. Este método combina a quantificação comportamental detalhada e automatizada de *high throughput* com um *screening* de manipulação neurogenética em grande escala para identificar populações neuronais envolvidas em diferentes aspectos da tomada de decisões das moscas durante a procura de alimentos. Especificamente, criámos uma configuração chamada *optoPAD*, um sistema optogenético em *closed-loop* para estudar os circuitos neuronais envolvidos no comportamento alimentar. Além disso, estendendo o paradigma da escolha alimentar a uma arena com múltiplas fontes de levedura e açúcar, estudámos as decisões de procura de alimentos tomadas pelas moscas enquanto exploram a arena. Usando esta configuração, realizámos um *screening* de comportamento neurogenético em grande escala para testar o efeito da inactivação de diferentes subconjuntos neuronais em aspectos da procura de alimentos. Descobrimos uma população neuronal que projecta

para uma região central do cérebro do insecto, o que é importante para a decisão de parar numa fonte alimentar. Mostrámos que um único par de neurónios dentro desta população é necessário para o impulso exploratório da mosca, e que a actividade neste neurónio é modulada pelo seu estado proteico. Finalmente, apresento conclusões deste estudo a fim de formar um quadro teórico integrador da tomada de decisões das moscas durante a procura de alimentos, com o objectivo normativo de equilibrar a ingestão de nutrientes.

Os nossos resultados revelam um substrato neural importante para a regulação de decisões complexas e etologicamente relevantes em matéria da procura de alimentos. Isto fornece um passo fundamental para uma explicação mecanicista das funções cognitivas necessárias para calcular os *trade-offs* entre explorar novas opções e utilizar recursos encontrados, e como tal, como combinar os resultados da procura de alimentos com as necessidades fisiológicas.

Palavras-chave: Neurociência de Sistemas, Comportamento, Procura de alimentos, Exploração, Computação Neuronal, Cognição

ZUSAMMENFASSUNG

Tiere passen ihre Entscheidungen bei der Nahrungssuche an die Verfügbarkeit von Nährstoffen und an ihre eigenen Bedürfnisse an. Diese beruhen auf komplexen Berechnungen, um zu entscheiden, ob eine Nahrungsquelle ausgebeutet oder die Erkundung von potenziell besseren Optionen fortgesetzt werden soll. Die neuronale Schaltkreislogik, die den dynamischen Berechnungen zur Abwägung von Erkundung und Ausbeutung während der Nahrungssuche zugrunde liegt, und die Art und Weise, wie diese durch innere Zustände beeinflusst werden, sind jedoch hauptsächlich unbekannt.

In dieser Arbeit stelle ich einen methodischen Ansatz vor, um zu untersuchen, wie die gemeine Fruchtfliege *Drosophila melanogaster* entscheidet, wo und was sie frisst, um ihren aktuellen und potenziell zukünftigen Nährstoffbedarf zu decken. Diese Methode kombiniert detaillierte und automatisierte Verhaltensquantifizierung mit einem groß angelegten neurogenetischen Manipulationsscreening, um neuronale Populationen zu identifizieren, die an verschiedenen Aspekten der Entscheidungsfindung der Fliegen während der Nahrungssuche beteiligt sind. Konkret haben wir einen Versuchsaufbau namens *optoPAD* als optogenetisches System mit geschlossenem Regelkreis entwickelt, um neuronale Schaltkreise zu untersuchen, die am Fressverhalten beteiligt sind. Darüber hinaus haben wir das Paradigma der Nahrungswahl auf eine Arena mit mehreren Hefe- und Zuckerfeldern ausgeweitet, um die von den Fliegen bei der Erkundung der Arena getroffenen Entscheidungen zu untersuchen. Mit diesem Aufbau haben wir ein groß angelegtes neurogenetisches Verhaltensscreening durchgeführt, um

die Auswirkungen des Ausschaltens der Aktivität in verschiedenen neuronalen Untergruppen auf Aspekte der Nahrungssuche zu testen. Wir entdeckten eine Population von Neuronen, die in eine zentrale Region des Insektengehirns projiziert, die die Entscheidung steuert, ob die Fliege an einem Futterplatz anzuhält oder nicht. Wir konnten zeigen, dass ein einziges Neuronenpaar innerhalb dieser Population für den Erkundungstrieb der Fliege erforderlich ist und dass die Aktivität dieses Neurons durch den Proreinstatus der Fliege moduliert wird. Abschließend stelle ich die Schlussfolgerungen dieser Studie vor, um einen integrativen theoretischen Rahmen für die Entscheidungsfindung der Fliegen bei der Nahrungssuche mit dem normativen Ziel einer ausgewogenen Nährstoffaufnahme zu schaffen.

Unsere Ergebnisse offenbaren ein neuronales Substrat, das für die Regulierung komplexer, ethologisch relevanter Entscheidungen bei der Nahrungssuche wichtig ist. Dies ist ein entscheidender Schritt hin zu einer mechanistischen Erklärung der kognitiven Funktionen, die für die Berechnung des Kompromisses zwischen Erkundung und Ausbeutung erforderlich sind, und damit auch für die Abstimmung des Erfolgs der Nahrungssuche auf die physiologischen Bedürfnisse.

Stichwörter: Systemneurowissenschaften, Verhalten, Nahrungssuche, Erkundung, Neuronale Berechnungen, Kognition

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ACRONYMS

AAs	Amino Acids (<i>p. 71</i>)
ACC	Anterior Cingulate Cortex (<i>p. 20</i>)
AL	Antennal Lobes (<i>p. 196</i>)
AMMC	Antennal Mechanosensory and Motor Center (<i>p. 196</i>)
ANTs	Advanced Normalization Tools software (<i>p. 112</i>)
CX	Central Complex (<i>pp. 27, 121</i>)
dFB	Dorsal Fan-shaped Body (<i>p. 31</i>)
EB	Ellipsoid Body (<i>pp. 27, 196</i>)
FB	Fan-shaped Body (<i>pp. 27, 196</i>)
GPIO	general-purpose input/output (<i>p. 39</i>)
GRs	Gustatory Receptors (<i>p. 25</i>)
GSNs	Gustatory Sensory Neurons (<i>p. 25</i>)
IRs	Ionotropic Receptors (<i>p. 25</i>)
LAL	Lateral Accessory Lobes (<i>p. 27</i>)
LH	Lateral Horns (<i>p. 196</i>)
LOP/LO	Lobular Complex (<i>p. 196</i>)

MB	Mushroom Bodies (<i>pp. 28, 196</i>)
MBONs	Mushroom Body Output Neurons (<i>p. 28</i>)
MoHiVE	Modular High-throughput Video-based Ethological tracking (<i>pp. 64, 67, 81</i>)
MOSFET	Metal-Oxide-Semiconductor Field-Effect Transistor (<i>p. 37</i>)
MVT	Marginal Value Theorem (<i>p. 17</i>)
NO	Noduli (<i>p. 27</i>)
OFT	Optimal Foraging Theory (<i>pp. 17, 73</i>)
OL	Optic Lobes (<i>p. 196</i>)
ORs	Olfactory Receptors (<i>p. 25</i>)
OSNs	Olfactory Sensory Neurons (<i>p. 25</i>)
PB	Protocerebral Bridge (<i>p. 27</i>)
PRW	Prow (<i>p. 196</i>)
RH	Relative Humidity (<i>p. 107</i>)
SEM	Standard Error of the Mean (<i>pp. 86, 111</i>)
SEZ	Subesophageal Zone (<i>pp. 26, 196</i>)
SMP	Superior Medial Protocebebrum (<i>p. 196</i>)
SNP	Superior Neuropils (<i>p. 28</i>)
Vaa3D	“3D Visualization-Assisted Analysis” software suite (<i>p. 113</i>)

PREFACE

All journeys with unimaginable goals start with taking a first step. While the results I present here were largely produced over the span of the last seven years. Nonetheless, without a doubt —my journey already started much earlier. Starting out my research career, I had to decide whether I am going to stick with a pure physics project for my bachelor thesis or branching out of traditional research topics into the world of biophysical phenomena. The brain was naturally a very fascinating system to me. Ever since I was a child, I often wondered what makes me "*me*", and how it would be to be in the body or rather mind of another person. After choosing to join the Computational Neuroscience department, I started to work on neuro-inspired robotics. Here I became captivated by the problem of traversing irregular terrain with walking leg and specifically how this can help a six-legged robot to walk over obstacle steps as high as its own body height. This interest drew me into reading seminal literature by Roy Ritzmann and others, which studied how the deathhead cockroach *Blaberus discoidalis* overcomes large obstacles. Lessons I have learnt from deepening my knowledge in this direction really imprinted on me the idea that the brain does not only generate behavior in isolation, but rather that brain-body interactions in an embodied way create a dynamical system that solves complex problems. And although I was working with lifeless machines, finding solutions in the biological blueprints made by evolution was a very fulfilling experience.

Nevertheless, my curiosity drove me further to not only try to understand how embodied behaviors are performed, but to understand more

deeply the complex computations the brain applies to generate those behaviors. I was especially fascinated by the navigational abilities of many animals, chiefly the eusocial insects of the order *Hymenoptera*. The seminal work by Rüdiger Wehner drew me to the desert ants' ability to perform path integration, and their ability to not only use this information to return to the nest on a straight path, but also to learn and store goal vector representations of discovered food sites. Uwe Homberg's work gave me insights into how locusts use polarization and how neurons in the central complex represent different orientations of polarization in a topological manner. The final inspiration for my computational model came from the Vivek Jayaraman's lab, which showed that neurons projecting to the ellipsoid body, a part of the central complex, represent an internal compass in fruit flies. These neurons do not only form this compass based on a visual feature in the environment, but also maintain its representation in the dark through self-motion cues. Further studies also from other labs have then elucidated how these signals are maintained as a ring attractor network, and how such directional representations are transformed into vector representations.

One missing piece in our computational model was the fact that animals behave completely different in different contexts or motivations. Not only are behavioral policies changed, but they also respond to same sensory stimuli differently depending on the context. In our model, we treated this internal context as a black box, literally a binary node that automatically switches between inward and outward navigation and affects the signalling of the network globally. Why? How could we have such an integral part of the computational model be a black box, which I (at that time) didn't know anything about. Fast forward, entering my PhD first-year coursework we studied the topic of "*Drive & State*" which felt like an epiphany to me. Indeed, many labs do study motivations and state, be it sexual drive, fear or hunger; and I was enthralled to learn more about it. I was naturally drawn towards hunger motivations, which Carlos Ribeiro taught us explaining that hunger and satiety are not just simply one-dimensional variables changing over time. In fact, there are multiple nutrient sensing mechanisms that send signals to the brain to represent the state of not only current, but also

future needs. The week felt truly transformative and therefore I decided to rotate and finally join Carlos' lab to further my understanding in this topic. Seven years went by, and I have learnt so many lessons, many of which are presented in this thesis.

This brings me finally to this point, where I am sitting here at the 6th Central Complex workshop marvelling at the keynote talk by Uwe Homberg, collaborating with and being integral part of this community. This is in the end what inspired the work of this thesis making this not just seven-year journey but rather a cumulative one of 10+ years. I feel humbled and grateful for this experience.

Oct 13, 2022

HHMI Janelia, Ashburn, VA, USA

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GENERAL INTRODUCTION

To understand how the brain generates complex behavior is one of the key questions in neuroscience (Gomez-Marín & Ghazanfar, 2019; Katz & Harris-Warrick, 1999; Krakauer et al., 2017). Specifically, the field of neuroethology tries to tackle this question by observing behaviors in a naturalistic setting (Dennis et al., 2021; Kennedy, 2022; Testard et al., 2021). The wide spectrum of complex, natural behaviors by animals in the wild is truly astonishing.

Not only do animals have to select appropriate actions dynamically, they also have to continuously sense and adapt to changes in internal and external states. Furthermore, behavior is also flexible as the same sensory input may lead to different behaviors. This is due to the fact that also internal factors or states affect which behavior is executed. How does the brain integrate many external and internal signals to form an appropriate output? But what is an appropriate output in terms of complex behaviors, like for example foraging for food?

I argue that a deeper and detailed understanding of complex behavior is the first step towards answering these questions. Moreover, connecting this

first step with systematic, large-scale neuronal manipulations and interrogations has the potential to decipher the underlying circuit logic and what computations are performed. This will form a comprehensive, integrative approach towards a mechanistic understanding of internal and external information processed by the brain to generate complex, and adaptive behavior, which is required for cognitive tasks such as foraging. Insights and knowledge from such studies will impact not only the field of neuroscience at large, but also others, such as psychology, socio-economics, medicine, ethics and philosophy (Damasio, 2007; Dezfouli et al., 2019; Mobbs et al., 2018; Shadlen & Kiani, 2013).

In this General Introduction, I first further elaborate how quantitative analysis and deeper study of behavior is important for understanding how the brain works. Then I describe how recent computational and technological advances led to a new and exciting way to quantify and analyze behavior at a high throughput. Secondly, I propose that such behavioral quantification can be used to study more complex cognitive functions, such as the decision-making during food foraging. Thirdly and finally, I introduce the fruit fly *Drosophila melanogaster* as a tractable model organism to study the underlying neuronal circuits that regulate foraging decisions, as well as what is known about brain structures that could support these computations.

1.1 Why is behavior important for understanding the brain

1.1.1 What is behavior

What does it mean when we say that an organism or system behaves in a certain way? Indeed, this is a complicated question, which many scientists have tried to answer (Bergner, 2011; Calhoun & El Hady, 2021). Here, I take an “agnostic approach” to the study of behavior — simply stating that behavior is defined by a sequence of dynamically changing, observable

1.1. WHY IS BEHAVIOR IMPORTANT FOR UNDERSTANDING THE BRAIN

variables, such as the movement of an arm for reaching an object (Shadmehr et al., 2010), or escaping a predatory danger (Evans et al., 2018; von Reyn et al., 2014). Observable means that we have ways of measuring and quantifying the behavioral variables. Interestingly, while many behaviors as defined above do not necessarily require a brain (Boussard et al., 2021; Calvo Garzón & Keijzer, 2011), it can be argued that the brain has evolved to generate, adapt and potentially optimize behaviors (Cisek & Hayden, 2022). Therefore, a deeper understanding of the evolved behaviors can help us understand how the brain implements its generation (Krakauer et al., 2017).

One way to better understand animal behavior is to identify its normative goal as to why certain aspects of behavior have evolved in a certain way. Animals ensure their survival by acting and reacting to their environments. As an embodied, physical system, animals therefore need to regulate their physiological states, which is referred to as homeostasis. Specifically, it refers to the ability of animals to maintain a stable internal environment despite changes in the external environment. This can involve physiological processes, such as regulating body temperature, or behavioral processes, such as finding shelter from the rain. Computationally, homeostasis is described through concepts from control theory involving negative feedback control. It suggests that animals' behavior is guided by internal goals or needs, and that animals compare their current state to their desired state in order to determine the best course of action. For example, an animal may be motivated to find food because it is hungry, and it will compare its current level of hunger to its desired level of fullness in order to determine the best way to obtain food.

However, feedback control has significant limitations. First, it is reactive, and as such, can only respond to perturbations that have already occurred, even if these are detrimental to the controlled system. Thus, in the context of nutrition, the animal must experience a lack thereof, which is detrimental to physiological systems, before mounting a regulatory response. Second, its responses are critically sensitive to the gain used for regulation (Sontag et al., 2013). While a high gain leads to fast responses

by the controller, it also renders the controller highly susceptible to sensory noise. Third, the time it takes for the controller to exert a response in the controlled variable provides an additional delay that could exacerbate the error (Richard, 2003). For example, appropriate food may not be immediately available, and foraging in a time of need uses up precious time and resources. Taken together, there is a speed-accuracy trade-off in how rapidly a feedback controller can compensate for errors in the controlled variable. In order to overcome these limitations of pure feedback control, control systems are often complemented by feed-forward mechanisms that are able to model and predict future states of the controlled variable (Carpenter, 2004; Sterling, 2012). This concept of feed-forward regulation is known as *allostasis*. This can involve physiological or behavioral changes, such as increased heart rate in response to stress based on predictive cues.

In order to achieve this normative goal, animals adapt to changes in their environment through adaptive behavior. As such, they are able to respond to the same external cues differently depending on context and internal state, and learn new associations over time. Examples of adaptive behavior include foraging for food, finding a mate, and protecting young. These behaviors help animals to find food, reproduce successfully, and avoid predators. Internal states or drives are important for understanding animal behavior because they help to explain why animals do what they do. These drives can be physiological, such as the need to eat or drink, or they can be psychological, such as the desire for social interaction or the need to explore. When an animal experiences a drive, such as hunger or thirst, it will be motivated to engage in behaviors that reduce the drive. For example, an animal that is hungry will be motivated to find food, and an animal that is thirsty will be motivated to find water.

One key aspect of behaviors is that some are mutually exclusive to others. For example, animals cannot simultaneously sleep and forage for food. As such, behavioral control by the brain likely does not only drive certain behaviors, but also suppresses other mutually exclusive ones (Cruz et al., 2022; Davis, 1979; Seeds et al., 2014). Furthermore, higher-order behaviors like foraging or courtship can be divided into smaller, subbehaviors that

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are performed sequentially or in parallel (Anderson, 2016; Pool et al., 2014). This hierarchical organization of behavior is one of the key properties of naturalistic behaviors and likely reflects hierarchical and modular structure of the underlying control system (Berman et al., 2016). Behavioral hierarchies are the ways in which animals organize their behavior into a series of actions or responses. These hierarchies can be simple or complex, and they may involve multiple behaviors that are triggered by specific stimuli. For example, a bird may first spot a worm, then approach it, peck at it, and finally eat it. Each of these behaviors is part of a hierarchical chain of actions that ultimately leads to the bird obtaining food.

All of these concepts are interconnected and help us to understand how animals think, act, and interact with their environments. Allostasis allows animals to adapt to changes in their environment, and adaptive behavior is one way in which animals respond to these changes. Drives motivate animals to engage in certain behaviors, and behavioral hierarchies help animals to organize their behavior in a logical and efficient way. By understanding all of these concepts, we can gain valuable insights into animal behavior and the ways in which animals think and act.

1.1.2 Quantifying behavior

The way how natural scientists tend to get a better understanding of the world is by quantifying variables which help them to discover underlying patterns that can finally form a mechanistic theory of the studied phenomena. Quantitative behavioral analysis is a relatively recent method of study in the field of animal behavior. The first systematic studies of animal behavior were conducted by Charles Darwin, Charles O. Whitman, and Oskar Heinroth in the 19th century (Darwin, 1872; Podos, 1994; Whitman, 1899), but it was not until the early 20th century that scientists began to develop more formal methods for measuring and analyzing animal behavior (Lorenz, 1965; Tinbergen, 1951; von Frisch et al., 1967). This involved the use of various techniques, such as behavioral experiments and observations, to study the actions and reactions of animals in controlled environments

(Morris et al., 1982; Tolman, 1948; von Holst, 1937). Over time, these methods have been refined and improved, allowing scientists to gain a better understanding of the underlying mechanisms of animal behavior and how it is influenced by various factors. Classically, low-dimensional descriptions of behaviors have been used to describe and quantify what the animal was doing. This included manual scoring of certain behavioral events or segments (Eibl-Eibesfeldt & Kramer, 1958), or automated setups that could track stereotyped or event-based, such as locomotor activity using beam crossings (Davis, 1957) or specifically for feeding automated dispensers (Gill et al., 1989).

More recently, computer vision and centroid tracking are two techniques that are commonly used in the study of animal behavior (Branson et al., 2009; Fontaine et al., 2008). Computer vision involves the use of specialized software and algorithms to analyze and interpret visual data, such as images and video footage, in order to identify and track specific objects or animals (Fig. 1.1A). Centroid tracking, on the other hand, is a method of measuring the movement of animals by tracking the position of their center of mass, or "centroid". By combining these two techniques, researchers can quantitatively measure and analyze the behavior of animals in a precise and objective manner. This can provide valuable insights into the behavior and movements of animals, and can be used to study a wide range of species, from insects (Branson et al., 2009; Crall et al., 2018) to mammals (de Chaumont et al., 2012). Measuring behavior directly by recording videos gives high spatial and temporal resolution, but also creates large and high-dimensional data to be analyzed. Simple tracking methods, such as color tracking or background subtraction allowed for fast centroid tracking when the background was static and the contrast between the moving animal and the background was strong enough. Only recently, advances in Machine Learning and Computer Vision have given rise to many powerful approaches to track many key points, like body parts, in a markerless way at high accuracy (Mathis et al., 2018; Pereira et al., 2022). This allowed for observing naturalistic behaviors that involve many moving joints or multiple animals, e.g. studying social behaviors. Furthermore, it

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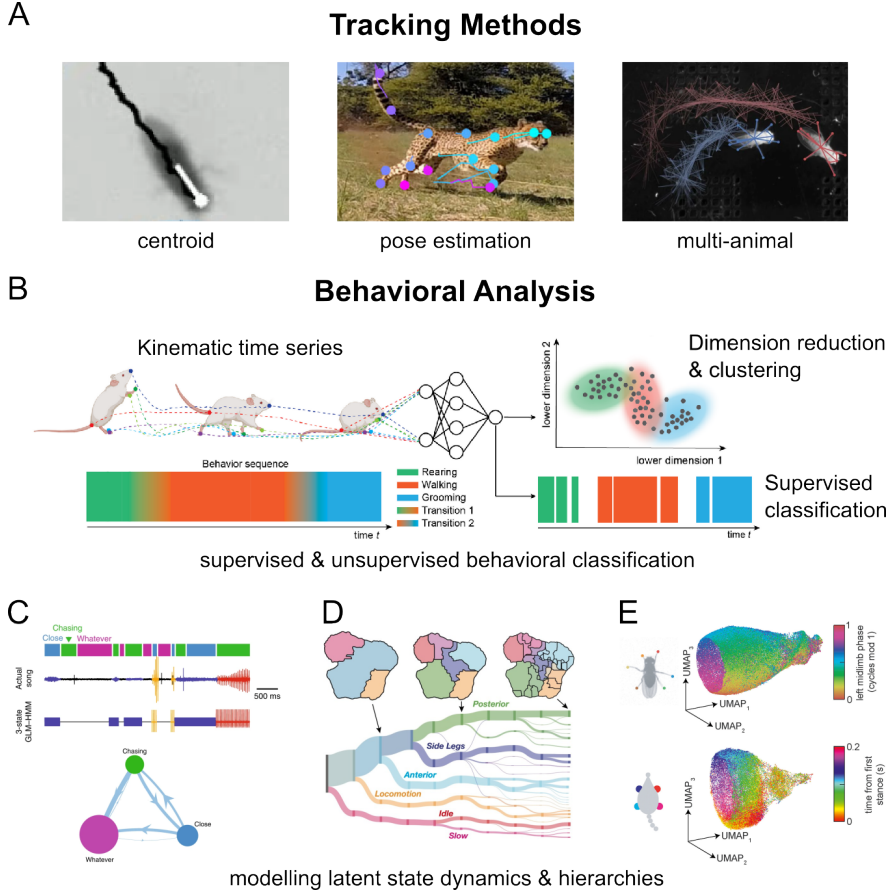


Figure 1.1: Methods for Quantitative Behavioral Analysis. (A) Tracking methods are applied to measure body centroids, multiple key points via pose estimation, and identifying multiple animals unambiguously. Images taken from Nath et al. (2019) and Pereira et al. (2022). (B) Measuring kinematic time series which describe a continuous sequence transitioning behavioral segments can be decoded using either unsupervised dimension reduction and clustering methods (learning from statistical properties of the data) or supervised classification, e.g. with neural networks or ensemble methods (learning from labeled data). Modified from Luxem et al. (2022). (C-E) Behavioral data can also be used to model and infer latent state dynamics and hierarchies. Images from Berman et al. (2016), Calhoun et al. (2019), and Gonçalves et al. (2022).

also improved high-throughput quantification in situations where simpler tracking methods were not sufficient, i.e. low contrast or variable dynamic backgrounds.

Besides tracking the location of body centroids and parts over time, machine learning methods have also been used for classifying and segmenting discrete behaviors (Fig. 1.1B), such as grooming or walking. Based on a supervised learning method, JAABA uses labeled data to infer predictive features from the multidimensional data obtained from tracking (Kabra et al., 2013). More recent work has focused on employing deep-learning architectures to solve behavioral classification and inference problems (Bohnslav et al., 2021). Unsupervised machine learning methods, such as clustering (Kanungo et al., 2002) and dimension reduction, like t-SNE (van der Maaten & Hinton, 2008), and UMAP (McInnes et al., 2018), can be used to analyze animal behavior and identify different behaviors or types of animals (Berman, 2018; Berman et al., 2016). These methods are useful for analyzing large datasets and finding patterns and relationships in the data, and have been shown to be effective at identifying different behaviors and distinguishing between groups of animals with different behavioral profiles. These techniques can provide objective and reproducible results, making them valuable tools for researchers studying animal behavior. Finally, behavioral analysis can also be used to reveal the structure and dynamics of latent states and underlying hierarchies. Calhoun et al. (2019) used a linear model together with a hidden Markov model to infer latent behavioral states during male courtship behavior in fruit flies (Fig. 1.1C). Unsupervised dimension reduction techniques have also been used to reveal hierarchical clusters in fruit fly behavior (Berman et al. (2016), see Fig. 1.1D). Similar methods were used by Gonçalves et al. (2022) to show conserved locomotor patterns in its low-dimensional, latent structure between flies and mice (Fig. 1.1E). These advances have also led to new insights into studying causal relationships between neuronal circuits and specific behaviors across different animal species (Fujiwara et al., 2022; Marques et al., 2020; Wimalasena et al., 2021).

1.1.3 The new age of high-throughput ethomics

As argued in the previous section, advances in hardware technology as well as software analysis tools have significantly improved the way to measure and quantify animal behavior. Not only have these new methods led to novel insights into the understanding of behavior, but also due to the standardization and automation of such methods, researchers are now able to scale up experiments to a level that has never been possible before. Namely, high-throughput screening of behaviors or as some defined it as high-throughput ethomics (Branson et al., 2009) – in reference to similar -omics approaches seen in molecular and cellular biology – has opened a door into systematically testing how behaviors are affected by certain manipulations, and even quantifying how behavioral variability arises in animals with the same genetic background (de Bivort et al., 2022; Werkhoven et al., 2021).

Smaller model organisms have often been favored for behavioral screening, due to easier handling and genetic targeting. For example in flies, silencing neuronal activity in genetically targeted, selective neuronal subset elucidated motor control programs of extending and retracting the proboscis for food intake (Pool & Scott, 2014; Shiu et al., 2022). Furthermore, a large-scale neurogenetic activation screen allowed the characterization of neuronal subsets or regions involved in locomotive control (Robie et al., 2017) as well as descending neurons projecting to the ventral nerve cord (Cande et al., 2018), the invertebrate analogue of the spinal cord. Besides flies, high-throughput ethomics and behavioral phenotyping have also been applied in other model organisms, ranging from *C. elegans* (Chalasani et al., 2007; Swierczek et al., 2011; Yemini et al., 2013) to mice (Weber et al., 2022; Wiltschko et al., 2020).

Challenges for behavioral screenings are manifold: Studying animal behavior on a large scale can generate huge amounts of data, and analyzing this data can be time-consuming and resource-intensive. Researchers must therefore develop efficient algorithms and data analysis techniques to effectively process and make sense of this data. Furthermore, handling time,

costs and resolution (spatial and temporal) of measurements are important factors to consider when designing behavioral screens, which recent technological work has impressively optimized (Alisch et al., 2018; Geissmann et al., 2017; Itskov et al., 2014). Another challenge is that animal behavior is complex and can be influenced by a wide range of factors, including genetics, environment, and previous experiences. This makes it difficult to predict how animals will behave in a given situation, and can make it challenging to identify and interpret patterns in behavioral data. Researchers must carefully control for these factors and use rigorous statistical methods to ensure that their findings are accurate and reliable.

Despite the many advances in high-throughput behavioral screening and the study of animal behavior, there are still many areas that remain largely unexplored. One such area is the study of higher-order behaviors, such as foraging. Foraging is a complex behavior that involves decision-making, problem-solving, and other cognitive abilities, and is an important aspect of animal behavior. However, to date, very few studies have used high-throughput behavioral screening to study foraging behavior in animals. This is likely due to the complexity of the behavior and the challenges of developing behavioral screens that can effectively test foraging ability. As a result, there is still much that we do not know about how animals make decisions and solve problems while foraging, and further research in this area could provide valuable insights into the brain and behavior.

1.2 Foraging

Evolution has led to incredible solutions to maximize fitness of animals via natural selection and genetic variation (Darwin, 1859; Shettleworth, 2009). All living organisms require and metabolize energy and nutrients from their environment. As such, a wide range of foraging behaviors have evolved for millions of years homo- and analogously in different species. Here, I define foraging as all behaviors and decision processes that allow an animal to gather food or information about it. Food or rather its nutrient content are not only sources of energy, but also important building blocks for the

animal to develop, grow and reproduce. Specifically, essential macronutrients – such as carbohydrates, proteins, and fats – are an important part of a healthy diet. Like with many essential variables, there is an optimal range of intake of a given nutrient. Specifically, overconsumption of proteins leads to reduced lifespan and negatively impacts fitness (Levine et al., 2014; Piper et al., 2011; Solon-Biet et al., 2019). Therefore, foraging provides solutions to the overarching, normative goal of nutrient homeostasis. Moreover, due to nutrient resources not necessarily being readily available, computations related to foraging cannot only act upon nutritional needs reactively, but rather combine reactive and predictive control in order to reach an optimal nutritional diet (Walker et al., 2017). Following this, I argue that the normative framework of foraging as defined above should be redefined as nutrient allostasis (Schulkin & Sterling, 2019; Sennesh et al., 2022).

Foraging observed in wild animals is highly complex and generally organized over multiple spatial and temporal scales (Amano & Katayama, 2009; Ehlers et al., 2021; Owen-Smith et al., 2010; Shipley, 2007; Stephens & Krebs, 1986). Furthermore, food resources within their habitat are scattered in heterogeneous and irregular patterns. As such, naturalistic foraging is not only difficult to study and quantify experimentally, but its high complexity makes it intractable for formulating a theory that could explain all aspects of foraging. I argue that due to these two reasons, different branches of studying foraging have been developed independently over time. In particular, there are three clearly distinct approaches which I will describe in the following sections. While these approaches have diverged from each other, there are parallel ideas and concepts that in the future will lead to a unified view of foraging.

1.2.1 Search and food navigation

The capabilities of many animal species in finding and navigating towards food have fascinated many ethologists since the late 19th and early 20th century (Fraenkel & Gunn, 1940; Lubbock, 1882; Turner, 1907). While many

navigational behaviors do not necessarily have to relate to foraging as defined earlier, it is trivial to understand that navigation is not only helpful but crucial in improving to search for and encounter food. Furthermore, once found navigational behaviors also guide the animal in returning back to known food locations. Finally, some animals have evolved navigational feats that include complex planning and internal maps, that allow foraging over large spatial – like fruit bats (Eliav et al., 2021; Tsoar et al., 2011) and wandering albatrosses (Jouventin & Weimerskirch, 1990) – or over large temporal scales, exemplified by food caching in squirrels (Robin & Jacobs, 2022) and birds (Payne et al., 2021). For the context of this thesis, I briefly describe the former two navigational types: search and returns.

How do animals find food? Searching for items is a highly relatable behavior, as many of us humans tend to forget about where exactly they left their keys or wallets. Similarly, animals have evolved multiple ways of finding important locations, such as food or nests. For example, hungry flies often perform a local search after encountering a drop of yeast or sugar – which they do so without apparent visual or chemosensory cues to guide them (Dethier, 1957; Kim & Dickinson, 2017). In fact, many insects (Müller & Wehner, 1988) and vertebrates (Etienne & Jeffery, 2004) perform such internal computations, called path integration, which allows them to return to earlier encountered locations by integrating oriented run distance vectors to compute a so-called global vector (Collett et al., 1998). Besides revisiting encountered locations, animals also exhibit strategies to efficiently search for food in a global sense (Bovet & Benhamou, 1988; Viswanathan et al., 1996). Internal states induced by starvation have shown to be modulators of such path integration-based searches (Dethier, 1957; Kim & Dickinson, 2017), and even other internal states, such as the mating state of the animal have shown to modulate its search pattern (Corrales-Carvajal et al., 2016).

Interestingly, many animals undergo transitions from local to global searches and vice versa (Bartumeus & Levin, 2008; Calhoun et al., 2014), in order to maximize information (Vergassola et al., 2007). It has also been shown that internal states are strong modulators of search strategies, where deprived animals tend to exhibit a more local search (Bell et al., 1985;

Corrales-Carvajal et al., 2016; López-Cruz et al., 2019). How search strategies are mechanistically implemented at a circuit level in the brain is largely unknown. Furthermore, while neuromodulation has been proposed as a possible mechanism for switching between global and local search (Calhoun et al., 2014), it is unclear whether internal states use a similar mechanism to regulate search strategies.

1.2.2 Optimal Foraging Theory

Life in highly dynamic and uncertain environments often presents complex, computational problems, and finding effective and efficient solutions ensures or improves the success of survival and reproduction. In mathematical terms, if a computational problem is described by one or multiple objective functions under certain constraints, there is an optimal solution which can be either analytically derived or numerically approximated (Boyd & Vandenberghe, 2015). Indeed, many real-life problems are optimization problems, for example legged and aquatic locomotion, in which both evolutionary processes as well as learning and adaptation during an individual's lifetime have optimized for factors such as speed, energy efficiency or stability (Alexander, 1989; Hildebrand & Hurley, 1985; Williams, 1999; Yang et al., 2016). Similarly, optimization methods can be applied to solve foraging problems. Specifically, Optimal Foraging Theory (OFT) is a normative approach to how an animal should make decisions in order to have a higher chance of survival (Pyke, 1984; Stephens & Krebs, 1986).

As animals forage for food or prey in an uncertain environment, encounters occur and an animal can either engage in feeding on this resource or catch the prey, or decide to ignore it for potentially better options. This is generally referred to as patch acceptance or engagement decisions. Similarly, as the animal engages in exploiting an encountered patch, the animal has to decide when to leave the patch in order to continue exploring the environment (patch leaving decision). This is especially crucial as patches are generally depleting during exploitation as well as over time. The Marginal Value Theorem (MVT) is a central theorem widely used in ecological models

of foraging behavior to describe the optimization of patch leaving decisions. First defined by (Charnov, 1976), it assumes that patch leaving decisions are governed by an optimal decision rule that balances the cumulative intake by diminishing returns (benefits) against the travel time (costs) between patches. Specifically, when the return rate (increments per time unit) or "marginal value" falls below the cumulative return divided by the sum of the average travel time between patches and the patch residence time, this optimal decision rule would instruct the animal to give up on the current patch to forage for potentially better options. As such, assuming constant travel speed, the tangent that crosses x-axis at the travel time between the spots and the curve of cumulative intake determines the time the animal will optimally spend exploiting the patch (Fig. 1.2).

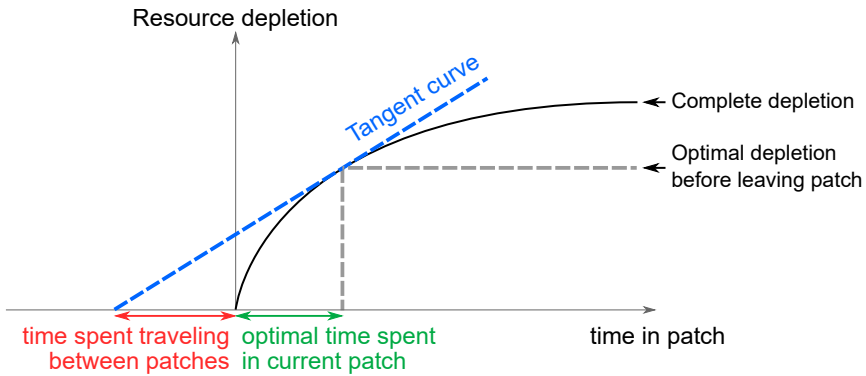


Figure 1.2: Scheme of the Marginal Value Theorem (MVT). As defined by Charnov (1976), the MVT describes the optimization of patch leaving problems based on relating patch depletion to patch density estimated by the travel time between patches. Mathematically, it relates the assumption of diminishing returns from exploiting the patch to the cost of time it takes to travel between patches. The optimal patch leaving time is when the instantaneous increase in patch intake (tangent of curve) falls below the cumulative patch intake per total time required for travel and exploitation (slope that crosses travel time between patches and the curve).

The MVT has been widely used in animal studies on foraging behavior, particularly in birds and small mammals (Cowie, 1977; Howell & Hartl, 1980; Kacelnik & El Mouden, 2013). For example, studies have shown that

chickadees (a type of small bird) will continue to forage in a patch until the net rate of resource intake in that patch is equal to the average net rate of resource intake across all patches (Krebs et al., 1974). This has been observed in experiments where chickadees were presented with a choice between two patches of food, and the patch with the higher net rate of resource intake was chosen more often. However, there are several critical deviations from the MVT that have been observed in animal studies. One deviation is the "satisficing" behavior of animals, where animals use a heuristic to not always maximize their net rate of resource intake but instead choose a patch that provides an adequate, though not optimal, rate of resource intake (Ward, 1992). Another deviation is the "patch residence time" effect, where animals will stay in a patch for a longer period of time if they have had a high rate of resource intake in that patch in the past (Cuthill et al., 1990).

There are also several general limitations of the MVT and OFT as a whole. One limitation is that OFT assumes that animals make foraging decisions based solely on resource intake, but in reality, animals often consider other factors such as predation risk and social interactions when making foraging decisions. Another limitation is that OFT assumes that resources are distributed uniformly in the environment, but in reality, resources are patchily and sparsely distributed, leading to deviations from the MVT. Finally, it is important to consider that besides uncertainty about nutrient availability, internal states that represent nutritional needs of the animal can modulate foraging strategies and decision-making (Corrales-Carvajal et al., 2016; Nonacs, 2001). For example, an animal with higher nutritional needs may be either more likely to take risks to obtain food during exploration (Caraco et al., 1980; Stephens, 1981) or increase exploitative decisions once an appropriate enough resource is found (Corrales-Carvajal et al., 2016; Leblond et al., 2011; Levy et al., 2013; Wunderle et al., 1987). Therefore, taking these variables into account is important for developing an accurate quantitative theory of foraging behavior.

1.2.3 Exploration-exploitation trade-offs & cognition

Animals often encounter a dilemma when they discover a resource, such as a food patch: should they take advantage of it right away, or continue searching for potentially better options? Should the grizzly bear go for the nearby fish or explore further to discover the lake with larger ones (Fig. 1.3)? This dilemma is known as the exploration-exploitation trade-off (Sutton & Barto, 1998) and has been



Figure 1.3: Illustrating the exploration-exploitation trade-off. Modified from Calhoun and Hayden (2015).

extensively studied in fields such as behavioral ecology, as well as computational and systems neuroscience. It is believed to be a key factor in decision-making and risk assessment (Addicott et al., 2017; Dall et al., 2005). Many animals have been found to adapt their behavior in order to effectively navigate this trade-off (Corrales-Carvajal et al., 2016; Kacelnik & El Mouden, 2013), even when faced with additional challenges such as the need for multiple or unpredictable nutrients, the presence of competitors or predators, and other risks. Systems neuroscientists tried to study the exploration-exploitation trade-off by “stripping down” decision variables to discrete and controlled options, which allowed them to test hypotheses in controlled experimental setups in the laboratory. The idea behind this reduction is to reduce behavioral & environmental variability, and specifically to control for and to manipulate variables relevant to the foraging decision, such as stimuli or rewards.

In vertebrates, cortical and specifically prefrontal brain regions have been shown to be involved in value-based decision-making as well as cognitive functions, such as planning and short-term memory (Goldman-Rakic, 1988; Rushworth et al., 2011). One key cortical region in vertebrates involved in foraging decision-making is the Anterior Cingulate Cortex (ACC). The

ACC is a part of the prefrontal cortex, which is responsible for higher-order cognitive processes such as decision-making, attention, and motivation. Studies of the ACC in animals have shown that it is involved in the evaluation of the risks and rewards of different foraging strategies, as well as the selection of the most appropriate behavior (Hayden et al., 2011; Kolling et al., 2012). One key concept in the study of foraging behavior is the exploration-exploitation trade-off. This refers to the tension between the need to explore new areas and resources in search of food, and the need to exploit known sources of food in order to maximize energy intake. Animals must strike a balance between these two strategies in order to optimize their foraging success. Studies of the ACC in primates have shown that it plays a key role in the exploration-exploitation trade-off (Blanchard & Hayden, 2014; Hayden et al., 2011; Kolling et al., 2012). Additionally, neuroimaging studies of the ACC in rats have shown that it is active when the animals are making foraging decisions, and that its activity is modulated by the availability and predictability of food. In particular, the ACC exhibit dynamic activity changes between segments when the animals are exploring new areas and resources and when they are exploiting known sources of food (Li et al., 2012). However, recent evidence from inactivating ACC neuronal activity in rats showed that this region is not required for performing optimal patch leaving decisions, and that the activity in the ACC represents other foraging decision variables (Kane et al., 2022). Interestingly, the ACC receives inputs from the locus coeruleus in the brainstem, known for norepinephrinergic control of arousal and convergence of hunger and thirst signals (Gong et al., 2020). Furthermore, its output projections connect to the striatum, which could facilitate the initiation and sustenance of foraging behaviors (Devinsky et al., 1995). However, whether this anatomical connectivity is indeed involved in naturalistic foraging, and how the ACC implements these computations mechanistically remains to be discovered. This highlights that while vertebrate brain regions important for guiding foraging decisions have been identified, their underlying mechanistic computations are yet unknown.

1.2.4 An integrative approach to foraging theory

While ecological studies focused on observing foraging behavior in naturalistic environments, such studies largely lacked quantitative accuracy and controlled conditions leading to large behavioral variability. As such, many studies reported suboptimal foraging decisions on one hand either argue to adjust MVT by additional parameters or on the other hand argue for the MVT to be invalid. Critically, MVT and optimal foraging theory in general have many assumptions that may be invalid in the context of naturalistic foraging as I described above. As such, this approach has led to relatively low yield of explainability of the wide variety of complex foraging decision-making observed in animals. In contrast, systems neuroscientists reduced foraging decisions to low-dimensional decision-making (e.g., two-alternative forced-choice tasks) in a controlled laboratory environment in order to reduce variability. This helped to gain insights into the neurobiological mechanisms of decision-making, it however does not describe how such decisions are performed in a natural environment, and what are the ecological and evolutionary forces that shaped these processes (Krakauer et al., 2017; Mobbs et al., 2018). It remains an open question whether the neural mechanisms for trained behavior are recruited for decisions made in natural settings.

In addition to analyzing naturalistic foraging behaviors in more detail using quantitative analysis, an integrative theory of foraging must also address how internal state which represent physiological needs of the animal are integrated and translated into foraging computations and decision-making (Nonacs, 2001). Hunger and satiety states dynamically modulate foraging decisions in a resource-specific manner (Corrales-Carvajal et al., 2016), and as such foraging theories must include these variables into their model formulations. Furthermore, the interactions of different internal states, such as nutrient deprivation, thirst, and mating, require a computational mechanism that organizes foraging for such mutually exclusive resources, e.g., a hierarchy of needs (Maslow, 1943). How such multitudes of internal states and models are then matched to the external availability of

resources adds further complexity to the cognitive computations that maximize foraging outcomes. Therefore, an integrative approach as described in this section is needed for a better understanding of how the brain performs cognitive computations to generate naturalistic foraging behaviors.

1.3 *Drosophila* as a Model for Studying Complex Behaviors

Systems neuroscience aims at an understanding of mechanistic and causal explanations of how the brain generates complex and adaptive behavior. Key to this is the ability to record and manipulate processes in the brain on a circuit, cellular and molecular level. The fruit fly *Drosophila melanogaster* has emerged as a powerful model organism in systems neuroscience (Kazama, 2015). This emergence is due to three major reasons: First, the fast developmental cycle and decoding of its genome (Adams et al., 2000) led to high genetic tractability. The GAL4/UAS system (Brand & Perrimon, 1993) has been developed to drive gene expression in a cell-targeted manner. This method allows to genetically express exogenous ion channels for neuronal activity perturbations (Klapoetke et al., 2014), or calcium reporters for measuring neuronal activity using confocal microscopy (Tian et al., 2009). Secondly, the fly brain is stereotyped and contains around 100000 neurons making it a simple enough system to study. This reduced complexity has made it feasible to target individual neurons through transgenic lines which are based on random insertions. For example, the Janelia Research Campus has created a library of more than 7000 GAL4 driver lines (Jenett et al., 2012), which have stereotyped expression in specific neurons¹ and are widely available through maintaining stock centers². This allows for systematic screening of specific neurons and neuronal populations by means of manipulating or recording neuronal activity. Thirdly, while insects are widely seen as behaving in simple perception-action cycles, they actually perform a wide

¹Flylight website: <https://www.janelia.org/project-team/flylight>

²e.g., Bloomington Drosophila Stock Center, <https://bdsc.indiana.edu/>

variety of complex and adaptive behaviors (Kinoshita & Homberg, 2017; Tastekin et al., 2018; Yamamoto et al., 2014).

1.3.1 Food search and foraging in *Drosophila*

As many might attest from their own experience with encountering fruit flies in their kitchen, fruit flies have remarkable food searching and foraging abilities. Indeed, their navigational abilities to locate food have been shown in an experiment, where marked *Drosophila pseudoobscura*, later also *Drosophila melanogaster*, have been released in a barren desert without apparent landmarks and have been found on feeders tens of kilometers from the release site just a few hours after (Coyne et al. (1982) and Jones et al. (1981), see review in Dickinson (2014)). Olfactory and visual senses are driving most of such long-range food search (Giraldo et al., 2018; Pang et al., 2018; van Breugel & Dickinson, 2014). During long-range food foraging, fruit flies largely travel by flying. As they encounter an appropriate food substrate, they land and start searching more locally while walking across the substrate. This transition from flying to walking is not only more energy efficient (Candy et al., 1997), but also allows for further sensing through leg gustatory receptors as well as the proboscis.

Walking *Drosophila* exhibit intricate search patterns and trajectories to locate and move towards food sources. Local searches around found food resources likely based on a combination of path integration and local chemical cues allow flies to return back to already visited food patches (Kim & Dickinson, 2017; Titova et al., 2022). Internal states, induced by starvation (Dethier, 1957; Kim & Dickinson, 2017), protein deprivation and mating state (Corrales-Carvajal et al., 2016) are strong modulators of these search patterns. Generally, needs induce more local exploration patterns that are characterized by increased turning and an overall smaller area covered. Likewise, the flies' engagement with a food resource also requires the evaluation of nutritional contents with respect to the nutritional needs. For example, protein-deprived flies are more likely to engage in feeding on protein-rich yeast patches and less likely to leave upon engagement,

while these decision variables are unaffected for carbohydrate-rich sucrose patches (Corrales-Carvajal et al., 2016).

1.3.2 Food sensing in *Drosophila*

As argued in the last section, chemosensors and chemosensory neuronal processing play a pivotal role in locating and evaluating nutrient resources during fly foraging. Chemosensory receptors can be grouped into three classes: Gustatory Receptors (GRs) (Scott et al., 2001), Olfactory Receptors (ORs) (Clyne et al., 1999), Ionotropic Receptors (IRs) (Benton et al., 2009). Gustatory processing is mediated through GRs and IRs on the labellum (i.e., sensilla and taste pegs), as well as post-ingestive pharyngeal sensilla, together with further receptors on all legs and wings. Olfaction is largely done through ORs and IRs on the antennae as well as maxillary palps. Note, that further chemoreceptors can be found internally in the brain, gut and reproductive organs.

Olfactory Sensory Neurons (OSNs) that express the same type of receptor are connected to the same spherical structure called a glomerulus in the antennal lobe, the first processing center for olfactory information in the brain. Depending on the composition of the involved ORs and IRs, odor discrimination is achieved through this glomerular segmentation (Fishilevich & Vosshall, 2005; Münch & Galizia, 2016) as well as lateral inhibition (Wilson, 2011; Wilson et al., 2004). Within the antennal lobe, these sensory neurons interact with local interneurons and projection neurons, which then transmit the olfactory information to higher brain regions such as the mushroom bodies and lateral horn.

Gustatory Sensory Neurons (GSNs) are responsible for detecting and discriminating between different tastes, including sweet, sour, salty, and bitter (Scott, 2018). They do this by expressing different combinations of GR and IRs, which are proteins that bind to specific chemicals and trigger a response in the neuron. When a gustatory sensory neuron is stimulated by a chemical that it can recognize, it generates an electrical signal, or action potential, which travels along the neuron's axon to the brain, where it

is interpreted by the gustatory centers of the Subesophageal Zone (SEZ) (Marella et al., 2006). In addition to detecting tastes, gustatory sensory neurons in insects also play a role in detecting and identifying potential food sources. For example, honey bees use gustatory sensory neurons to detect the presence of nectar in flowers, while predatory insects such as praying mantises use gustatory sensory neurons to detect the presence of prey. Downstream processing of second-order taste neurons in SEZ have been recently identified and studied to test how their processing and computations of taste signals is translated into feeding behavior (Münch et al., 2022; Shiu et al., 2022; Sterne et al., 2021; Yapici et al., 2016). Indeed, neurons involved in the motor control and commands for feeding, both regarding controlling the proboscis and inhibiting locomotion have been identified in the SEZ (Flood et al., 2013; McKellar, 2016; Pool et al., 2014).

Most of what is known about internal state relates to gain modulation of chemosensory responses (Marella et al., 2012; Root et al., 2011; Steck et al., 2018; Youn et al., 2018). How deeper processing stages may integrate internal states has been recently studied on a neuropil-wide level (Münch et al., 2022; Shiu et al., 2022), but how higher-order, potentially cognitive circuits may integrate internal states remains largely unknown. Furthermore, while neuromodulatory and -peptidergic signaling has been identified to regulate appetite, feeding motivation, satiety and nutritional homeostasis (Al-Anzi et al., 2010; Audsley & Weaver, 2009; Chen et al., 2016; Kim, Su, et al., 2017). However, the connections between SEZ circuits and peptidergic neurons in the brain are not well established.

1.3.3 Higher-order brain areas in *Drosophila*

The complex and cognitive may require involving higher-order brain areas, that receive and integrate not only multisensory input, but also internal states that represent metabolic and physiological needs (Chittka, 2017; Haberkern & Jayaraman, 2016; Shettleworth, 2009). Furthermore, cognitive functions often involve internally generated signals that relate to internal

and external representations and models that help to form and control appropriate and adaptive action selection and behavioral policies. As foraging is related to such higher-level cognitive computations, it is important to review *Drosophila* higher-order brain areas, that could support these functions.

The Central Complex (CX) is a highly conserved, unpaired neuropil in the insect brain (Pfeiffer & Homberg, 2014). It consists of four substructures: The Protocerebral Bridge (PB), the Fan-shaped Body (FB), the Ellipsoid Body (EB), and the Noduli (NO) (Hanesch et al., 1989; Wolff et al., 2015). A large body of work has linked the CX to several complex behaviors, ranging from gap-crossing (Triphan et al., 2010) to orientation behaviors (Neuser et al., 2008). Furthermore, specific neurons that send projections to the EB have shown to carry a visually evoked representation of orientation, like a compass, which is maintained ideothetically as visual input is removed (Seelig & Jayaraman, 2015). More recently, neurons that project to a specific part in the fan-shaped body have shown to integrate information about the orientation with information about the forward speed to form a representation of the travel direction (Hulse et al., 2021; Lu et al., 2022; Lyu et al., 2022). Interestingly, this requires complex vector computations that are achieved by the remarkable anatomical properties and connectivity of these neurons. Furthermore, the CX also sends projections to downstream neuropils, such as the Lateral Accessory Lobes (LAL) that connect to descending neurons that control steering and locomotion behaviors (Hulse et al., 2021; Rayshubskiy et al., 2020). As such, the CX sits at the interface of sensory processing and behavioral control, where external information is transformed into decisions about what to do next. To ensure that appropriate behaviors are carried out in the right context, the central complex also receives and controls metabolic internal states related to sleep (Donlea et al., 2018), and feeding (Musso et al., 2021; Park et al., 2016; Sareen et al., 2021). Furthermore, it has been shown to be required for cognitive tasks that involve memory formation, in particular visual place memory (Ofstad et al., 2011). Thus, the CX presents a tractable substrate for integrating sensory and internal signals (e.g., hunger) to drive appropriate decisions

and behaviors during foraging. However, the neuronal circuitry and the underlying computations performed for solving complex explore-exploit trade-offs remains unknown.

The Mushroom Bodies (MB) is another widely studied, higher-order neuropil in the insect brain. Located in the superior protocerebrum, they have been extensively studied in the fruit fly, *Drosophila melanogaster*, due to their involvement in a range of cognitive processes, including memory, learning, and decision-making. Traditionally, the mushroom bodies have been studied in the context of the olfactory processing pathways and receive inputs from so-called projection neurons that carry information about the identity of odors. These form synapses with the so-called Kenyon cells, a population of thousands of neurons that are able to form associating between sparsely encoded odors and reinforcement signals (reward or punishment). In addition to their role in the detection and evaluation of food, the mushroom bodies are also involved in the learning and memory of food-related information. For example, studies have shown that the mushroom bodies are involved in the formation of long-term memories of food sources, as well as the modulation of these memories based on the availability and predictability of food. This allows the fly to remember and use past experiences to guide its foraging behavior. Several Mushroom Body Output Neurons (MBONs) have been shown to be required and sufficient for food search (Tsao et al., 2018). Additionally, dopaminergic neurons projecting to specific compartments of the MB have been shown to affect feeding (Lan-dayan et al., 2018). Furthermore, MB and specifically MBONs have also been implicated in odor navigation and send projections to the CX (Hulse et al., 2021; Matheson et al., 2022).

The Superior Neuropils (SNP) notably receives inputs and outputs from both CX and MB, and other higher-order processing areas (Hulse et al., 2021; Li et al., 2020). The so-called SAG neurons is an ascending neuron that sends projections to the superior neuropils. Furthermore, the pars intercerebralis, which is the anterior dorsal part of the SNP contains several neurosecretory cells that have been involved in the regulation of feeding. Another set of four neurons that release SIFamide modulates processing in

1.3. *DROSOPHILA* AS A MODEL FOR STUDYING COMPLEX BEHAVIORS

olfactory centers to promote appetitive behaviors (Martelli et al., 2017).

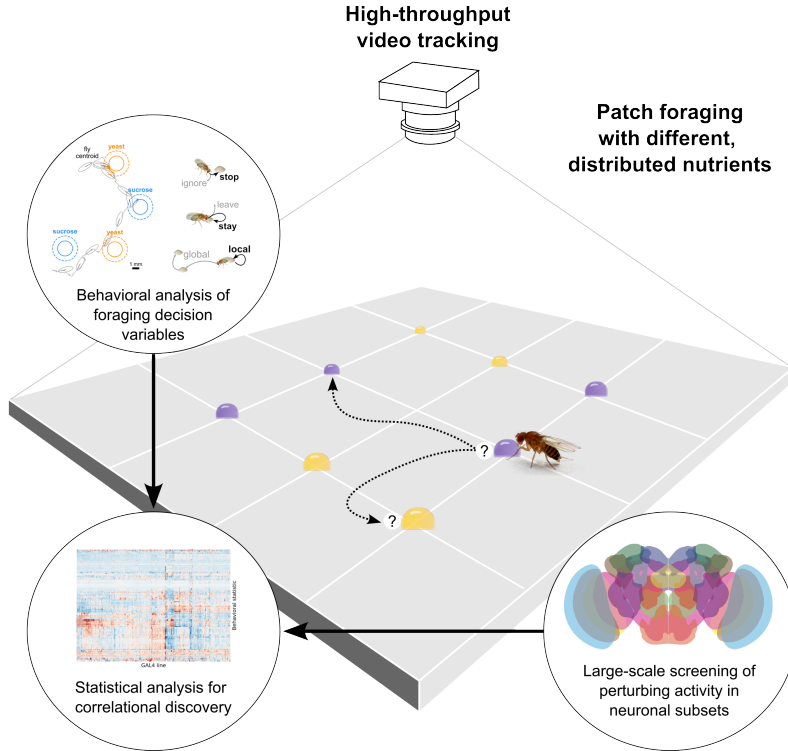


Figure 1.4: A proposed approach to study neuronal circuits involved in foraging behavior in natural environments. This thesis proposes to use high-throughput video tracking to record trajectories of an individual fly to analyze parameters that quantify patch foraging decisions and exploration of distributed nutrients. Using this behavioral analysis, we perform a large-scale screening of manipulating the activity of different neuronal subsets in order to discover statistical relationships between the manipulations, and as such the importance of specific neuronal subset, for controlling specific behavioral parameters related to foraging.

Here, I have outlined how higher-order brain areas in the *Drosophila* brain could potentially support and facilitate foraging behaviors. However, the underlying circuit elements and neuronal subsets that implement these cognitive computations remain unknown. An entry point into the study of

such elements would not only elucidate how internal state-dependent foraging decisions are regulated in the *Drosophila* brain, but could also serve as basis for cognitive algorithms to solve complex foraging problems that may be conserved across different phyla. Therefore, in this thesis I set out to study neuronal elements involved in foraging behavior in natural environments by using a proposed approach illustrated in Fig. 1.4. At its core, it combines high-throughput and high-resolution behavioral analysis of freely foraging flies in a naturalistic environment with distributed nutrient patches with large-scale genetically targeted perturbation of the activity in different neuronal subsets. Through this large-scale screening, we can apply statistical methods to correlate and discover specific neuronal subsets that are involved in certain behavioral aspects during foraging.

1.4 Aims and Structure of the Thesis

In this thesis, I aimed at identifying neural circuits involved in exploratory foraging and nutrient decision-making in *Drosophila melanogaster*. Furthermore, we investigated how internal states modulate neural activity in these circuits. The thesis is divided into three results chapters followed by a general discussion which concluded the results as a whole and discusses how the implications of this thesis fit and extend the understanding of how foraging behaviors are generated by the brain.

Specifically, the results chapters are structured as follows:

Chapter 2: In order to study the neural circuit logic of feeding decisions and nutrient choice, we created a closed-loop, optogenetic system called *optoPAD* which is aimed at flexible and robust high-throughput experimental quantification of feeding microstructure combined with closed-loop control of a high-power RGBA LED for either activation or inactivation of targeted neuronal populations depending on the opsins genetically encoded.

Chapter 3: We further extended a foraging assay for modular high-throughput video tracking of individual flies exploring an arena with distributed

yeast and sucrose patches to investigate which neuronal subsets are involved in different decision parameters and behavioral aspects during nutrient foraging. Specifically, we employed a large-scale neurogenetic silencing screening to test more than 500 different driver lines which target different neuronal subsets in the fly brain – to show what effect the neuronal silencing of these subsets has on the resulting foraging behavior.

Chapter 4: Based on this silencing screen, we identified a neuronal population which is key in the decision to transition from exploration to exploitation in order to initiate feeding at a food patch. We showed that the neurons involved project to the Dorsal Fan-shaped Body (dFB), which is part of the central complex – a series of neuropils shown to be involved in orientation and higher-order sensorimotor processing. Strikingly, we further showed that a single pair of neurons, a subset of the dFB population, is required for increasing the exploratory drive in the decision to stop at food patches. Furthermore, the activity in these neurons is modulated by the protein state of the fly, suggesting that these neurons could regulate the decision to stop at food patch by integrating internal state signals with sensory signals related to nutrient availability. Finally, we showed that the activity in dFB neurons is required for adapting foraging decisions for patch engagement to resource density as predicted by the MVT, but not for patch leaving decisions.

Chapter 5: In the last chapter of this thesis, I discussed the conclusions and implications of the results presented in this thesis in a wider context. Specifically, I aimed at circling back to the themes introduced here, and discussed how the presented thesis addressed them. Finally, I discussed open questions from these studies and how to tackle these in future work.

optoPAD, A CLOSED-LOOP
OPTOGENETICS SYSTEM TO STUDY THE
CIRCUIT BASIS OF FEEDING
BEHAVIORS

2.1 Contributions

This chapter was published in *eLife* (Moreira et al., 2019), in which I (DG) co-first authored together with JM Moreira (JMM) and PM Itskov (PMI). The other co-authors on this publication were Célia Baltazar (CB), Kathrin Steck (KS), Ibrahim Tastekin (IT), Samuel J Walker (SJW), and Carlos Ribeiro (CR, corresponding author).

Detailed contributions are as follows:

	JMM*	PMI*	DG*	CB	KS	IT	SJW	CR
Conceptualization		■	■					■
Data curation	■	■	■		■			
Formal analysis	■	■	■		■	■	■	■
Investigation	■		■	■	■	■	■	
Methodology	■	■	■		■		■	■
Project Administration			■					■
Resources	■	■						
Software	■	■	■					
Supervision		■	■			■		■
Validation	■		■	■	■	■	■	
Visualization	■	■	■		■			■
Writing—Original Draft		■	■					■
Writing—Reviewing & Editing	■	■	■	■	■	■	■	■
Funding acquisition								■

* these authors contributed equally

2.2 Summary

The regulation of feeding plays a key role in determining the fitness of animals through its impact on nutrition. Elucidating the circuit basis of feeding and related behaviors is an important goal in neuroscience. We recently used a system for closed-loop optogenetic manipulation of neurons contingent on the feeding behavior of *Drosophila* to dissect the impact of a specific subset of taste neurons on yeast feeding. Here, we describe the development and validation of this system, which we term the *optoPAD*. We use the *optoPAD* to induce appetitive and aversive effects on feeding by activating or inhibiting gustatory neurons in closed-loop - effectively creating virtual taste realities. The use of optogenetics allowed us to vary the dynamics and probability of stimulation in single flies and assess the impact on feeding behavior quantitatively and with high throughput. These data demonstrate that the *optoPAD* is a powerful tool to dissect the circuit basis of feeding behavior, allowing the efficient implementation of sophisticated behavioral paradigms to study the mechanistic basis of animals' adaptation to dynamic environments.

2.3 Introduction

The ability to experimentally manipulate the activity of neurons with cellular resolution has revolutionized our understanding of how circuits generate behavior (Luo et al., 2018). This ability has gone hand in hand with an improvement in technologies allowing for the automated and quantitative analysis of behavior (Anderson & Perona, 2014; Branson et al., 2009; Brown & de Bivort, 2018; Calhoun & Murthy, 2017; Egnor & Branson, 2016; Mathis et al., 2018; Wiltshcko et al., 2015). While most methods for analyzing behavior quantitatively rely on video recordings, alternative methods play important roles in neuroscience research (Davis, 1973; McLean & Kinsey, 1964). Such approaches have become especially important to study feeding in *Drosophila melanogaster* (Murphy et al., 2017; Ro et al., 2014; Yapici et al., 2016). We have recently established a new method for quantifying feeding

behavior in the fly which relies on capacitance measurements (Itskov et al., 2014). The *flyPAD* allows automated, high-throughput, quantitative analysis of feeding behavior with high temporal resolution. This high temporal resolution enables the dissection of feeding behavior at the level of the motor pattern and the microstructure of feeding. Using this framework, we have shown that different circuit and molecular mechanisms impinge on food intake by modulating two key variables of the feeding microstructure: the probability of initiating a feeding burst and the length of feeding bursts (Corrales-Carvajal et al., 2016; Itskov et al., 2014; Steck et al., 2018; Walker et al., 2015).

At the neural circuit level, gustation plays a pivotal role in regulating food intake. Classically, gustation has been shown to allow animals to both detect suitable food sources as well as to reject harmful foods (Dethier, 1976; Jaeger et al., 2018; Scott, 2018; Yarmolinsky et al., 2009). A key feature of gustation when compared to olfaction or audition is that it requires the physical interaction of taste organs with the sampled substrate. As such, gustatory information is outstandingly contextual and depends critically on the behavior of the animal as it actively explores substrates. Ideally, therefore, manipulations of circuit function during feeding should be tightly coupled to the ongoing behavior. Techniques allowing for the real-time analysis of behavior have enabled neuroscientists to trigger neuronal manipulation specifically when the animal is performing specific behaviors. We have previously used such an approach to show that in *D. melanogaster*, taste peg sensory neurons specifically control the length of yeast feeding bursts (Steck et al., 2018).

Here, we describe the design and implementation of such a high-throughput system allowing optogenetic manipulation of neurons in *Drosophila* contingent on the feeding behavior of the fly: the *optoPAD*. We show that the *optoPAD* system allows for the specific, bidirectional manipulation of sweet and bitter neurons thereby triggering or suppressing appetitive or aversive feeding behaviors. We furthermore demonstrate the ability of our system to implement dynamic as well as probabilistic stimulation protocols. These protocols significantly expand the scope of the *optoPAD*, allowing

for complex experimental designs. These additions significantly extend the toolset available to study complex behaviors in high throughput in *Drosophila*.

2.4 Results

2.4.1 The optoPAD system

We set out to develop a system allowing the optogenetic manipulation of circuit activity in *Drosophila* conditional on specific aspects of its ongoing feeding behavior (Fig. 2.1A). To develop such a device, it is essential to be able to measure specific parameters of feeding behavior in real time. We previously developed the flyPAD system, which reliably measures feeding behavior in high throughput using capacitive proximity sensors from two food sources (Itskov et al., 2014). To allow for optogenetic manipulation of neurons, we designed an LED board housing a high-power multicolor LED (four colors), as well as Metal-Oxide-Semiconductor Field-Effect Transistor (MOSFET) gates and current limiting resistors, that fits on top of the flyPAD arenas (Fig. 2.1B).

In the original flyPAD system, the relevant aspects of feeding behavior are extracted by offline processing of the capacitance signal after the behavioral experiment. We took advantage of the low complexity of the capacitance signal paired with the real-time data processing capacities of the Bonsai data stream processing language (Lopes et al., 2015) to analyze feeding behavior of flies in real time. Bonsai is a powerful visual programming framework especially designed for the acquisition and on-line processing of complex data streams such as those generated during behavioral experiments. We focused on using Bonsai to extract the periods in which the fly was actively interacting with food ('activity bouts'), as we previously showed that the total time of these interactions correlated well with total food intake (Itskov et al., 2014). After a series of simple signal processing steps (Fig. 2.1C), we could obtain the onset time of activity bouts in real time. In order to precisely control the LED illumination,

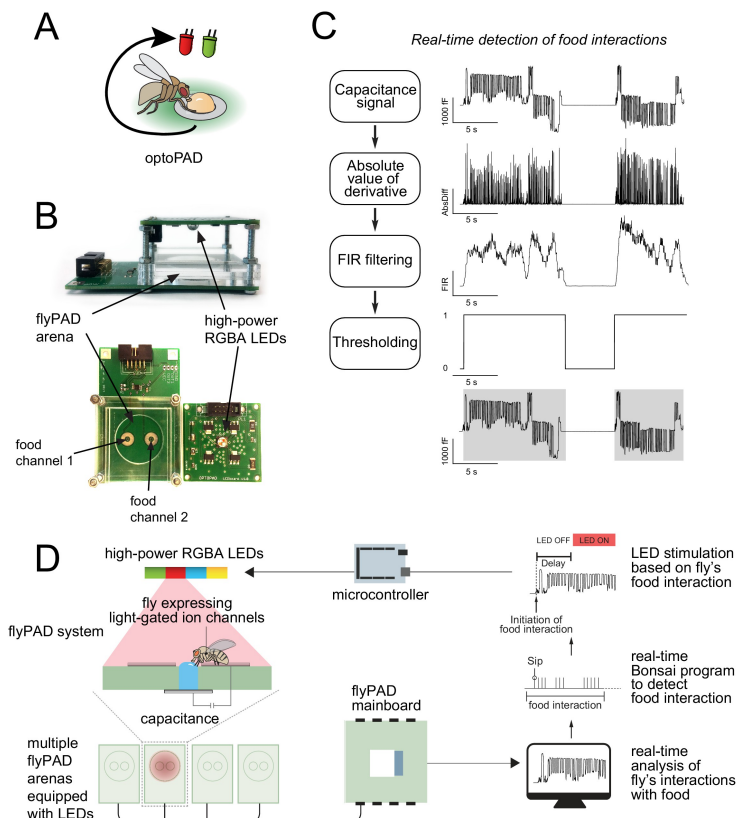


Figure 2.1: The optoPAD system. (A) Concept for the use of closed-loop capacitance measurement of feeding with optogenetic manipulation of neurons in behaving flies. The interaction of the fly with the food source triggers the activation of the LED. (B) Overview of the components of the optoPAD, the flyPAD arena and the high-power RGBA LEDs. (C) Algorithm for real-time detection of food interactions. Extracted food interaction bouts (activity bouts) are shaded in gray. (D) Schematic overview of the optoPAD experimental dataflow.

we designed a control breakout board (a standard 32-arena flyPAD system requires three of these boards), each of which uses one microcontroller (Arduino Mega) which operates as an IO device controlling the gates of the MOSFETs. This included a power distribution circuit to distribute power to the 128 channels of the LEDs ($32 \text{ LEDs} \times 4 \text{ colors}$, blueprints available

at <https://github.com/ribeiro-lab/optoPAD-hardware>). The Arduino Mega runs a standard Firmata software, allowing it to function as a digital general-purpose input/output (GPIO) board from within the Bonsai environment. This allowed us to write all the controlling software, including the finite state machine controlling the experiments for all of the 64 channels of the flyPAD, in Bonsai.

The resulting optoPAD system has the following dataflow (Fig. 2.1D): The flyPAD system uses a capacitance-to-digital converter to measure the interaction of the fly with the food. The capacitance information is sent to a computer via the flyPAD mainboard, and the real-time Bonsai algorithm detects when the fly starts to interact with one of the food electrodes. The software sends a signal to one of the digital pins of the microcontroller, which in turn controls the opening of the MOSFET on the LED board, leading to the illumination of a predefined LED color channel and thereby corresponding activation/inactivation of genetically identified neurons.

Importantly, five parameters of LED activation can be easily controlled using this software: which food source triggers LED activation; which LED color is activated; the delay between the detection of the initiation of an interaction with the food and light onset; the duration the LED remains on during the light stimulation; and the probability with which an onset of food interaction leads to LED illumination. Furthermore, the user can set how many times the light is triggered, can use a script which allows the delivery of the light after an activity bout is terminated, and can choose to deliver the light in an open-loop mode using predefined set intervals.

In order to ensure that the LED can be rapidly activated following the initiation of food interactions, we measured the latency of this system. The latency is defined as the time between food contact and LED illumination (with the 'delay' parameter set to 0). Our measurements revealed that it takes between 50 and 120 ms for the LED to be activated upon contact with the food. The latency is likely to originate in delays inherent to the serial communication bus, while the range of the latency is likely due to the buffer length of the flyPAD's system communication. Importantly, this latency is very short relative to the time it takes the fly to initiate a feeding burst after

touching the food. This delay is quite reproducibly in the range of 400 ms (Fig. 2.2). It is therefore very unlikely that the fly starts feeding before the LED is activated. By setting the delay parameter to 250 ms one can even time the light onset to coincide even better with feeding onset. Thus, our system is fast enough to 'close the loop' within the duration of a single sip.

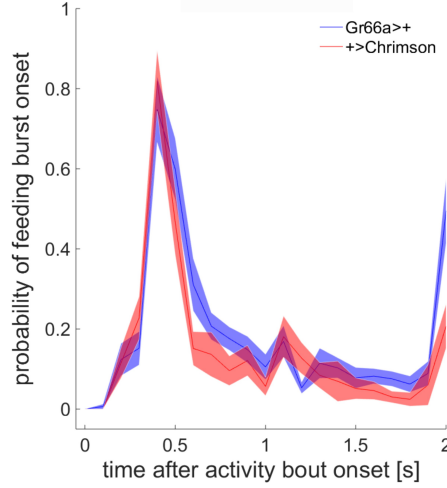


Figure 2.2: Quantification of the probability of the occurrence of a feeding burst onset depending on the time after activity bout onset. Bins are 0.1 s wide and probabilities are plotted for two different genetic background controls feeding on yeast, on an optoPAD channel triggering light activation.

To test how well the online activity bout detector works, we compared the performance of the new online algorithm with the previously validated offline algorithm (Itskov et al., 2014). We wrote a MATLAB script that replicates the online Bonsai workflow and classified each sample of a raw capacitance trace as belonging to an activity bout or not and compared this classification to the already validated flyPAD offline detector (Itskov et al., 2014). We then performed an ROC analysis to confirm the accuracy of the online detector. The detection of activity bouts by the online method correctly identified 91.5% of the capacitance trace samples as belonging to an activity bout (8.5% false negatives) and misclassified 1.6% of the samples

as activity bouts (false positives). These data show that the online activity bout detector operates within the range of accuracy observed for state-of-the-art offline methods (Itskov et al., 2014).

2.4.2 Optogenetic gustatory virtual realities

To validate the ability of the optoPAD system to manipulate neuronal activity in closed-loop, we decided to use it to create 'virtual gustatory realities' and test their impact on feeding behavior. To be able to better control the stimulation parameters, we used a switching DC power supply (40 A) to regulate the intensity of the LEDs. The luminous flux of the LEDs increased linearly with the forward voltage on the LEDs above 2 V for the red and amber LEDs and 2.5 V for the green and blue LEDs (Fig. 2.3A), allowing us to vary the intensity of stimulation over a significant range. It is important to note that the maximum level of irradiance which can be achieved strongly depends on the wavelength of the LED.

To test if we can induce appetitive consummatory behaviors using the optoPAD, we used the Gr5a-GAL4 line, which drives expression in sugar-sensing neurons of the labellum (Marella et al., 2006; Thorne et al., 2004). These neurons have been shown to be sufficient to initiate feeding (Zhang et al., 2007). Starved male flies expressing the red-shifted channelrhodopsin CsChrimson (Klapoetke et al., 2014) in Gr5a neurons were given the choice to feed from two 5 mM sucrose sources in an optoPAD arena. One food source in each arena was programmed to trigger the activation of the red LED upon interaction (ON channel) while interactions with the other food source in the same arena never led to light activation (OFF channel). Even very low stimulation intensities (2 V) led to a clear and strong increase of feeding from the food source paired with optogenetic stimulation compared to the unstimulated source (Fig. 2.3B). Interestingly, increasing the stimulation intensity did not lead to an increase in appetitiveness, indicating that a maximal behavioral impact can be achieved at low irradiances, thereby minimizing possible side effects caused by light.

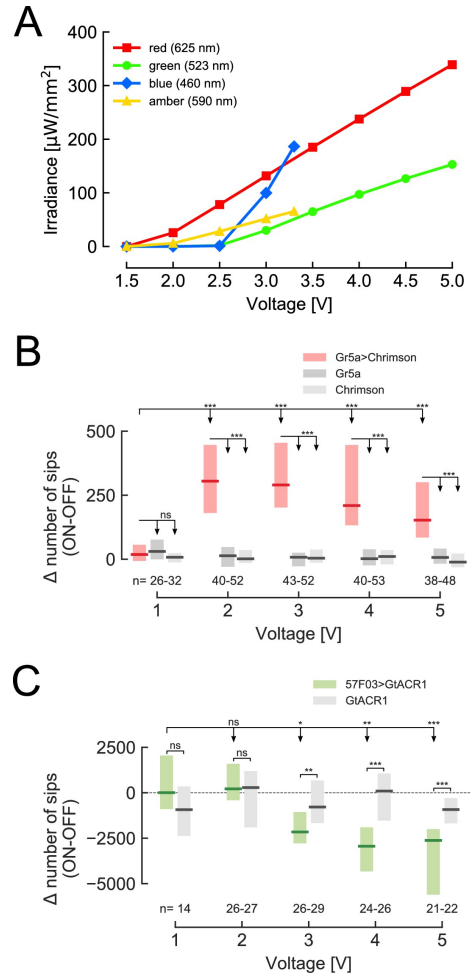


Figure 2.3: Increasing light intensity affects the feeding behavior of flies expressing different optogenetic effectors. (A) Irradiance of all four LEDs increases linearly with increasing voltage (for red and amber above 2 V, for green and blue above 2.5 V). The average value of the three measurements is shown and error bars indicate standard error of mean. (B) Difference in total number of sips on the stimulated (ON) and unstimulated (OFF) food patches of the same arena for 24 hr starved male flies expressing *CsChrimson* under the control of *Gr5a-GAL4*, and corresponding genetic controls. Both food sources contained 5 mM sucrose solution in 1% agarose. (Caption continued on the next page.)

Figure 2.3: (continued from page 42) (C) Difference in total number of sips on the stimulated (ON) and unstimulated (OFF) food patches of the same arena for 3 days yeast-deprived, mated female flies expressing GtACR1 under the control of *57Fo3-GAL4*, which labels taste peg GRNs, and corresponding genetic control. For genotypes, see Materials and methods and key resources table. Both food sources contained 10% yeast solution in 1% agarose. The numbers below the graphs indicate the number of flies tested in each condition. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns non-significance. Boxes represent median with upper/lower quartiles; groups compared by Wilcoxon rank-sum test, followed by Bonferroni multiple comparison test when more than two groups were compared.

Mated female flies deprived of protein for 3 days develop a robust appetite for yeast - their main protein source (Carvalho-Santos & Ribeiro, 2018; Leitão-Gonçalves et al., 2017; Ribeiro & Dickson, 2010; Steck et al., 2018). This appetite is driven by an increase in the length of feeding bursts, which is controlled by the activity of specific subsets of yeast gustatory neurons (taste pegs gustatory neurons) located on the labellum (Corrales-Carvajal et al., 2016; Steck et al., 2018). We previously demonstrated that silencing taste peg neurons with the anion channelrhodopsin GtACR1 (Mohammad et al., 2017) using the optoPAD system is sufficient to terminate feeding on yeast (Steck et al., 2018). To better characterize this silencing effect, flies were given the choice between two sources of yeast, one of which was paired with green light illumination. As we increased the intensity of light by increasing the voltage of the LED to 3 V, flies expressing GtACR1 in taste peg neurons fed significantly less from the channel paired with light compared to control flies (Fig. 2.3C). In contrast to the Gr5a activation experiments (Fig. 2.3B), the decrease in yeast feeding was accentuated with the increase in the applied voltage. This can be easily explained by the fact that the irradiance for the red and green lights reach the same intensity at different voltages (Fig. 2.3A). Furthermore, it is important to note that in both experiments, we observed no behavioral effect of light in control genotypes. This indicates that the illumination itself has minimal effects on

fly feeding behavior. Importantly, the optoPAD can also be used to stimulate neurons using open-loop experimental designs. Indeed, silencing taste peg neurons by delivering the light in a fixed pattern not contingent on the behavior of the animal led to a specific decrease in the length of feeding bursts (Fig. 2.4). Such experiments can be very useful when no hypothesis exists as to how the activity of a neuronal population of interest relates to the microstructure of the behavior. By correlating the relationship between light onset and specific behavioral phenotypes post-hoc, it should be possible to generate specific hypotheses of how the activity of a neuron relates to specific features of feeding behavior, guiding the design of specific follow-up closed-loop experiments.

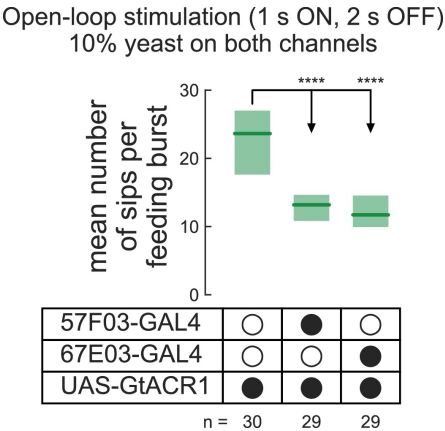


Figure 2.4: Mean number of sips per feeding bursts during open-loop stimulation Both channels were measured during open-loop stimulation (1 s ON, 2 s OFF) in 3 days yeast-deprived, mated female flies expressing GtACR1 under the control of *57F03-GAL4* or *67E03-GAL4*, which both label taste peg GRNs, and corresponding genetic control (Steck et al., 2018). Both food sources contained 10% yeast solution in 1% agarose. The numbers below the graphs indicate the number of flies tested in each condition. **** $p < 0.0001$. Boxes represent median with upper/lower quartiles; groups compared by Wilcoxon rank-sum test, followed by Bonferroni multiple comparison test for two comparisons.

To show that the optoPAD can also be used to manipulate sparse sets of neurons located deeply within the brain, we chose to activate giant fiber

neurons in closed-loop upon initiation of feeding. The rationale of this proof-of-concept experiment was to use a sparse line labeling few (2) neurons deep in the brain of the fly, which has a readily observable phenotype (jumping), and which should have a clear effect on feeding behavior (termination of feeding). Indeed, upon the initiation of feeding and concomitant light activation, flies expressing CsChrimson in escape neurons (Namiki et al., 2018) jumped, leading to the termination of feeding. This led to a drastic decrease in feeding from the food source triggering light when compared with the control food source (Fig. 2.5). These results show that the optoPAD can be used to manipulate very sparse, centrally located neurons, and monitor the impact of the manipulation on feeding behavior.

The mechanistic dissection of specific neurons' contribution to a behavior often requires the observation of opposite behavioral effects upon increases and decreases in their activity. Gustatory neurons are an ideal test case for this as they elicit both appetitive (e.g. sweet neurons) as well as aversive behavioral responses (e.g. bitter neurons). We tested the ability of the optoPAD system to both induce and suppress appetitive feeding responses using the *Gr64f-GAL4* line, which labels appetitive sugar-sensing neurons previously shown to be important to sustain carbohydrate feeding (Jiao et al., 2008). As observed for *Gr5a* neurons, closed-loop activation of *Gr64f* neurons using CsChrimson led to increased feeding (Fig. 2.6A, left panel). This effect was absent in control genotypes (Fig. 2.6A, right panel). Conversely, hyperpolarization of *Gr64f* neurons using GtACR1 led to a loss of appetitive behavior and hence a decrease in feeding from the sugar source paired with green light activation (Fig. 2.6B).

To characterize the effect of closed-loop optogenetic manipulation of aversive neurons on feeding, we used *Gr66a-GAL4*, which labels bitter-sensing neurons (Marella et al., 2006; Thorne et al., 2004). In contrast to the depolarization of sweet gustatory neurons, flies expressing CsChrimson in bitter gustatory neurons immediately terminated feeding from an appetitive food source upon light activation (Fig. 2.6C). This strong effect clearly mimics the potent aversive effect bitter substances have on feeding behavior. To test if we could suppress the aversive effect of bitter food using the

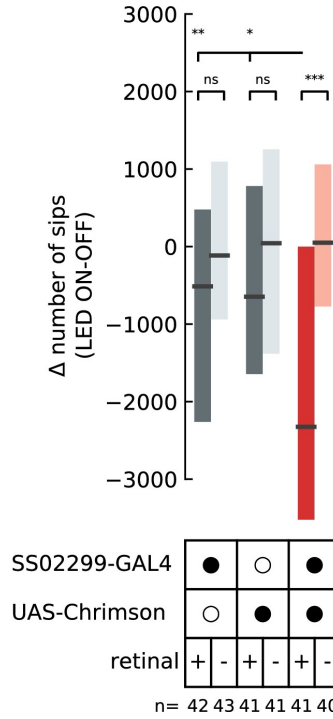


Figure 2.5: Difference in total number of sips on the stimulated (ON) and unstimulated (OFF) food patches of the same arena for 3 days yeast-deprived, mated female flies expressing CsChrimson under the control of *SS02299-GAL4*, and corresponding genetic controls. All genotypes have been supplemented either with (+) or without (-) 5 ml of 400 mM all-trans retinal for 6 days. Both food sources contained 10% yeast solution in 1% agarose. The numbers below the graphs indicate the number of flies tested in each condition. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns non-significance. Boxes represent median with upper/lower quartiles; groups compared by Wilcoxon rank-sum test, followed by Bonferroni multiple comparison test when more than two groups were compared.

optoPAD setup, we expressed GtACR1 in *Gr66a* neurons and observed the effect of green light activation on feeding from a quinine-laced sucrose solution. Indeed, flies exhibited higher feeding from the bitter food source paired with light stimulation than from the unpaired food source (Fig. 2.6D). These experiments demonstrate the ability of the optoPAD to induce and suppress both appetitive and aversive effects on feeding behavior using

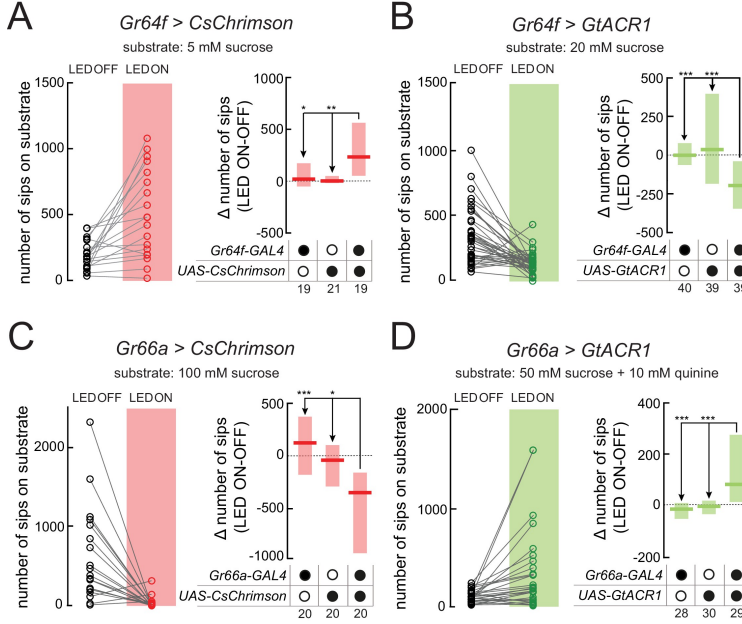


Figure 2.6: Creating virtual taste realities for *Drosophila* using the op-toPAD. (A-D) Total number of sips from the unstimulated (LED OFF) and the light-stimulated (LED ON) food patches of the same arena by flies expressing CsChrimson (A and C) or GtACR1 (B and D), under the control of *Gr64f*-GAL4 (A and B) or *Gr66a*-GAL4 (C and D) (left side of the panels). Difference in total number of sips on the stimulated (ON) and unstimulated (OFF) food patches for flies expressing CsChrimson or GtACR1 (A and C), under the control of *Gr64f*-GAL4 (A and B) or *Gr66a*-GAL4 (C and D), and corresponding genetic controls (right side of the panels). All flies were 24 hr starved male flies (for genotypes, see Materials and methods and key resources table). The food substrate is indicated in each panel. The numbers below the graphs indicate the number of flies tested in each condition. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Boxes represent median with upper/lower quartiles; groups compared by Kruskal-Wallis test, followed by Dunn's multiple comparison test.

either optogenetic activation or inhibition of different neuronal populations.

2.4.3 Creating dynamic gustatory virtual realities

One of the unique features of virtual realities is the ability to generate stimuli that can change dynamically in a way that might be impossible with natural stimuli. The high temporal precision of optogenetics makes it ideal to both tightly link changes in activity of specific neurons to the behavior of the animal, as well as to change this contingency in a flexible while precise manner. We therefore implemented the ability to arbitrarily predefine the conditions upon which the behavior of the animal triggers light activation. As a proof of concept, we first set out to determine how flies would respond to dynamically changing the identity of the food source triggering gustatory stimulation. When flies expressing CsChrimson in bitter neurons (using *Gr66a-GAL4*) are given the choice between two identical appetitive food sources, they avoid feeding from the one paired with light activation (Fig. 2.6C). Bonsai allows us to change the variables controlling this stimulation in a dynamic fashion. We programmed the system to switch the identity of the food source paired with light activation every 5 min (Fig. 2.7A). From the beginning of the assay, the behavior of the flies appears to follow the stimulation pattern, with flies feeding less from the food source paired with light activation (Fig. 2.7B). Only after 10 min of exploration, however, did these preferences reach statistical significance. As clearly visible in the raster plots of the feeding behavior, some flies always interact with both channels, but in the two first blocks of the experiment (0-5 min and 5-10 min) overall few flies interact with the food (Fig. 2.8). This likely reduces the statistical power to detect a possible preference and might be due to an 'acclimatization' period from the moment the animals are introduced into the chambers (Corrales-Carvajal et al., 2016).

Next, we tested if we can alter the length of the interaction of the fly with food by increasing the delay between the initiation of food interactions and LED activation (Fig. 2.7C). By initiating the optogenetic activation of *Gr66a* neurons 1.5, 3 or 6 s after the fly starts interacting with the food, we could make the flies terminate their interaction with food after precisely 2.66, 4.25 or 7.34 s, respectively (Fig. 2.7D). This was a dramatic shortening

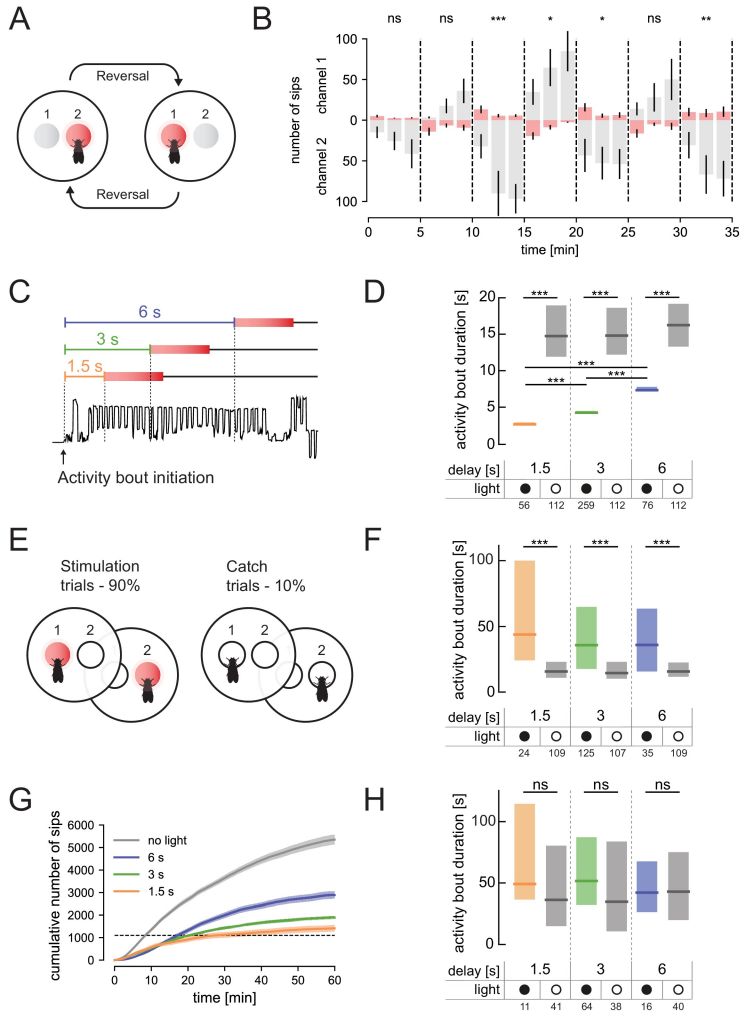


Figure 2.7: The optoPAD allows for complex dynamic closed-loop experimental designs. In all experiments, 5-7 days old female *Gr66a-GAL4* > *CsChrimson* flies were used. (A) Schematic overview of the dynamic virtual taste reality experiment: every five minutes the contingency of the experiment is reversed (in each experimental block the fly's interaction with a different channel triggered light stimulation). (Caption continued on the next page.)

of their activity bout, as control flies displayed reproducibly long bouts with a median of around 15 s. These experiments show that the optoPAD system

Figure 2.7: (continued from page 49) (B) Number of sips from channel 1 (upper half of the plot) and channel 2 (lower half of the plot) across time in the changing virtual taste reality setting described in A. Columns and lines represent mean and the standard error of the mean, respectively. The trials leading to LED activation are shaded in red. (C) Onset of light stimulation (red box) can be freely set to occur at different times after the initiation of an interaction with food (delay of 1.5, 3 and 6 s). The lower part of the diagram represents a representative capacitance trace with the onset of food contact marked with an arrow. (D) Duration of activity bouts in flies exposed to light after different delays relative to the initiation of food interactions and corresponding controls (experimental design described in C). Plotted are the duration of activity bouts for the stimulated flies (light) and for the same number of trials that were longer than 1.5, 3 and 6 s (from left to right) performed by the 'no light' control flies. (E) Schematic of the experimental design in which light activation was set to happen in a probabilistic manner. (F) Duration of activity bouts of the catch trials. Plotted are the duration of activity bouts for the stimulated flies (light) and for a selection of 10% of all the trials that were longer than 1.5, 3 and 6 s (from left to right) performed by the 'no light' control flies. (G) Cumulative feeding for the four different groups of the experiment described in (E). Line represents the mean and the shading the standard error of the mean. Dotted line indicates the 1100 sips threshold used to calibrate the data for the internal state of the animal. (H) Duration of activity bouts of the catch-trials for sip-calibrated flies (trials performed until the flies had reached a total of 1100 sips). Plotted are the duration of activity bouts for the stimulated flies (light) and for a selection of 10% of all the trials that were longer than 1.5, 3 and 6 s (from left to right) performed by the 'no light' control flies. For genotypes, see Materials and methods and key resources table. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns non-significance. The numbers below the graphs in D, F and H indicate the number of flies tested in each condition. In D, F, and H, boxes represent median with upper/lower quartiles. In D, F and H, groups were compared by Kruskal-Wallis test, followed by Dunn's multiple comparison test. In B, the total number of sips for all bins in each channel during each period of 5 min was compared by Wilcoxon rank-sum test.

can be used to dynamically change the contingency between the behavior of the animal and the optogenetic stimulation.

2.4.4 Creating probabilistic gustatory virtual realities

While the optogenetic experiments described up to this point have been deterministic in nature, behavioral experiments in which the behavior of the animal is linked to a probabilistic delivery of a reward or punishment have been very powerful in probing the neuronal substrates of complex learned behaviors (Fiorillo et al., 2003). Such designs can be either used to allow the animal to learn specific statistical properties of the environment (Lottem et al., 2018), or unstimulated trials (catch trials) can be used as controls within a task (Lak et al., 2014). We tested the ability of the optoPAD system to implement such probabilistic experimental designs. We used Bonsai to set the probability of red light activation upon food interactions to 90% for both food sources (Fig. 2.7E). Therefore, 10% of food interactions (trials) did not result in LED activation. Importantly, these 'catch trials' were randomly selected and therefore could not be predicted by the fly. Protein-deprived female flies expressing CsChrimson in bitter taste neurons were subjected to such a probabilistic experimental design for an hour. Similarly to the experiments described in Fig. 2.8C-D, for each fly red light was either triggered after 1.5, 3, or 6 s. As expected from Fig. 2.8D, in stimulation trials, the length of activity bouts was shortened to different extents under the three different delays. Intriguingly however, in the catch trials, the food interaction bouts were much longer than in control experiments where no light was triggered throughout the experiment (Fig. 2.8F). This effect was independent of the length of the interval between food contact and light onset.

2.5 Discussion

We describe an experimental setup which allows the closed-loop optogenetic manipulation of specific neurons in *Drosophila melanogaster* contingent on the feeding behavior of the animal. The system extends the ability of the flyPAD to efficiently detect interactions of flies with food using capacitive sensing, by implementing real-time analysis of feeding behavior

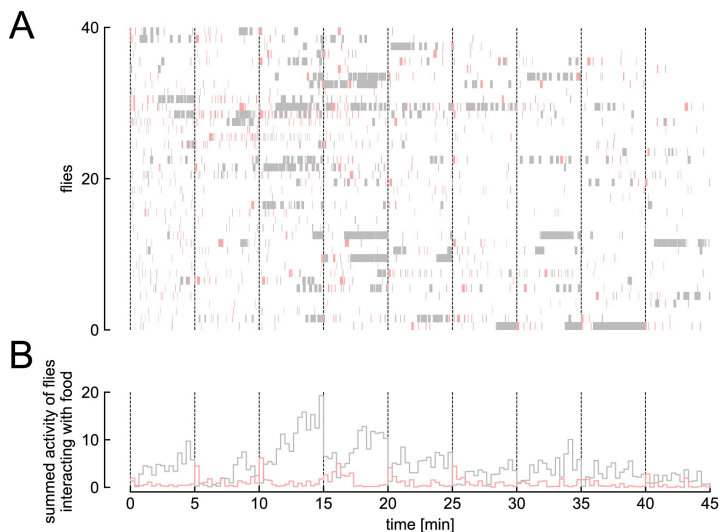


Figure 2.8: Activity bouts of individual flies in a dynamic experimental protocol experiment. (A) Raster plot of the data shown in Fig. 2.7B. Every line corresponds to a single fly undergoing the dynamic experimental protocol. Gray marks activity bouts with the food source not triggering light stimulation and red marks activity bouts triggering light activation and hence likely activation of bitter neurons. Dashed lines symbolize the time point at which the channel triggering light activation was changed. (B) Summed time flies are interacting with the food in a 20 s wide bin for non-light triggering channel (gray) and light triggering channel (red). Data in the bin are normalized by the total number of flies in the assay.

and the ability to activate light sources of different wavelengths depending on this behavior. This has allowed us to explore the effect of activating and silencing specific gustatory neurons on feeding behavior. Importantly, by activating and inhibiting sweet and bitter neurons, we were able to produce both phagostimulatory and phagoinhibiting effects in a coherent manner. This demonstrates that our closed-loop optogenetic manipulations are able to induce all phenotypes expected from the known biology of these neurons. Given that the exposure of the animal to gustatory stimuli is highly dependent on its behavior, it is essential that the design of gustatory circuit manipulations takes into account the behavior of the animal. We therefore

envisage that the optoPAD will be especially valuable when exploring the involvement of sensorimotor circuits in feeding behavior. As the modular regulation of feeding microstructure plays a key role in nutrient homeostasis, the high temporal sensitivity of the flyPAD makes the optoPAD ideally suited to study its circuit basis.

The ability to analyze feeding behavior in a quantitative, high-resolution, and high-throughput fashion using the flyPAD technology has been leveraged to significantly advance our understanding of *Drosophila* feeding behavior. The optoPAD highlights a further advantage of this technology: its flexibility and expandability. After we described the first use of the flyPAD technology to manipulate behavior using optogenetics (Steck et al., 2018), Jaeger and colleagues also described an adaptation of the flyPAD technology to manipulate taste neurons during feeding (Jaeger et al., 2018). Interestingly, their approach is fundamentally different from the one we described, attesting to the flexibility of the flyPAD system. They define at the level of the hardware, specific features of the capacitance signal to trigger light activation. This approach allows the rapid triggering of light when the capacitance signal reaches a certain level, which is likely to be highly correlated with the time periods the fly touches the food. Given that the analysis of the behavior is implemented at the level of the hardware, it is however not possible for the user to readily modify the analysis of the behavior leading to the triggering of the light or to modify how and when the light stimulus gets triggered (delays, probabilities of stimulation, dynamic changes to the stimulation protocol). Furthermore, while this system is fast, the signal is not processed to detect sips and the light onset is therefore unlikely to reliably coincide with these feeding events. While our experiments clearly show that the current optoPAD system is able to modulate feeding behavior using closed-loop optogenetic manipulations, the ability to process the behavioral data in a flexible and complex way is likely to introduce a longer latency than that of the STROBE system. This latency can be counteracted using the existing knowledge of the very stereotyped parameters underlying feeding behavior, which allow us to tune the stimulation parameters in order for the light to better coincide with sip onset.

It should also be possible to further optimize the efficiency with which the hardware handles the incoming signals and the speed at which the software processes these, leading to a significant improvement in the temporal precision of the delivery of the light stimulus. This might be important when dissecting the relationship between circuit activity and behavior at the scale of a single action potential. There will, however, always be a conflict between flexibility in experimental design, requiring computation time to analyze the behavioral signal, and minimizing the delay between behavior detection and light stimulation. We propose that as long as the delay is not beyond the behaviorally meaningful scale, the advantages of flexibility outweigh small improvements in the speed of the system. In any case as shown by both the *optoPAD* and the *STROBE* system, the *flyPAD* technology is flexible enough to allow the implementation of either priority.

Given its flexible design, the *optoPAD* system not only allows for a fixed closed-loop manipulation of neurons, but confers substantial flexibility to define how feeding behavior is linked to light activation. The researcher can for example predefine how many light stimulations will happen in one session, stimulate after the termination of an activity bout, or perform experiments in an open-loop mode. An important further feature is the possibility to design experiments in which light activation is altered to be probabilistic, while occurring at a fixed rate. This allows the experimenter to probe the effect of neuronal manipulations altering feeding behavior using interleaved control trials (catch trials). We expect that the ability to alter the dynamics and probabilities of optogenetic manipulations will be most useful to manipulate the behavior of *Drosophila* in order to probe its ability to learn complex contingencies. By activating 'reward' and 'punishment' neurons in closed-loop using different behavioral contingencies, the *optoPAD* system should allow the design of new operant conditioning paradigms in which the animal learns to associate the consequences of its own behavior with specific outcomes or the statistical structure of its environment. Learning such abstract environmental structures is a fundamental ability animals use to optimize their foraging strategies (Chittka,

2017; Glimcher & Fehr, 2014). The extent to which *Drosophila* is able to perform such proto-cognitive computations is currently an important frontier in fly systems neuroscience. We expect that the flexibility of the optoPAD as well as its high-throughput design will allow researchers to explore how flies adapt their behavior to complex environmental features and identify the underlying neuronal circuits and computations.

2.6 Materials & Methods

2.6.1 Fly stocks and rearing conditions

Flies were reared at 25°C, 70% relative humidity (RH) in the dark to prevent non-specific activation of neurons. Flies were reared at standard density and were matched for age and husbandry conditions. The yeast-based fly medium (YBM) contained 80 g cane molasses, 22 g sugar beet syrup, 8 g agar, 80 g corn flour, 10 g soya flour, 18 g yeast extract, 8 ml propionic acid, and 12 ml nipagin (15% in ethanol), per liter. All data are from 24 hr wet-starved male flies unless otherwise stated. After hatching flies were aged for 5-7 days, then groups of 12-15 males were transferred to new vials and approximately 10 wild-type female flies were added. They were kept on YBM medium containing 0.4 mM all-trans-retinal (Sigma-Aldrich, #R2500, made using a stock solution of 100 mM all-trans-retinal dissolved in ethanol) for 2 days. To induce starvation, the flies were then transferred to vials containing tissue paper soaked with 5 ml Milli-Q water containing 0.4 mM all-trans-retinal 24 hr before the experiment. The starvation was chosen to increase carbohydrate appetite and to ensure robust feeding on sucrose.

For the experiments described in Fig. 2.2, Fig. 2.3B, Fig. 2.4, Fig. 2.7 and Fig. 2.8, mated female flies were deprived of yeast for 3 days on a tissue soaked with 6.5 ml of 100 mM sucrose, and 0.4 mM all-trans-retinal.

2.6.2 Hardware design and real-time data analysis

The optoPAD system is a new generation of the flyPAD, that was previously described in Itskov et al. (2014). Additional hardware to the flyPAD was

designed to fit on top of each of the 32 behavioral arenas and allow independent activation of 32 high-power (10 W) RGBA LEDs (ref. no. LZ4-00MA10; LED Engin, San Jose, CA, USA). Printed circuit boards were designed using Eagle CAD software (Cadsoft - version 6.2.0). The designs were then sent to Eurocircuits (<https://www.eurocircuits.com/>) for production.

The PCBs hosting the LED (LED board) measure 5×5 cm and fit exactly on top of the flyPAD arenas supported by screws and nuts that align the two boards. The LED is placed at the center of each board directly facing the middle of the flyPAD arena. An additional electrical circuit on each LED board includes four MOSFET N-ch (one for each LED color) that serve as switches for the LEDs and a set of five resistors connected in series to set the correct voltage to the pole of each individual color LED (3.3 and 0.5 Ω for red; 1 and 0.5 Ω for amber; 2 Ω for green, no voltage drop resistor was used for the blue color).

Three groups of twelve LED boards ($3 \times 12 = 36$, four were not used) receive power and LED control signals from a control breakout board, which hosts the microcontroller (Arduino Mega 2560). These boards receive and distribute power from an external power supply unit (Corsair CSM 550W 80 + Gold Certified Semi-Modular ATX) through SATA power cables, allocating a maximum of 3.3 V to both amber and blue LEDs and of 5 V to both red and green LEDs. The microcontroller sends activation signals of 5 V to the correct transistor to be activated based on the information from serial communication with the computer. To alter the LED brightness, we used the Voltage dial of a switch mode bench power supply (Circuit Specialists CSI3060) to set the voltage applied to the LED.

All PCB designs can be found on the following GitHub repository: <https://github.com/ribeiro-lab/optoPAD-hardware> (Goldschmidt, 2019a) (copy archived at <https://github.com/elifesciences-publications/optoPAD-hardware>).

2.6.3 Real-time detection of food interactions and LED activation

The detection of food interactions in real time as well as the closed-loop control of LED illumination was performed using a custom-written Bonsai workflow (Lopes et al., 2015). This visual programming framework allows for real-time analysis of the flyPAD capacitance data sampled at 100 Hz and is capable of online communication with actuators for closed-loop experiments.

Unlike the data processing described in Itskov et al. (2014), the optoPAD system detects when the fly interacts with a food source in real time. To do so, the Bonsai workflow continuously takes the absolute difference of two consecutive samples and applies a finite impulse response (FIR) filter with a running window of 50 samples. The filtered signal is finally thresholded with a value of 120 (a.u.), resulting in a binary signal representing if activity bouts were detected (Fig. 2.1C). We chose the values for the running window size and threshold based on optimizing the accuracy of the activity bout detection using the output of the offline detection algorithm as a standard (Itskov et al., 2014).

The protocol for LED activation implemented in Bonsai is a series of conditional nodes that can be programmed to perform flexible experiments.

Through this Bonsai workflow, each experiment can be programmed to run with the same protocol for as long as the user desires. The protocol used to control LED activation can be programmed independently for each flyPAD capacitance channel. Every time the fly starts interacting with food, a trial is initiated. From there, two possible behavioral outcomes are possible: 1. the fly stops interacting with food before the set 'delay' period (such activity bouts are classified as short trials, with no LED activation); 2. the fly keeps eating for longer than the 'delay' period. When the latter occurs, and depending on the experimental design, LED activation may or may not occur. This feature is dependent on the setting of the 'probability of stimulation' value. Bonsai pseudo-randomly samples a real number between 0 and 1 from a uniform distribution and compares it to a given previously defined

probability. If this number is less or equal than the defined probability, the fly receives light stimulation for a period of time that is set by the user prior to the experiment. Else no stimulation occurs and the trial is considered a 'catch-trial', which only terminates when the fly stops interacting with the food at the end of the activity bout. Upon termination of light stimulation, the protocol is restarted, and the system will wait for the fly to start interacting with food again (if the fly continues to interact with food during the light stimulation, the ongoing activity bout is considered a new activity bout). Additionally, the user can define how many stimulations will occur on each channel.

For dynamic virtual taste environment experiments (Fig. 2.7A-B), in which the channel controlling LED activation is changed every five minutes, a timer was added to the Bonsai workflow, which after a set time instructs the software to change the experimental parameters by reading from another configuration file.

Software can be found on the following GitHub repository:
<https://github.com/ribeiro-lab/optoPAD-software> (Goldschmidt, 2019b), copy archived
at <https://github.com/elifesciences-publications/optoPAD-software>.

2.6.4 Irradiance measurements

We performed the irradiance measurements shown in Fig. 2.3A using an optical power meter (Thorlabs PM100D) and a standard photodiode power sensor (Thorlabs S121C). In order to accurately measure how much irradiance reaches the fly upon LED illumination, we placed the sensor at the same distance from the LED as the arena. We varied the voltage applied to the LED between 1.5 and 5 V in 0.5 V steps, and measured the peak value of power on the optical power meter upon LED illumination. Measurements were started at 1.5 V as no measurable optical power was detected below this voltage. We carried out each measurement three times. The irradiance was computed by dividing the optical power measurements by the effective sensor area (14.923 mm²).

2.6.5 Behavioral experiments

Behavioral experiments were performed at 25°C, 70% RH. We used flies expressing either GtACR1 (Mohammad et al., 2017) or CsChrimson (Klapoetke et al., 2014) in subsets of gustatory neurons. The genotypes of the lines used in the manuscript are listed in the key resources table. optoPAD assays were performed following a protocol previously described (Itskov et al., 2014). Briefly, both wells of the optoPAD were filled with solutions containing different concentrations of sucrose (Fig. 2.3B and Fig. 2.6AA: 5 mM; Fig. 2.6B: 20 mM; Fig. 2.6C: 100 mM; Fig. 2.6D: 50 mM + 10 mM quinine) or 10% yeast solution (Fig. 2.3C and Fig. 2.7). All solutions were in 1% agarose. Single flies were transferred to optoPAD arenas by mouth aspiration and allowed to feed for 1 hr in a light shielded box.

To test how different light intensities affect feeding behavior (Fig. 2.3B & C), red (625 nm) LED activation was set to occur 0 s after the initiation of each activity bout for the experiment shown in Fig. 2.3B and green (523 nm) LED activation was set to occur 0.5 s after each bout initiation for Fig. 2.3C. For both experiments, light stimulation was sustained for 1.5 s independent of the flies' behavior. Based on the dose-response curves in Fig. 2.3, we chose to use 3.5 V for the green LED and 2.25 V for the red LED for the experiments in Fig. 2.6.

For the experiments aiming at inducing and repressing aversive and appetitive behaviors (Fig. 2.6), LED activation was set to occur 0.01 s (Fig. 2.6A), 0 s (Fig. 2.6B), and 0.5 s (Fig. 2.6C-D) after an activity bout was detected in the light-triggering channel. In Fig. 2.6A & C the red LED was used and in Fig. 2.6B & D the green LED was used. Light stimulation was sustained for 1.5 s independent of the behavior of the fly.

For the dynamic virtual taste environment experiment (Fig. 2.7A-B), red LED activation was set to occur 0.5 s after the initiation of each activity bout in the light-triggering channel (channel 1 or two depending on the experimental block), and the light activation was sustained for 2 s independently of the activity of the fly. For the 'control' channel, interactions of the fly with the food did not trigger any LED activation.

For the experiments testing the flies' behavioral response to activation of bitter-sensing neurons following a delay in relation to the initiation of the activity bout (Fig. 2.7C-H), the red LED was set to occur 1.5, 3 or 6 s after an activity bout was detected in 90% of the trials on both food sources. Light stimulation was sustained for 2 s. A new trial was initiated when a new interaction with food was detected. The 10% of trials with no LED activation were terminated at the end of the respective activity bout.

2.6.6 Statistics

Results of optoPAD experiments were compared using the Kruskal-Wallis test, followed by Dunn's multiple comparison test when more than two groups were compared or Wilcoxon rank-sum test, followed by Bonferroni correction when multiple comparisons were made. All tests were two-tailed.

2.6.7 Data availability

Data used to generate the plots in the figures are included in the manuscript and supporting files. The optoPAD hardware designs and software are made available on GitHub at <https://github.com/ribeiro-lab/optoPAD-hardware> (Goldschmidt, 2019a) and <https://github.com/ribeiro-lab/optoPAD-software> (Goldschmidt, 2019b), respectively. Copies have been archived at <https://github.com/elifesciences-publications/optoPAD-hardware> and <https://github.com/elifesciences-publications/optoPAD-software>.

2.7 Acknowledgments

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CHAPTER



LARGE-SCALE NEUROGENETIC
SCREENING TO IDENTIFY NEURONAL
SUBSETS INVOLVED IN FORAGING

3.1 Contributions

This chapter was mainly written by me with largely unpublished results, but also contains parts written together with Ibrahim Tastekin and Carlos Ribeiro, in preparation for publication together with the results from the next chapter. The experiments and interpretation of the data shown in this chapter were obtained with the help of Célia Baltazar, Lúcia Serra, Ibrahim Tastekin, Daniel Münch, Johanna Schweizer and Carlos Ribeiro.

3.2 Summary

Mapping behavioral function and programs to specific neuronal circuitry has long been a central goal in neuroscience and is required towards a circuit-level understanding of how the brain generates naturalistic behaviors. While there have been systematic approaches to correlate high-throughput behavioral analysis and neurogenetic manipulations, only few have studied complex, naturalistic behaviors, like nutrient foraging. Furthermore, while studies have provided insights into how foraging behaviors may be modulated by internal states via sensory gain modulation, how these affect more cognitive functions in higher-brain areas to maximize foraging outcomes still remains unknown. Here, we present an experimental framework for **Modular High-throughput Video-based Ethological tracking (MoHiVE)** to study nutrient foraging decisions and exploration. We first validated this modified assay based on a foraging assay presented in Corrales-Carvajal et al. (2016) where individual flies explore an arena with six yeast and sucrose patches, and optimized the setup for scalability, modularity and robustness. This allowed us to run a total of 12 setup simultaneously, each recording four individual flies at the same time. I also created an automated, efficient analysis pipeline that tracks the movement of all flies, extracts kinematic parameters, and classifies specific behaviors related to exploitation (feeding micromovements) and exploration (runs and turns). Using this framework, we employed a large-scale neuronal silencing screen of more than 15000 flies to identify neuronal subsets involved in specific

decision-making processes and behavioral programs during nutrient foraging. Specifically, we quantified the behavioral effects of silencing more than 500 different GAL4 driver lines in a one-hour foraging assay. This chapter presents the whole-screen analysis as an entry point into investigating and mapping neuronal subsets to foraging behaviors and decision parameters as outlined below in Chapter 4.

3.3 Introduction

The goal of neuroscience is to understand how the brain's circuitry affects behavior, which is the response of a living organism to internal or external stimuli. In recent years, advances in technology such as the ability to target specific neurons genetically (Pfeiffer et al., 2010), image brain activity in awake animals (Ahrens et al., 2013), and manipulate brain circuits with precision (Klapoetke et al., 2014) have allowed researchers to better understand the role of individual neurons in behavior. Moreover, methods for accurately measuring and quantifying behavior, which includes defining specific actions and motivation states, have advanced significantly in recent years with the advent of machine learning methods to solve markerless tracking of movements (Mathis et al., 2018; Pereira et al., 2022), as well as automated behavioral detection and segmentation (Bohnslav et al., 2021; Kabra et al., 2013; Wiltchko et al., 2015). While the combination of these two advances in Systems Neuroscience gave rise to several studies using high-throughput screenings of correlating neuronal activity or manipulation to behavioral analysis to discover how neural circuits link to specific behaviors (Cande et al., 2018; Robie et al., 2017), only a few were able to apply them to naturalistic behaviors (Marques et al., 2020; Vijayan et al., 2022; Wiltchko et al., 2020). Naturalistic behaviors are difficult to capture and analyze for multiple reasons: First, they often involve complex movements, as well as many, potentially external and internal dependent variables, making the dimensionality of state and action space very high. Secondly, naturalistic behaviors are often performed in naturalistic environments that are different from controlled laboratory environments. Finally,

while some behaviors can be described by a discrete set of distinct and identifiable motifs, there are naturalistic behaviors too complex to be described by distinct motifs or additionally may be performed over many hierarchically organized temporal and spatial scales.

Foraging is a class of behaviors that presents all of these challenges in the study of naturalistic behaviors, as it is hierarchical and high-dimensional in nature and often organized over multiple spatial and temporal scales (Amano & Katayama, 2009; Ehlers et al., 2021; Owen-Smith et al., 2010; Shipley, 2007; Stephens & Krebs, 1986). Furthermore, decision-making in a foraging context is highly cognitive and may be supported by complex neuronal computations: For example, once an animal encounters a resource, such as a food patch, it faces the crucial decision of whether to exploit it or further explore to find possibly better ones. This problem is known as the exploration-exploitation trade-off (Sutton & Barto, 1998). This trade-off has been studied extensively in behavioral ecology and computational neuroscience and is thought to be central to decision-making and risk assessment in all animals (Addicott et al., 2017; Dall et al., 2005). As such, many animals have shown to optimally adjust their behavior to solve this trade-off (Corrales-Carvajal et al., 2016; Kacelnik & El Mouden, 2013), even with additional risks, such as requiring multiple or unreliable nutrients, and the presence of competitors and predators. Despite its relevance, the neuronal circuit mechanisms underlying exploration-exploitation trade-off computations are poorly understood.

Foraging effectively enables animals to explore and exploit food resources in the environment. Therefore, foraging can be formulated as part of the normative goal to achieve nutrient homeostasis. Nutrient intake has shown to be tightly regulated by animals, and this balance is key to their health and well-being (Simpson & Raubenheimer, 2012). Proteins are key nutrients and their balanced intake has been shown to optimize overall mortality, fecundity, and lifespan in many animals across phyla (Carvalho-Santos et al., 2020; Piper et al., 2017; Solon-Biet et al., 2019; Solon-Biet et al., 2014). While there has been significant progress on identifying circuit elements that regulate feeding decisions for nutrient homeostasis (Flood et al.,

2013; Gordon & Scott, 2009; Inagaki et al., 2014; Steck et al., 2018), including neuropil-wide sensory processing and internal state modulation (Münch et al., 2022; Shiu et al., 2022), circuit mechanisms regulating exploration-exploitation trade-offs in the context of nutrition are mostly unknown.

A key idea of systems neuroscience is to identify the molecular, cellular and computational mechanisms which operate within neuronal circuits to generate and regulate behavior. This requires a mapping of the extensive connections between neurons, perturbing and recording activity in specifically targeted neurons, and measuring its output on behavior in an ecologically relevant context. The fruit fly, *Drosophila melanogaster*, and its rich genetic toolkit is one of the best established models for behavior and its underlying neural basis (Kazama, 2015; Oswald et al., 2015). While their brains contain approx. 100000 neurons, which makes it more feasible for comprehensive studies on a cellular level than vertebrate brains, flies display a complex repertoire of innate and adaptive behaviors (Berman et al., 2016; Kacsoh et al., 2015; Kacsoh et al., 2013; Kim, Su, et al., 2017; Ofstad et al., 2011; Zacarias et al., 2018).

In this chapter, we aimed at identifying neural circuits involved in exploratory foraging and nutrient decision-making in *Drosophila melanogaster*. We developed an experimental framework, named MoHiVE, to study the foraging choices made by walking *Drosophila* to achieve nutrient homeostasis. The high-throughput setup combined video-tracking foraging arenas (Corrales-Carvajal et al., 2016), with the ability to perform opto- and thermogenetic manipulations (Hampel & Seeds, 2016; Zacarias et al., 2018) to accurately measure foraging and feeding behaviors while manipulating genetically targeted neural circuits. We applied computer vision and machine learning methods to analyze and extract relevant information about the spatio-temporal dynamics of *Drosophila* foraging decisions. Secondly, we performed a large-scale screen using genetic manipulations to identify neural circuits involved in exploratory foraging and nutrient decision-making. Specifically, we used a total of 589 driver lines genetically targeting central-brain interneurons to silence them while the fly is foraging for food. We identified multiple lines labeling different neuronal populations affecting

different aspects of nutrient-specific exploitative behaviors with a bias towards inducing phenotypes resembling nutrient deprivation. This implies that many of the neuronal populations targeted by our screen mediate a satiety state. This framework will allow us to explore hypotheses of how exploration-exploitation strategies may play out in more complex environments with variable food density and quality, a key step on the path to understanding the neural basis of navigation in naturalistic settings.

3.4 Results

3.4.1 High-throughput image-based tracking for quantifying foraging decisions

Naturalistic behaviors performed by freely moving animals are notoriously challenging to capture and describe quantitatively (Dennis et al., 2021; Kennedy, 2022). Recent advances in integrated hardware and software solutions (e.g., computer vision and machine learning) enabled scientists to capture the behavior of animals using videos in unprecedented detail (Mathis et al., 2018; Pereira et al., 2020). Furthermore, automated quantitative analysis has become the method of choice to describe naturalistic behaviors and dissect the underlying circuit mechanisms (Labuguen et al., 2021; Wiltshko et al., 2015). Inspired by an existing setup (Corrales-Carvajal et al., 2016) built to quantify foraging decisions performed by *Drosophila melanogaster*, we built an automated image-based tracking setup that captures trajectories of an individual fly in a foraging arena with radially distributed 6 yeast patches (amino acid source) and 6 sucrose patches (carbohydrate source) of equal volume (Figs. 3.1 and 3.2, also see section 3.6 Materials & Methods). As the goal of the setup was to perform high-throughput genetic screens we optimized it for scalability, robustness, and modularity (Fig. 3.2, see section 3.6 Materials & Methods for details).

From the tracked trajectories, we computed kinematic time series, such as translational speeds of head and body as well as rotational speed, and the distance to the closest food patch (Fig. 3.1B, see also Section 3.6 Materials &

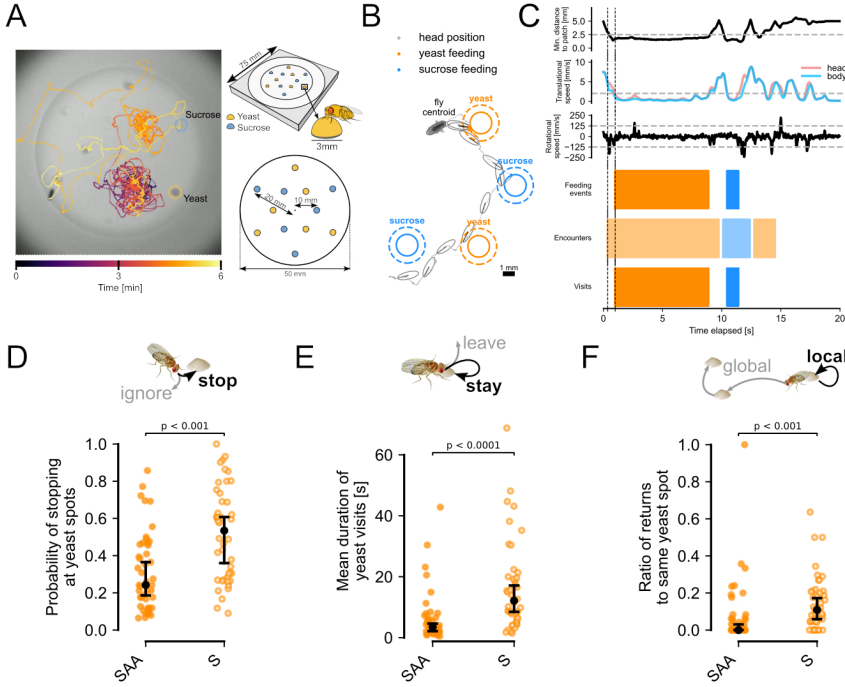


Figure 3.1: High-throughput experimental setup to dissect foraging decisions enables us to quantify that flies adjust foraging decisions according to their internal amino acid (AA) state. (A) Individual flies are recorded for one hour and their trajectories are measured in real-time as they explore an enclosed arena with distributed yeast and sucrose patches. (B) Example trajectory of a fly. (C) Automated detection of feeding micromovements and patch visits based on thresholding speeds and distance to patches (see Section 3.6 Materials & Methods). (D) Probability of stopping at yeast patches for SAA (fully fed) and S (AA deprived) flies. (E) Mean duration of yeast visits for fully fed and AA deprived flies. (F) Ratio of return to the same yeast patch for fully fed and AA deprived flies. P-values were computed based on the non-parametric Wilcoxon ranksum test.

Methods for details) (Corrales-Carvajal et al., 2016). These kinematic parameters allow us to extract parameters relevant to foraging behavior (Fig. 3.1C). We, for example, automatically detect feeding events by thresholding these kinematic time series. As feeding is characterized by slow, but non-resting micromovements of the head close to the food patch, micromovements on

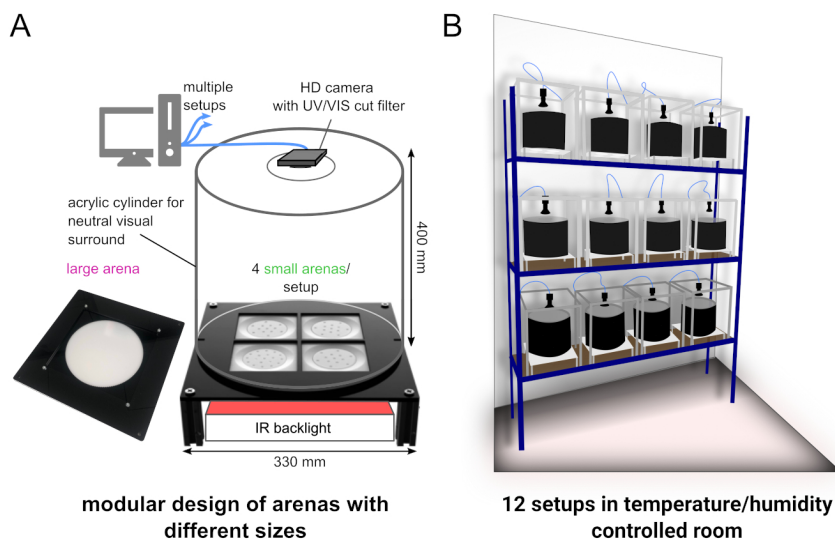


Figure 3.2: Detailed scheme of high-throughput experimental setup to study foraging. (A) Modular structure of individual setup. (B) We built 12 exact copies of the tracking setups for high-throughput experimentation.

food serve as a reliable proxy for feeding (Corrales-Carvajal et al., 2016). This approach was validated in earlier studies by multiple labs as capturing feeding events with high confidence (Corrales-Carvajal et al., 2016; Geissmann et al., 2019). We furthermore grouped multiple of these feeding event segments that occur on the same patch and defined these as a "visit" of a food patch. We also computed segments, in which a fly is close enough to a food patch so that it likely touches it (distance < 3 mm), and defined these as "encounters". Encounters differ from a visit in that the fly might touch the food patch but not engage in feeding during these segments.

3.4.2 Flies adapt different foraging decisions to their nutrient state

Foraging can be broadly divided into exploration and exploitation phases (Stephens et al., 2007; Stephens & Krebs, 1986; Wolfe, 2013). While exploration is key for the animal to identify the distribution and content of

different food patches, it prevents the animal from staying on a food patch to ingest large amounts of food (i.e., exploitation). The ability of animals to balance this exploration-exploitation trade-off is therefore key to matching foraging outcomes with physiological needs and hence efficiently achieve nutrient homeostasis (Corrales-Carvajal et al., 2016; Rowland, 2007). In simple terms, the animal balances this trade-off by making a decision to either stop and engage in feeding upon encountering a food patch, or to continue exploring. This decision is therefore key to balancing the exploration-exploitation trade-off. To capture the decision to stop at a food patch, we calculated the ratio dividing the total number of visits by the total number of encounters. This defines the probability of stopping at a food patch, which we will use as the variable describing how the animal balances the exploration-exploitation trade-off (Corrales-Carvajal et al., 2016). Within foraging theory, the budget theory predicts that foraging behavior should be modulated by the nutrient state of the animal (Kearney et al., 2013). In order to specifically deprive flies from specific nutrient, we employed holidic media as a diet (Piper et al., 2014; Piper et al., 2017). SAA indicate flies with a fully fed diet, while S are flies that have been fed with a diet that lacks Amino Acids (AAs). In an earlier study, we have shown that upon AA deprivation, flies specifically alter different foraging parameters to increase their intake of foods containing the required nutrient with the aim to restore nutrient homeostasis (Corrales-Carvajal et al., 2016). Using our setup we also observed that AA deprived animals specifically increase their feeding on yeast, a source of AAs (Fig. 3.3A-C). Accordingly, the probability to stop at yeast patches (Fig. 3.1D) is also increased by AA deprivation and AA-deprived flies are significantly less likely to leave yeast patches (as quantified by an increase in their mean duration of yeast visits) (Fig. 3.1E). These effects are nutrient specific as the behavior of AA-deprived flies towards sucrose patches remains unaltered (Fig. 3.5A-C), while carbohydrate-deprived flies, specifically alter their behavior towards sucrose patches and not yeast (Fig. 3.5D-F). As such, we can quantify different aspects of exploitative decisions during foraging and their modulation by diet.

Furthermore, our assay also allows for quantifying how the fly explores

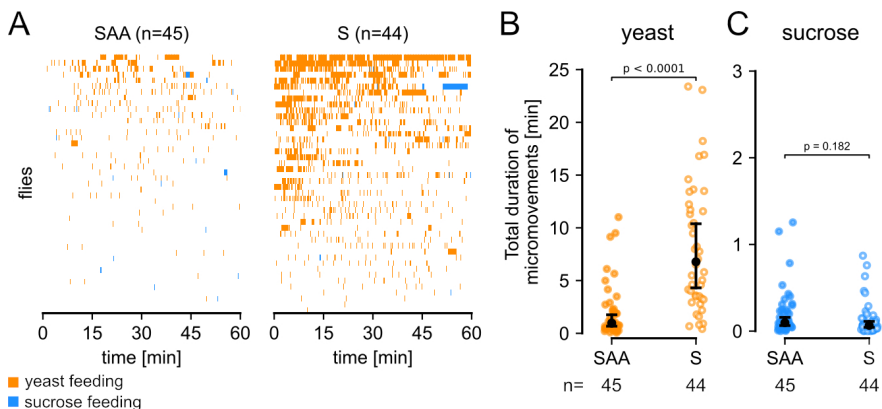


Figure 3.3: Amino acid (AA) deprivation increases exploitation of yeast patches, and not sucrose patches. (A) Ethograms of yeast and sucrose feeding micromovements for fully fed (SAA, $N = 45$ flies) and AA-deprived (S, $N = 44$ flies) conditions, where each row corresponds to a fly over the course of the one-hour-long assay. (B) Total duration of yeast feeding micromovements of SAA vs. S, and (C) total duration of sucrose feeding micromovements of SAA vs. S. Wilcoxon ranksum tests have been used to calculate p-values indicated.

the arena. For this, we analyzed how the flies transition from one yeast spot to the next. We computed the ratio of returns that lead to the same yeast patch divided by the total number of transitions between yeast patches as a measure of how local the exploratory pattern of a given fly is. Similarly to what we have described before, we observed that AA deprivation increases the ratio of returns to the same yeast spot (Fig. 3.1F). The fly therefore exhibits a more local exploration pattern, which can be interpreted as the fly reducing its risk-taking to remain close to a known food source, while still allowing for some exploration for better food sources. Further evidence for this modulation is given by quantifying the area extent explored over certain time bins, which is decreased in AA-deprived flies (Fig. 3.6). These results demonstrate that our high-throughput setup allows for quantifying various aspects of decision-making regulating foraging and their modulation by the nutrient state of the animal.

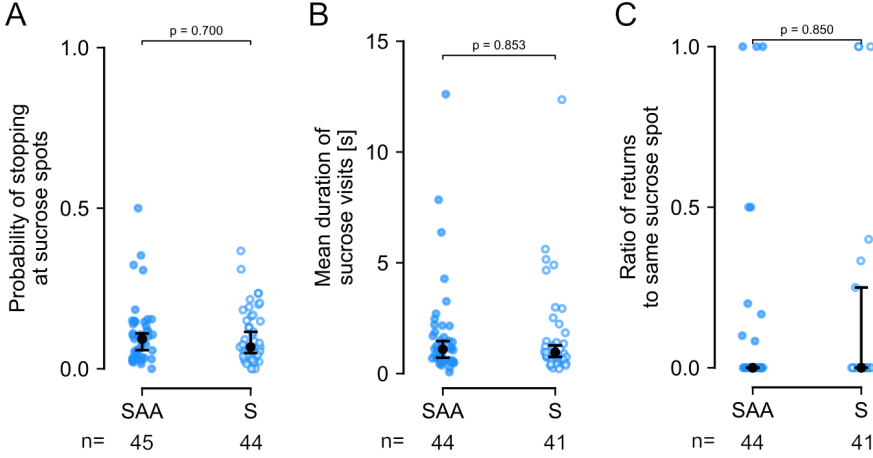


Figure 3.4: AA deprivation does not affect foraging parameters towards sucrose patches. (A) Probability of stopping at sucrose patches for fully fed (SAA, $n = 45$ flies) and AA-deprived (S, $n = 44$ flies) conditions. (B) Mean duration of sucrose visits of SAA vs. S, and (C) ratio of returns to the same sucrose spot of SAA vs. S. Wilcoxon ranksum tests have been used to calculate p-values indicated.

3.4.3 Flies adapt foraging decisions to nutrient availability as predicted by optimal foraging theory

As described above in the General Introduction, animals do not only adapt their foraging decisions to the internal nutrient needs, but also to the environmental statistics of nutrient availability. OFT, and specifically the MVT formalized the relationship between resource density to patch leaving decisions as a cost-benefit analysis, in which the travel time between resource patches are proportional to the dwelling time of the animal exploiting the patch (Charnov, 1976). As such, we set out to test this hypothesis for foraging decision-making in *D. melanogaster*. Specifically, we used our modular setup of a single, large arena to increase the area, thus effectively increasing the distances between the patches without changing the concentration, volume, and number of patches (Fig. 3.2A). This way of decreasing the patch density reveals an increase in both the mean duration of yeast visits (Fig. 3.7A) and the probability of stopping at yeast spots (Fig. 3.7B). We

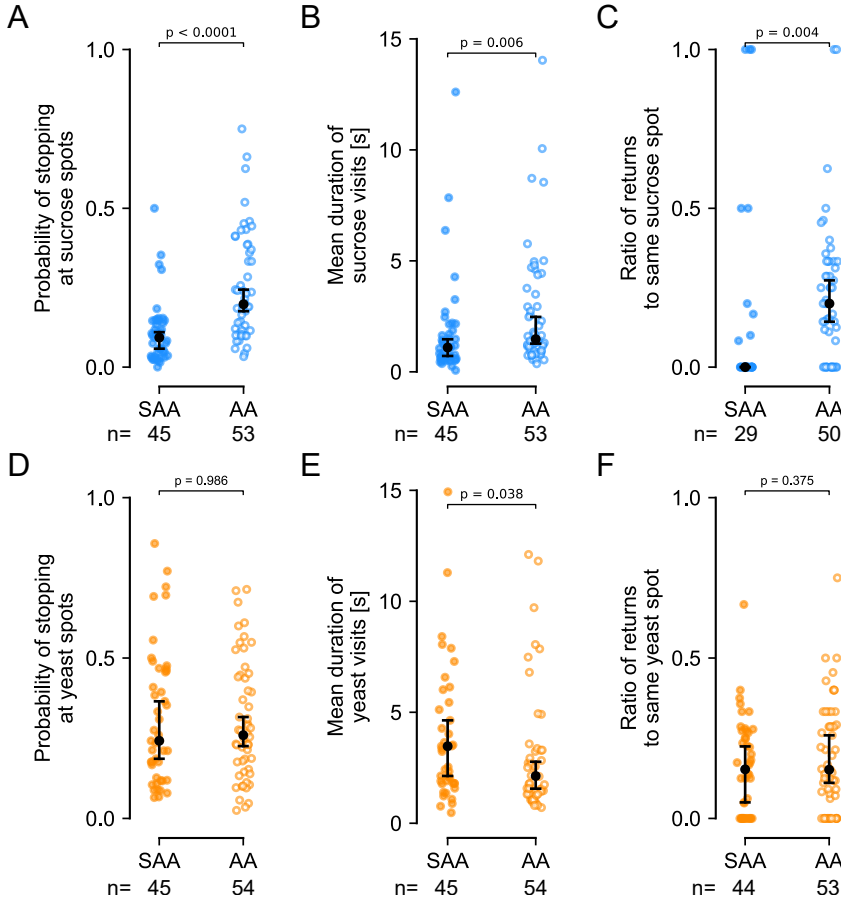


Figure 3.5: Sucrose deprivation modulates foraging parameters towards sucrose patches, but not yeast. (A) Probability of stopping at sucrose patches for fully fed (SAA, $n = 45$ flies) and sucrose deprived (AA, $n = 54$ flies) conditions. (B) Mean duration of sucrose visits of SAA vs. AA, and (C) ratio of returns to the same sucrose spot of SAA vs. AA. (D-F) Probability of stopping, mean duration of visits, and ratio of returns of yeast patches for SAA vs. AA. Wilcoxon ranksum tests have been used to calculate p-values indicated.

showed that by increasing the distances between the patches, flies travel on average for longer durations between the patches (Fig. 3.8). Therefore, these data suggest that flies adapt foraging decisions to nutrient availability

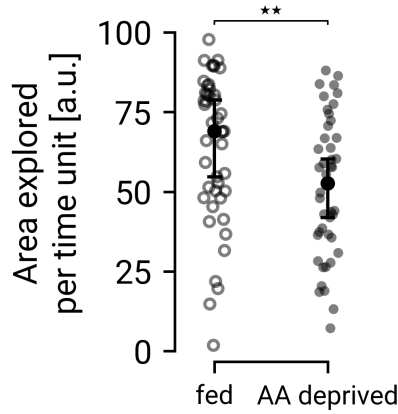


Figure 3.6: AA deprivation modulates area-restricted search. Average area extents calculated over 2-min bins across the whole one-hour assay for SAA and S conditions. Wilcoxon ranksum test has been used to calculate statistical significance. **: $p < 0.01$

as predicted by optimal foraging theory (Charnov, 1976). While previous literature has indicated similar results for patch leaving decisions in a more simplified assay (Koganezawa et al., 2009; Shimada et al., 1993), we showed here that they also adapt patch engagement decisions depending on the patch density in their environment.

We furthermore asked whether single travel times could predict and account for foraging decisions made for patch engagement and leaving, and found that there is indeed a monotonously increasing relationship between travel times and decision parameters (Fig. 3.9), where longer travel times led to more exploitative patch engagement and leaving decisions. Single travel times were defined as the duration from the last patch encounter to the current patch encounter.

3.4.4 Performing a large-scale silencing screen

In order to identify neural circuits that control foraging decisions, we will genetically target and manipulate the activity of central brain neurons. An

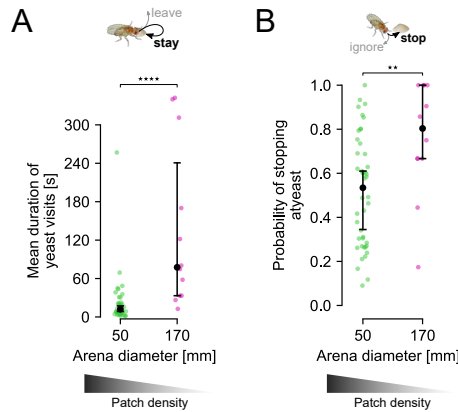


Figure 3.7: Patch density modulates foraging decisions for patch leaving and engagement. (A) Mean duration of visits for flies ($N = 12 - 45$) in foraging arenas with different patch density. (B) Probability of stopping at yeast for flies in foraging arenas with different patch density. Wilcoxon ranksum test has been used to calculate statistical significance. **: $p < 0.01$. ****: $p < 0.0001$.

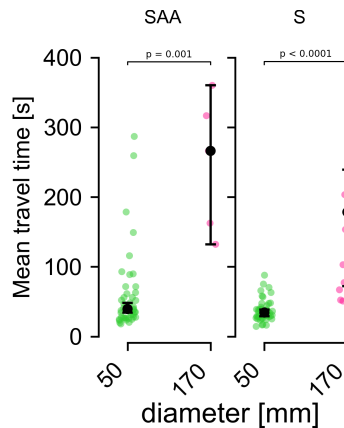


Figure 3.8: Mean travel times in arenas with different patch density. Mean travel times for both SAA and S flies increase in arenas with smaller patch density. Wilcoxon ranksum test has been used to calculate p-values.

extensive collection of GEN1 GAL4 lines expressed in central brain neurons is freely available from the HHMI Janelia Research Campus, including a detailed characterization of the anatomy of these neurons (Fig. 3.10A). From

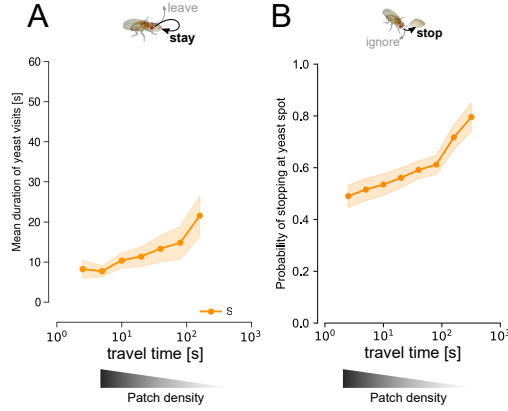


Figure 3.9: Time travelled between patch encounters (travel time) modulates foraging decisions. (A) Mean duration of yeast visits binned by travel time ($N = 44$, S). (B) Probability of stopping at yeast patches binned by travel time ($N = 44$, S). Each datapoint is the mean value for the respective time bin and shaded areas indicate $\text{mean} \pm \text{SEM}$.

this collection and other driver libraries (see Section 3.6 Materials & Methods), we chose 589 driver lines based on sparsity, anatomy, and existing literature. Specifically, we avoided lines with extensive expression in sensory and motor projections. Furthermore, we included lines with interneurons which are known to affect aspects of feeding, to test whether these might have additional functions in foraging. We decided to use genetic silencers to suppress neural activity in the neurons labelled by the chosen driver lines. Specifically, we combined the inward rectifier potassium channel Kir2.1 (Baines et al., 2001) with a tubGAL80ts construct, which allows us to temporally control when the silencer is expressed in the neurons.

For each day, we tested 5-10 GAL4 lines together with a day control, which is a GAL4 insertion that does not label any neurons (Empty-GAL4). From our analysis pipeline, we computed per-fly statistics for 216 behavioral parameters (Fig. 3.10B), that describe foraging, feeding and general locomotor behaviors. Most of these parameters have been defined as in Corrales-Carvajal et al. (2016). The strength of our setup is that we can use

both thermo- and optogenetic manipulations together with a detailed, unbiased behavioral characterization, which provides not only more flexibility for temporal control, but can also be used for further validation while allowing us to also uncover unexpected phenotypes induced by the targeted neuronal manipulations. From the screen, we generated a large dataset consisting of more than 15000 flies, for which we computed the behavioral parameters (Fig. 3.10C). Our focus is on detecting phenotypes affecting specific exploratory and exploitative parameters altered upon amino acid deprivation changes towards the proteinaceous resource.

3.4.5 The whole-screen distribution for total duration of micromovements on yeast is skewed towards more exploitation

We first analyzed the screen distribution of the total duration of micromovements for both of the substrates (10% yeast and 100 mM sucrose). This parameter is correlated to the total amount of food intake observed on the given nutrient during the duration of the one-hour foraging assay (Corrales-Carvajal et al., 2016). We sorted GAL4 lines according to their median value of total duration of micromovements on yeast in ascending order (Fig. 3.11A). The day control lines exhibit some variation in the median value, but they populate around the mean of observed values for median total duration of yeast micromovements. Interestingly, the distribution has a skew towards increases in yeast feeding, indicating that the neurons targeted by our manipulations mediate a satiety state. We also analyzed the screen distribution for total duration of sucrose micromovements (Fig. 3.11B) sorted in the same order as in Fig. 3.11A. It reveals that many of the effects of the neuronal silencing on total duration of micromovements are nutrient-specific. In order to effectively detect lines with statistically significant and large effects for given behavioral parameters, estimated by large and robust effect sizes, we applied statistical methods to threshold and as such filter the dataset (Fig. 3.11C) to detect candidate lines for retesting. Specifically, we employed a multi-layered decision tree (Fig. 3.11C), in which we first test for strong

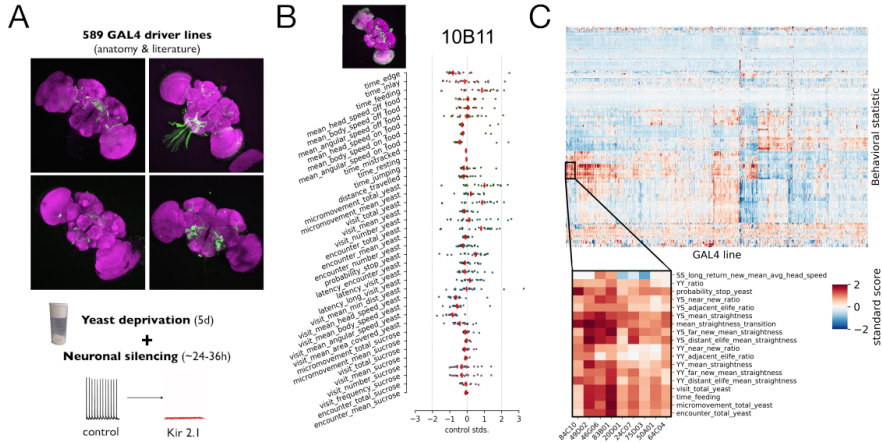


Figure 3.10: Large-scale neuronal silencing screen of sparse Janelia Generation 1 GAL4 driver lines and big-data statistical analysis pipeline for rapid automated discovery of hit driver lines. (A) 589 GAL4 driver lines created by the HHMI Janelia Research Campus have been chosen based on sparsity, anatomy and existing literature. Each of these driver lines was crossed to an effector Kir2.1TubGAL8ots, which silences neuronal activity in the neurons labelled by the driver line. Per line, we tested 16-24 mated female flies, which were yeast-deprived on 100 mM sucrose for 5 days. (B) Standardized summary statistics for an example GAL4 driver line 10B11. Using automated behavioral analysis Fig. 3.1C, we computed 216 behavioral parameters for each line tested (not all shown here). We normalized the population statistics of the behavioral parameters, so that we can not only compare each line to its day control, but also to other tested lines across the whole screen. (C) Heatmap of standardized effects of silencing 589 GAL4 lines on 216 behavioral parameters. The inlet shows details of specific GAL4 lines and behavioral parameters. See methods for details on quantifying behavioral parameters.

effect sizes, and then test whether these effects of silencing were significant compared to its day control. Finally, we further retested and validated the detected lines to confirm their behavioral effects upon silencing.

CHAPTER 3. LARGE-SCALE NEUROGENETIC SCREENING TO IDENTIFY NEURONAL SUBSETS INVOLVED IN FORAGING

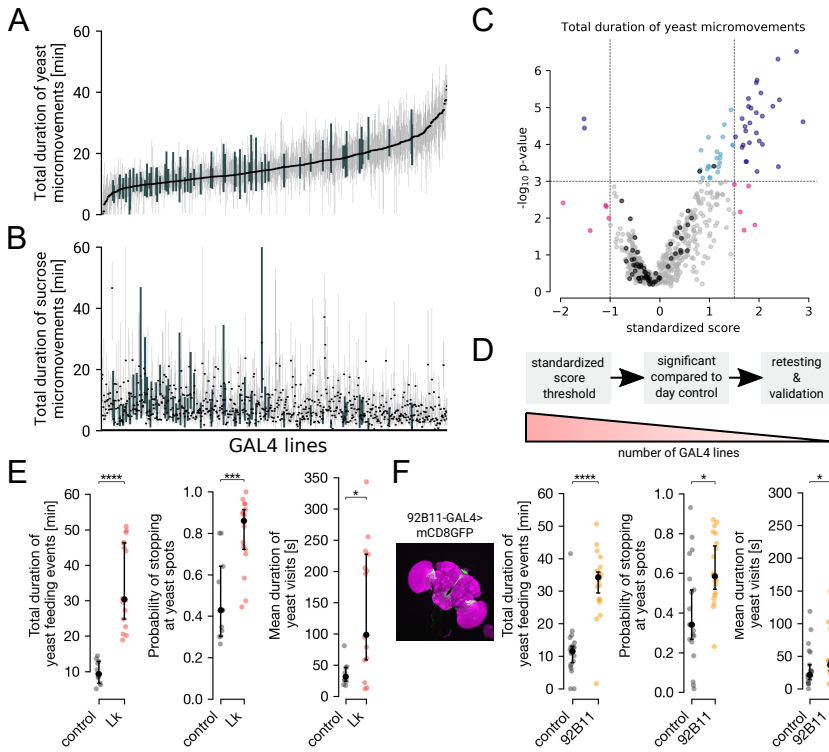


Figure 3.11: Analysis of total yeast micromovements of silencing screen reveals increased exploitation in neurons representing satiety signals. (A) Screen curve of all tested GAL4 lines sorted by the median value of total duration of yeast micromovements. Day controls are shown in dark-gray. (B) Screen curve for the total duration of sucrose micromovements across all tested GAL4 lines ordered the same as in (A). (C) Volcano plot displaying the thresholding to detect hit lines. We adjusted the thresholds of p-valued significance and standardized scores to discover robust and strong effects in the tested lines (purple dots). (D) Decision tree for detecting hit lines. (E) Total duration of yeast micromovements, probability of stopping at yeast and mean duration of yeast visits for Lk-GAL4>UAS-Kir2.1TubGAL8ots. (F) GFP expression pattern of 92B11-GAL4. Graphs as in (E) for 92B11-GAL4>UAS-Kir2.1TubGAL8ots. We used Wilcoxon rank sum test for calculating p-values to assess statistical significance. ns not significant. * $p < 0.05$. *** $p < 0.001$. **** $p < 0.0001$.

3.4.6 Silencing broad neuromodulatory populations leads to specific effects

Using this detection pipeline, we showed specific lines which increase food intake upon silencing. Both of these lines are known to be satiety signalling neurons. Leucokinin (Lk) signalling has been shown to regulate the sustenance of feeding bouts (Al-Anzi et al., 2010), as well as postprandial sleep (Zandawala et al., 2018). We show that silencing Lk-expressing neurons leads to an increase in general exploitation, measured by total duration of yeast micromovements, probability of stopping at yeast, and mean duration of yeast visits (Fig. 3.11E). The line 92B11 is a sparse line that labels neurons of the dorsally located pars intercerebralis (Fig. 3.11F). This region is known to house insulin-producing cells (IPCs), which have been shown to regulate various aspects of feeding behavior, and expression levels of insulin-like peptides is increased in the fed state (Sudhakar et al., 2020). Indeed, we showed that the neurons labelled by 92B11-GAL4 were overlapping with immuno-positive signals of dILP2, a specific subtype of IPC-secreted peptides (data not shown here). Like for Lk, silencing 92B11-expressing neurons leads to an increase in total duration of yeast micromovements, probability of stopping at yeast, and mean duration of yeast visits. Our results indicate that both Leucokinin- and 92B11-positive neurons are also involved in regulating exploitation during nutrient foraging (Fig. 3.11E-F) and might act as satiety signals upon different downstream circuits controlling different foraging behaviors.

3.5 Discussion

In this chapter, we created an experimental setup for MoHiVE tracking to study the foraging choices made by walking *Drosophila* to achieve nutrient homeostasis. We used this setup to leverage the genetic toolkit of *Drosophila melanogaster* in order to perform a large-scale neurogenetic silencing screen to test the effect of inactivating different neuronal subsets on different aspects of foraging behavior.

In order to perform the large-scale screen, we optimized an existing setup Corrales-Carvajal et al. (2016) for scalability, robustness and modularity. Specifically, we scaled the setup to run a total of 12 setups simultaneously connected to four independent PCs. I developed an automated analysis pipeline, that tracks the movements of the flies within the arena to quantify foraging decisions. While there have been many setups that have been built for large-scale, high-throughput behavioral testing and screening (Alisch et al., 2018; Geissmann et al., 2017), our framework is novel by not only including spatially distributed food patches, but also record behavior in a larger arena at high spatial and temporal resolution. Furthermore, recent advances in hardware and optics technology allowed for even higher spatial and temporal resolution in a large arena (Thomson et al., 2021), but these rely on multiple imaging sensors and objectives reducing the potential scalability of such a system for high-throughput behavioral testing.

3.6 Materials & Methods

3.6.1 Fly husbandry & standard diet

Flies were reared on yeast-based medium (per liter of water: 8 g agar [NZYTech, Portugal], 80 g barley malt syrup [Próvida, Portugal], 22 g sugar beet syrup [Grafschafter, Germany], 80 g cornflour [Próvida, Portugal], 10 g soya flour [A. Centazi, Portugal], 18 g instant yeast [Saf-instant, Lesaffre, France], 8 ml propionic acid [Argos], and 12 ml nipagin [Tegospet, Dutscher, UK] [15 % in 96% ethanol] supplemented with instant yeast granules on the surface [Saf-instant, Lesaffre, France]) and kept at 25°C, 70% relative humidity in a 12 hr-12 hr light-dark cycle.

3.6.2 Internal state manipulations & holidic media

To study the effects of nutrient-specific deprivation on foraging behavior, we employed diets based on holidic media, developed in collaboration with the laboratories of Linda Partridge at UCL and Matthew Piper at Monash University (Piper et al., 2014; Piper et al., 2017). Detailed protocols have been

published and described in detail (Leitão-Gonçalves et al., 2017; Santos et al., 2017). We specifically used the media named *FLYAA* with following conditions:

- **SAA**: Holidic media with all ingredients as indicated in the protocol (Santos et al., 2017),
- **S**: Same as **SAA**, but without any amino acids (removing isoleucine, leucine, tyrosine, eAA & neAA mix, glutamate, and cystein),
- **AA**: Same as **SAA**, but without any sugars (removing sucrose),
- **O**: Same as **SAA**, but without any amino acids and sugars (removing all parts described for **S** and **AA**).

For the large-scale silencing screening, yeast deprivation was induced by transferring 2-6 days old flies to vials containing a half paper tissue soaked with 5 ml of 100 mM sucrose solution for 10 days. Fully fed flies were age-matched. All flies were transferred to vials with the respective fresh medium every two days. We ensured a mated state in females by collecting 10 female flies together with 5 male flies upon sorting.

3.6.3 Fly stocks & genetics

The list of all stocks and genotypes used in this study are shown in Table A.1. We obtained stocks from Bloomington Drosophila Stock Center (NIH P40ODO18537), HHMI Janelia Research Campus, Vienna Drosophila Research Center, and Kyoto Stock Center.

3.6.4 Behavioral assays in the large-scale screen

We sorted 10 0-4 days old, female flies and transferred them to vials containing fresh standard yeast-based medium (YBM) together with 5 males to ensure that females are mated. A day later, all vials were transferred to fresh YBM. Another day later, all vials were transferred to vials filled with

a paper tissue soaked with 5 ml of 100 mM sucrose water. 36-48 hours before the behavioral assays, we induce the genetic expression of the inward rectified potassium channel *Kir2.1* (Baines et al., 2001) using *TubGAL80_{ts}* as temperature-sensitive induction by transferring flies to an incubator at 30°C and 70% humidity.

Single flies were tested by in individual arenas that contained two kinds of food patches: 10% yeast and 100 mM sucrose with 1% agarose. Flies were individually transferred to the arenas by mouth aspiration and allowed to feed for 1 hour. Tracking videos were acquired using the Bonsai framework (Lopes et al., 2015) and analyzed in Python v3 using custom-written software. To avoid patch exhaustion before the end of the tracking assays, each circular patch contained 5 μ l of food with a diameter of approximately 3 mm. After each assay, the tracking arenas were washed with soap, and rinsed with a pressured stream of distilled water.

3.6.5 Image-based tracking setup

We built a high-throughput tracking setup that can record four flies in individual arenas using a custom-built acrylic arena holder. Arenas were placed above an IR backlight for video recording using a FLIR Flea3 camera (FL3-U3-32S2M-CS) acquiring videos at 30 frames per second and a resolution of 1408 px $\times$ 1408 px. This resolution resulted in an average real-world pixel resolution of 7.8 px per mm.

3.6.6 Tracking algorithm

We used a custom, real-time tracking algorithm using the Bonsai framework (Lopes et al., 2015). Fly centroids and trajectories were extracted using a background subtraction method, and for each recorded frame we measured the x- and y-position of the centroid, major and minor axis as well as the angle of the centroid. We also developed custom-written Python scripts to determine the head position of the fly in an offline algorithm. This algorithm compares the pixel intensity of both extreme points of the centroid to determine the head, which is darker than the tail position due to the

transparency of the wings. We then propagated the head position by applying a proximity rule (Gomez-Marín et al., 2011), in which the position closer to the previous head position is assigned as head position. This rule becomes invalid when flies jump, therefore we detected jumps as segments to reapply the pixel intensity comparison. We further validated correct head position assignment, by comparing the movement vector to the heading vector, which should largely align because flies mostly walk forwards. Using this head detection method, we validated the accuracy by manual visual inspection to be correct in more than 98% of frames.

3.6.7 Behavioral classification

From the head and body trajectories, we calculated the displacements between each of the consecutive frames. By dividing these displacements by the time interval between the frames, we obtain the translational speeds of head and centroid positions. We subsequently filtered the speed time series using a Gaussian kernel with a window length of 36 frames and a standard deviation of 3.6. Similarly, we calculated the rotational speed from displacements in the angle of the centroid to head relative to the reference frame. Based on these kinematic variables, we used threshold values to classify behavioral segments. "Walking" segments are defined by instances, in which the fly's head speed is above 2 mm/s, while "Jumps" are above 20 mm/s. "Turns" are defined by instances, in which the fly's rotational speed is larger than 125 °/s. "Resting" segments are instances, in which the fly's head speed is lower than 0.2 mm/s. "Micromovements" are instances, which have not been so far classified otherwise, and as such the head speed falls into the range of 0.2 to 2 mm/s. Micromovements which are performed closer than 2.5 mm to a yeast patch are considered "yeast micromovements", while those closer than 2.5 mm to a sucrose patch are called "sucrose micromovements". We filtered out spurious high body speeds that rarely occur during micromovements.

3.6.8 Patch encounters and visits

"Encounters" are segments, in which the fly is closer than 3 mm to a given food patch, regardless of whether feeding occurs or not. "Visits" are all micromovements that occur on the same food patch without the fly leaving a distance larger than 5 mm from the patch. The "probability of stopping at a food patch" is calculated by dividing the total number of visits by the total number of encounters. Since each encounter can at most have one visit, the resulting ratio is between 0 and 1, and therefore can be described as a probability. For patch leaving decisions, we calculated the "mean duration of visits" for all patches of each substrate. The "ratio of returns to the same food patch" is calculated by dividing the number of returns to the same patch divided by the total number of subsequent visits of the same substrate.

3.6.9 Travel time analysis

We calculated the travel time for each resource encounter as the time duration from the end of the last encounter of a resource of the same substrate, regardless of whether a visit occurs, to the beginning of the current encounter. We binned the observed travel times to obtain the probability of stopping. Bins were determined by a $\log_{10}$ scale and overlapping to increase sampling across all bins. The final lineplots are plotted with markers indicating mean values for the respective bin (placed on the left side of the bin) as well as shaded areas indicating the Standard Error of the Mean (SEM) around the mean.

3.6.10 GAL4 lines

GAL4 lines were constructed and characterized at the Janelia Research Campus as described in (Jenett et al., 2012). We selected GAL4 lines based on anatomical neuropil targeting and sparseness based on publicly available confocal imaging data¹. All GAL4 drivers tested in this study are listed in

¹Flylight link: <https://flweb.janelia.org/cgi-bin/flew.cgi>

Table A.1. For testing the GAL4 lines, we used an Empty-Gal4 driver line (BDSC #68384) as control.

3.6.11 Split-GAL4 lines

Split-GAL4 lines were constructed and characterized by the Rubin lab as in previous work (Pfeiffer et al., 2010). Briefly, we tested candidate AD and DBD hemidriver pairs (Dionne et al., 2018; Tirian & Dickson, 2017) for expression in cell types of interest and assembled successful combinations into stable fly strains that were then used for subsequent experiments. Split-GAL4 lines generated in this study and images of their expression patterns are available online (<https://www.janelia.org/split-GAL>). All Split-GAL4 drivers created in this study are listed in Table A.1. For testing the Split-GAL4 lines, we used an Empty Split-Gal4 driver line (BDSC #79603) as control.

3.6.12 Statistical analysis

For all statistical analyses, if not otherwise stated, we use non-parametric, two-sided hypothesis testing (i.e., Wilcoxon-Mann-Whitney ranksum tests). Specifically, we used the SciPy (v.1.8.0, Virtanen et al. (2020)) implementation of the function `stats.ranksum`.

3.7 Acknowledgments

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CHAPTER 3. LARGE-SCALE NEUROGENETIC SCREENING TO IDENTIFY NEURONAL SUBSETS INVOLVED IN FORAGING

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CHAPTER

4

DORSAL FAN-SHAPED BODY NEURONS
INTEGRATE NUTRIENT STATE TO
REGULATE THE TRANSITION FROM
EXPLORATION TO EXPLOITATION

Contributions

This chapter is a manuscript in preparation for submission and has been written by me, Carlos Ribeiro and Ibrahim Tastekin. I (DG) am the main first author, and co-authors are Ibrahim Tastekin (IT), Jin-Yong Park (JYP), Daniel Münch (DM), Hannah Haberkern (HH), Célia Baltazar (CB), Lúcia Serra (LS), Ann M Hermundstad (AMH), Vivek Jayaraman (VJ), Gerald M Rubin (GMR), and Carlos Ribeiro (CR, corresponding author).

Detailed contributions are as follows:

	DG	IT	JYP	DM	HH	CB	LS	AMH	VJ	GMR	CR
Conceptualization	■	■		■							■
Data curation	■	■	■	■						■	
Formal analysis	■	■	■	■	■						■
Investigation	■	■	■	■	■	■	■				
Methodology	■	■	■	■	■					■	■
Project Administration	■	■									■
Resources	■	■								■	
Software	■	■									
Supervision	■	■						■	■	■	■
Validation	■	■	■	■	■	■	■				
Visualization	■	■	■							■	■
Writing—Original Draft	■	■									■
Writing—Reviewing & Editing	■	■	■	■	■	■	■	■	■	■	■
Funding acquisition								■	■	■	■

Summary

Foraging animals must balance the costs of exploring their surroundings with the potential benefits of finding nutritional resources. Upon finding a food source, they have to make the central decision of whether to stop and initiate feeding or to continue searching for potentially better options. Foraging theory predicts that this decision should be dependent on an estimate of the density of available resources in the environment. How this complex computation is algorithmically implemented in the brain remains poorly explored. Here, we use video-based tracking to show that *Drosophila melanogaster* regulates the decision to stop at food spots based on the density of food patches in the environment. To answer how underlying neuronal circuits may implement this computation algorithmically, we performed a large-scale neurogenetic silencing screen of more than 500 different genetic driver lines with sparse expression patterns in the brain. We identified a population of neurons in the fly central brain that acts as a key regulator of the decision to stop. Specifically, we show that manipulating the activity in these neurons increases not only the probability of stopping at food spots, but also alters their ability to adjust this foraging variable to the estimated resource density. Our results reveal a neuronal substrate involved in modulating ethologically relevant foraging decisions, a key step towards a mechanistic explanation of cognitive functions required to solve complex cost-benefit trade-offs.

4.1 Introduction

Animals live in dynamic and uncertain environments, and survive by gathering vital resources, such as food, mates and shelter (Hills et al., 2015). Most of them do so by means of foraging their environment. Importantly, foraging decisions must be guided not only by external cues, but also by internal needs of the animal in order to attain homeostasis (Corrales-Carvajal et al., 2016; Dethier, 1976; Dietrich et al., 2015). Therefore, they developed nutrient-specific appetites where current and predicted future nutritional needs are

translated into foraging and feeding decisions (Chen et al., 2015; Corrales-Carvajal et al., 2016; Itskov et al., 2014; Walker et al., 2017). It has been shown that deviations in nutrient-specific appetites have serious implications on health and reproductive fitness (Grandison et al., 2009; Solon-Biet et al., 2014). While recent work has investigated internal state-dependent modulation of gustatory sensory neurons (Inagaki et al., 2014; Marella et al., 2012; Steck et al., 2018) and sensorimotor neuropil-wide processing (Münch et al., 2022; Shiu et al., 2022; Snell et al., 2022), how nutrient-specific appetites are implemented by higher-order processing and cognitive computations are largely unknown.

As animals encounter resources, such as a food patch, they face the key decision of whether to exploit it or further explore to find possibly better ones, a problem commonly referred to as exploration-exploitation trade-off (Sutton & Barto, 1998). This trade-off has been studied extensively in behavioral ecology and computational neuroscience and is thought to be central to decision-making and risk assessment in most animals (Addicott et al., 2017; Dall et al., 2005). As such, animals have shown to optimally adjust their behavior to solve this trade-off (Kacelnik & El Mouden, 2013). Despite its relevance, the neuronal circuit mechanisms underlying exploration-exploitation trade-off computations remain unknown. During foraging, animals make decisions, such as whether to engage in a found resource (patch engagement) and when to leave a resource (patch leaving). The idea that animals may adjust foraging decisions in an “optimal” way in order to maximize fitness has led to the formulation of the Marginal Value Theorem (MVT) (Charnov, 1976), which postulates that an animal adjusts the time when to leave a patch depending on the time that it takes to travel between patches. In doing so, the theorem implies that an animal internally estimates resource density to adjust its foraging decisions. Indeed, many studies have shown this qualitative relationship in animals ranging from vertebrates — like mammals (Cassini et al., 1990) and birds (Cowie, 1977; Kacelnik, 1984) — to insects, including flies (Koganezawa et al., 2009; Parker, 1992; Shimada et al., 1993). However, other studies also showed quantitative deviations from optimal foraging (Pyke, 1984), where animals either

stay longer (Howell & Hartl, 1980) or shorter (Pleasants, 1989) than predicted by the MVT, and generally exhibit high variability. Such deviations might be due to variability in internal states and environmental factors (Nonacs, 2001). Furthermore, while behavioral and ecological studies have shed light on potential algorithmic explanations of how optimal foraging could be computed, its mechanistic implementation in terms of neuronal circuit computations is largely unknown.

Recent advances in software and hardware technologies allow for versatile behavioral and physiological assays ensuring both high throughput and high resolution (Alisch et al., 2018; Barlow et al., 2022; Mathis et al., 2018). Combined with unprecedented neurogenetic tools (Klapoetke et al., 2014; Pfeiffer et al., 2010) and connectomes at synaptic resolution for *Drosophila melanogaster* (Dorkenwald et al., 2022; Scheffer et al., 2020), high-resolution behavioral analysis enables rapid identification of neuronal circuits that are involved in complex, naturalistic behaviors (Ache et al., 2019; Coen et al., 2014; Hindmarsh Sten et al., 2021; Tastekin et al., 2015). Foraging decisions require the integration of information from multiple sensory modalities, past experiences and internal states. The central complex (CX), a higher-order brain neuropil in the insect brain, is a suitable candidate region for such complex decision-making to occur, because it does not only integrate multisensory inputs (Okubo et al., 2020) but also generates cognitive behaviors such as learning and navigation (Ofstad et al., 2011; Seelig & Jayaraman, 2015). Furthermore, it has been shown that CX also integrates metabolic states to regulate sleep (Donlea et al., 2014; Donlea et al., 2018; Liu et al., 2016) and feeding choices (Musso et al., 2021; Park et al., 2016; Sareen et al., 2021). Anatomically, the CX consists of four unpaired, conserved neuropils: the protocerebral bridge (PB), the fan-shaped body (FB), the ellipsoid body (EB) and the noduli (NO). Recent studies have extensively identified and characterized cell types and anatomical connectivity in the fly CX (Hulse et al., 2021). As such, the CX has become a tractable neuropil for integrating sensory and internal state signals and generating adaptive behavioral policies that help solve the complex decision-making underlying foraging

decisions. However, which neurons and circuits are involved in these computations are still unknown.

In Chapter 3, we performed a large-scale neuronal silencing screen to identify neuronal subsets involved in foraging decisions. To achieve this, we built an experimental setup for high-throughput video-based tracking of individual flies foraging in an arena with yeast and sucrose patches (Corrales-Carvajal et al., 2016). We chose and tested more than 500 GAL4 driver lines and more than 15000 flies, which revealed a neuronal population projecting to the dorsal fan-shaped body (dFB) important for the transition from exploration to exploitation. Silencing these neurons leads to an increase in the probability of stopping at food patches, while other foraging parameters and the overall food intake remains unaffected. Likewise, acute optogenetic activation while the animal approaches the food patch lead to a suppression of initiating feeding. We further investigated which neuronal subsets of this population are responsible for the phenotype and showed that a single pair of dFB neurons (FB6A) is required to suppress the initiation of feeding upon food encounter. More strikingly, whole-cell patch-clamp recordings showed that the firing rate in the FB6A neurons is modulated by the protein state of the fly, such that protein deprivation decreases the firing rate of these neurons. Finally, we showed that environmental changes of the nutrient density modulates the probability of stopping at food patches in wild-type flies, and that silencing dFB neurons abolishes the underlying relationship between the travel time between food patches and the resulting probability of stopping upon food encounter as predicted by the MVT (Charnov, 1976). Our work unravels a higher-order neuronal substrate that is key in integrating both internal nutrient needs and external nutrient availability to regulate a behavioral policy underlying transitions from exploring the environment to exploiting an encountered food patch. Both foraging and nutrient homeostasis are crucial computational processes existing across animal phyla, and underlying computational and algorithmic principles by which they are regulated are likely to be conserved. Therefore, our work provides novel insights into the circuit basis of foraging decisions, and how they aid the animal

to maintain nutrient homeostasis that can be generalized to other animals including humans. Furthermore, many brain processes underlying specific aspects of foraging, such as the balance between exploration and exploitation can be seen as the blueprints of cognition. Our and future work will therefore provide mechanistic insights into how complex computations are implemented at the circuit level and will provide a basis to understand how proto-cognitive functions might have evolved.

4.2 Results

4.2.1 A neurogenetic silencing screen identifies neuronal subsets required for the decision of transitioning from exploration to exploitation

In order to identify neuronal elements that regulate foraging decisions, we performed a large-scale silencing screen, in which we inactivated neuronal subsets labeled by different GAL4 driver lines in the central brain and measured the effects on foraging parameters defined in Chapter 3. Given the importance of understanding how the animal balances the exploration-exploitation trade-off, we decided to focus on the decision to stop and start feeding at a food patch (captured by the probability to stop at a yeast patch). Specifically, of the 589 tested GAL4 lines in total, we queried 434 GAL4 driver lines from the Janelia Generation 1 library (Jenett et al., 2012), which were selected with a focus on lines with a sparse expression pattern and together targeting a wide repertoire of neuropils (Fig. A.1). We inactivated neurons labeled in the GAL4 lines by inducing the expression of the hyperpolarizing ion channel Kir2.1 in these neurons using a temperature-inducible strategy (Baines et al., 2001). Protein-deprived flies with silenced neurons, were recorded using our setup for one hour while foraging in the arena containing yeast and sucrose patches (Fig. 3.1A). We calculated the probability to stop at a yeast food patch for each line as well as corresponding genetic controls tested within the same batch. The resulting distribution for this behavioral parameter was centered around the median of all control flies

CHAPTER 4. DORSAL FB NEURONS REGULATE THE TRANSITION FROM EXPLORATION TO EXPLOITATION

tested and spanned a wide phenotypic range of values with an enrichment for lines showing an increase in the probability to stop (Fig. 4.1A). We then performed a bootstrapping analysis for phenotypes deviating significantly from the whole-screen population distribution leading to the identification of 49 lines (Fig. 4.1B-C, see Methods for details). 32 of these showed a significant difference to the control lines tested within the same batch (Fig. 4.1C). Based on multiple rounds of retests, 3 lines were selected as showing a reproducible increase in the probability of stopping at a yeast patch while also displaying a sparse expression pattern in the brain (Fig. 4.1D).

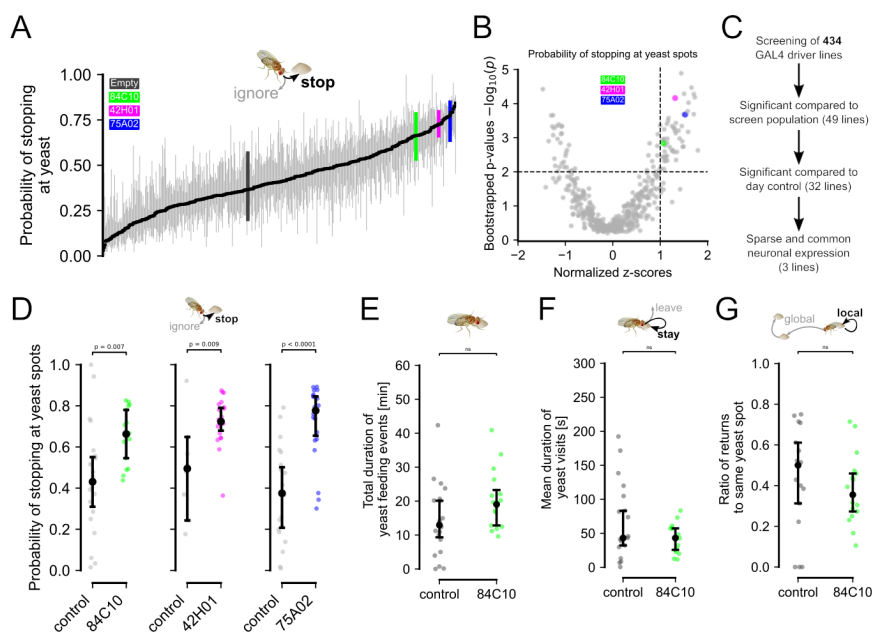


Figure 4.1: Screen distribution and statistical pipeline to identify lines of interest for increased probability of stopping at yeast patches.

We next wondered if silencing the labeled neurons in these three lines led to other foraging phenotypes. Strikingly, other foraging parameters, such as the mean duration of yeast visits, i.e. the decision to leave a patch (Fig. 4.1F), and the ratio of returns to the same yeast spot, i.e. the spatial organization of exploration (Fig. 4.1G) were not affected when silencing the neurons labeled in these GAL4 driver lines. This suggests that the

silencing phenotypes are specific to the transition from exploration to exploitation, but not other aspects of the exploration or exploitation phases in general. Furthermore, since the total duration of micromovements (a proxy for total food intake) (Fig. 4.1E) are not affected by the silencing, it strongly suggests that the labeled neurons are not involved in setting the overall nutrient state of the fly. These results were further confirmed by using the flyPAD assay (Itskov et al., 2014), which directly measures the total amount of feeding (Fig. 4.2). We therefore identified three Gal4 lines which label neurons important for mediating the decision to stop at a food spot and hence balancing the exploration-exploitation trade-off. Given the specificity of the phenotype these data also suggest that different foraging decisions are controlled independently by different neuronal populations.

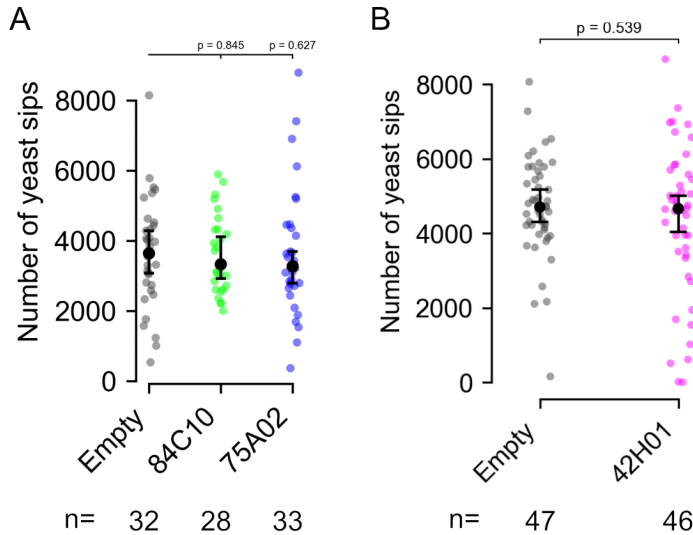


Figure 4.2: Number of sips on yeast are unaffected upon silencing dFB neurons. (A) Total number of sips on 10% yeast for dFB-silenced (75A02- and 84C10-GAL4) flies compared to control flies (Empty-GAL4) after yeast deprivation. (B) Experiments of silencing 42H01-GAL4 have been carried out on another day, therefore has been compared same-day control flies (Empty-GAL4). Wilcoxon ranksum test has been used to compute shown p-values.

4.2.2 Dorsal fan-shaped body neurons involved specifically control the decision to stop at food patches during foraging

Close inspection of the labeling pattern of the three identified Gal4 lines in the brain, suggested that they overlap in the dorsal fan-shaped body (dFB) (Fig. 4.3A-C). Registering these expressions onto a standard template brain (JFRC2) revealed that they have overlapping projections to the dorsal FB layer 6 and the Superior Neuropils (SNP) with cell bodies located in the lateral posterior part of the protocerebrum (Fig. 4.3D). The dFB is part of the central complex (CX), an insect higher-order brain region involved in orientation and adaptive navigation behaviors (Ofstad et al., 2011; Seelig & Jayaraman, 2015). Intriguingly, the FB and the CX in general have also been involved in regulating sleep (Donlea et al., 2018), feeding (Musso et al., 2021; Sareen et al., 2021), and other behaviors (Matheson et al., 2022). This suggests that its role extends beyond navigation and that its computations facilitate higher-order internal, spatio-temporal representations orchestrating sensorimotor transformations and state-dependent action selection (Adden et al., 2022; Rayshubskiy et al., 2020). The types of computations the dFB performs and how they control behavioral decisions in general and specifically foraging remains poorly understood.

Previous literature suggests a role of dFB neurons in regulating sleep. Importantly, silencing other dFB lines like 23E10-, C205- and 104y-GAL4, which have been shown to alter the sleep state of flies (Donlea et al., 2014; Donlea et al., 2011; Wang et al., 2022) did not lead to a phenotype in the probability of stopping at yeast (Fig. 4.4A). This suggests that changing or perturbing the sleep state through dFB neuronal silencing is not sufficient to induce the foraging phenotypes we observed. Furthermore, confocal imaging of GFP expression revealed that these driver lines do have extensive genetic targets outside the dFB that could potentially lead to the reported phenotypes (Fig. 4.4B-D). Altogether, these findings suggest that the neurons labeled in these three dFB lines specifically control the probability that the fly transitions from exploration to exploitation without affecting other foraging decisions. They also strongly suggests that modifying the

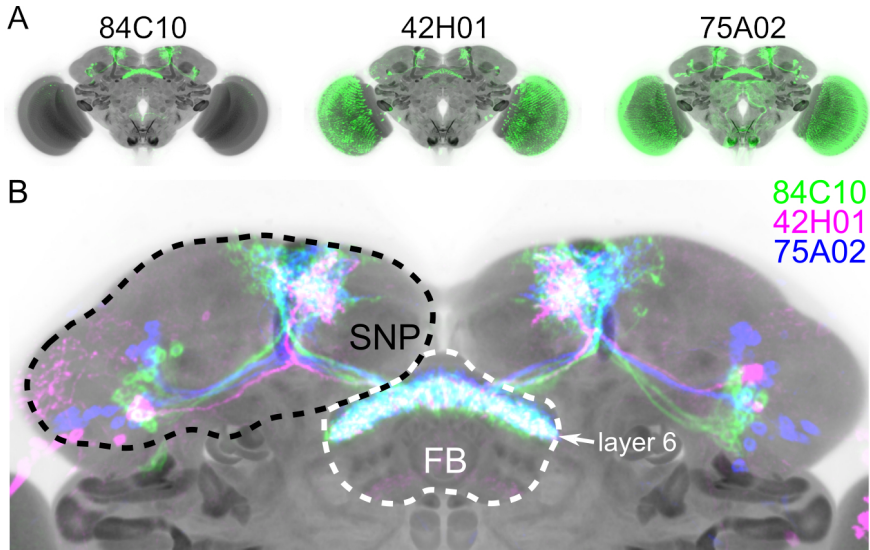


Figure 4.3: Anatomy of dFB GAL4 lines.

activity of these neurons does not alter the general nutritional state of the animal, which would lead to an overall change in most analyzed foraging parameters including the total amount of feeding.

4.2.3 A single pair of FB6A neurons is necessary for adapting the probability to stop at food

Our data raise the exciting possibility that the three identified lines label a common set of neurons projecting to the FB layer 6, which specifically controls the probability of the fly to stop at a yeast patch. However, because the genetic lines also exhibit expression outside the dorsal fan-shaped body, such as the SEZ and VNC, there remains the possibility that these off-target expressions could lead to the phenotypes observed. Nonetheless, this is unlikely as the off-targets are not overlapping between our three driver lines. Furthermore, as these lines contain multiple, possibly heterogeneously functioning neuron types, we therefore set out to test if that there could be subsets of FB6 neurons which produced the observed silencing foraging phenotype. We employed a split-GAL4 strategy (Dionne

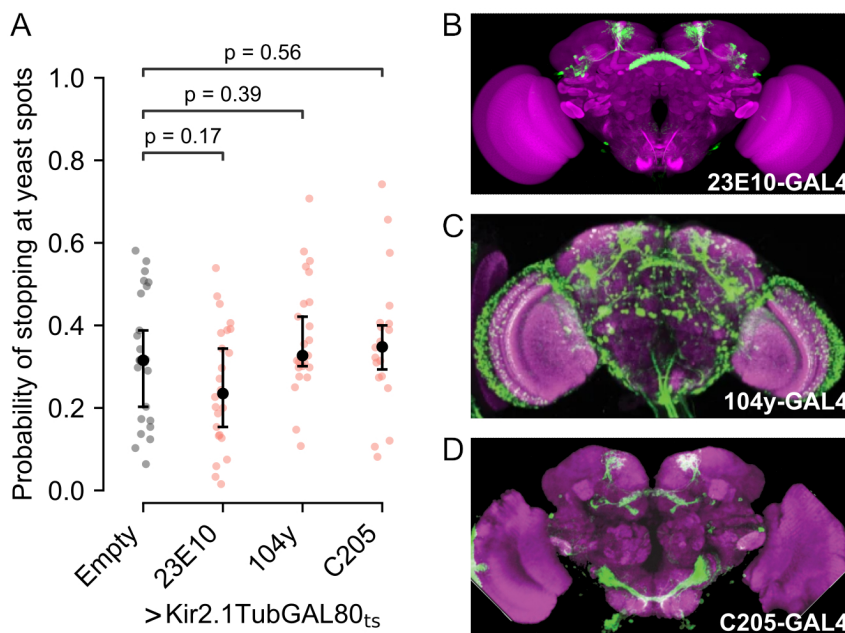


Figure 4.4: Silencing sleep-inducing GAL4 lines exhibits no changes in probability of stopping at yeast. (A) Probability of stopping at yeast spots for Empty- (control), 23E10-, 104y-, and C205-GAL4 expressing Kir2.1 silencing. P-Values were computed using Wilcoxon ranksum test. GFP expression pattern driven for (B) 23E10-, (C) 104y-, and (D) C205-GAL4. Confocal imaging data has been obtained and modified from Hu et al. (2018), Jenett et al. (2012), and Yap et al. (2017).

et al., 2018) to sparsen the number of dFB neurons expressed by the GAL4 lines identified in our silencing screen. For this, we created intersectional Split-GAL4 combinations based on our three identified dFB GAL4 lines.

Strikingly, two lines based on the intersection of 42Ho1- and 84C10-GAL4 resulted in a strong expression in a single pair of neurons projecting to the dFB layer 6 (Fig. 4.5A-B). Silencing these neurons led to a significant increase in the probability of stopping at yeast spots, comparable to the results obtained from silencing either 42Ho1- or 84C10-GAL4 lines (Fig. 4.5C & E). Of note, the effect size of the phenotype was slightly smaller, likely due to the sparser and weaker expression of GAL4 in the split-GAL4 lines.

Furthermore, the silencing phenotype in the decision to stop is specific, as other foraging decisions, such as deciding to leave a food spot were not affected (Fig. 4.5D & F).

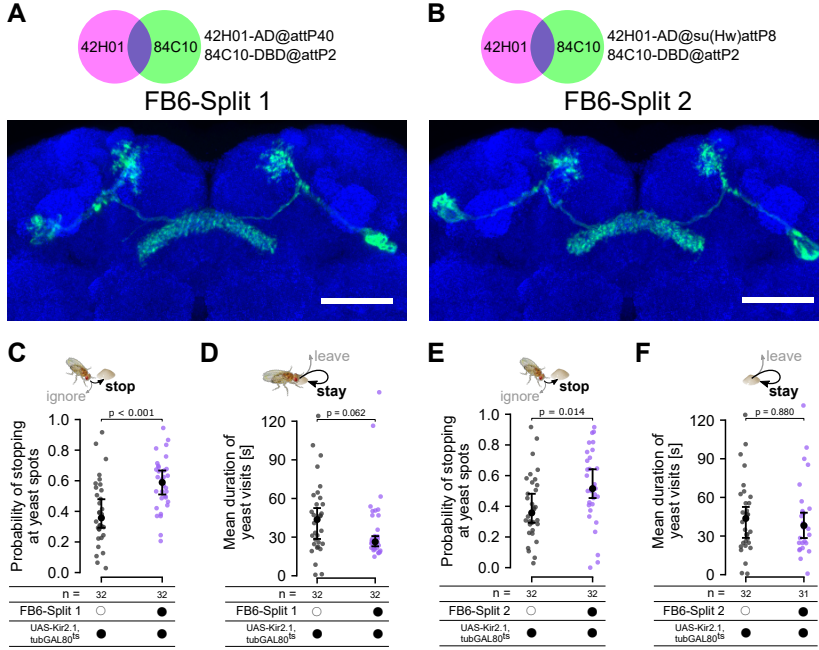


Figure 4.5: Split-GAL4 silencing reveal a single pair of FB layer 6 neurons required to decrease probability of stopping at yeast. (A-B) Using two hemidriviers of the 42H01- and 84C10-GAL4 lines at different landing sites, we created two split-GAL4 lines that label a single pair of neurons (one per brain hemisphere) with projections to SNP and FB layer 6. (C) Probability of stopping at yeast for FB6-Split 1 silenced by Kir2.1. (D) Mean duration of yeast visits (in s) for FB6-Split 1 silenced by Kir2.1. (E) Probability of stopping at yeast for FB6-Split 2 silenced by Kir2.1. (F) Mean duration of yeast visits (in s) for FB6-Split 2 silenced by Kir2.1. P-Values were computed using Wilcoxon ranksum test.

To further analyze the morphology of the two single-neuron split-GAL4 lines, we employed a computational pipeline to compare light microscopy (LM) images to defined cell types in the Janelia electron microscopy (EM) hemibrain dataset for identification (Fig. 4.6A). To do so, we performed

LM using a genetic tool called multi-color flip-out (MCFO) enabling us to stochastically label individual neurons and hence giving us access to fine anatomical and morphological features (Fig. 4.6B). These experiments revealed a class of neurons with characteristic branching in the SNP and FB layer 6 as well as characteristically the large somata. These neuroanatomical characteristics allowed us to identify these neurons as of the neuron type FB6A (Fig. 4.6E). We further validated our identification by applying NBLAST scoring (Costa et al., 2016) on a skeletonized neuron from our MFCO confocal images compared to FB6A as well as other FB layer 6 candidate neurons (Fig. 4.6C-D) classified by the Janelia & Google EM hemibrain dataset (version 1.2.1, Hulse et al. (2021) and Scheffer et al. (2020)). Note, that while there are 3 FB6A neurons per hemisphere, we only ever observed one of them labeled by either of the two FB6-Split GAL4 lines we identified. This suggests that while anatomically very similar, at the genetic, and therefore functional level, these might not be identical. We have therefore identified a subset of neurons projecting to the SNP and FB layer 6 as regulators of the probability of the fly to transition from exploration to exploitation. While our genetic dissection supports the importance of a specific neuronal cell type in mediating this computation, it is important to highlight that such complex foraging decisions are unlikely to be fully dependent on a single neuron, explaining for example more subtle silencing phenotypes. A more plausible explanation might be that FB6A is an important neuronal element, and part of a larger neuronal population, which perform the computations required for complex foraging decisions in a distributed way.

4.2.4 The firing rate and excitability of FB6A neurons is modulated by the internal protein state of the fly

What could be the processes necessitating the modulation of the probability to transition from exploration to exploitation and hence FB6A function? Within foraging theory, the budget theory predicts that foraging parameters should be dependent on the internal nutritional state of the animal (Kearney et al., 2013). Furthermore, internal states such as hunger, reproductive state,

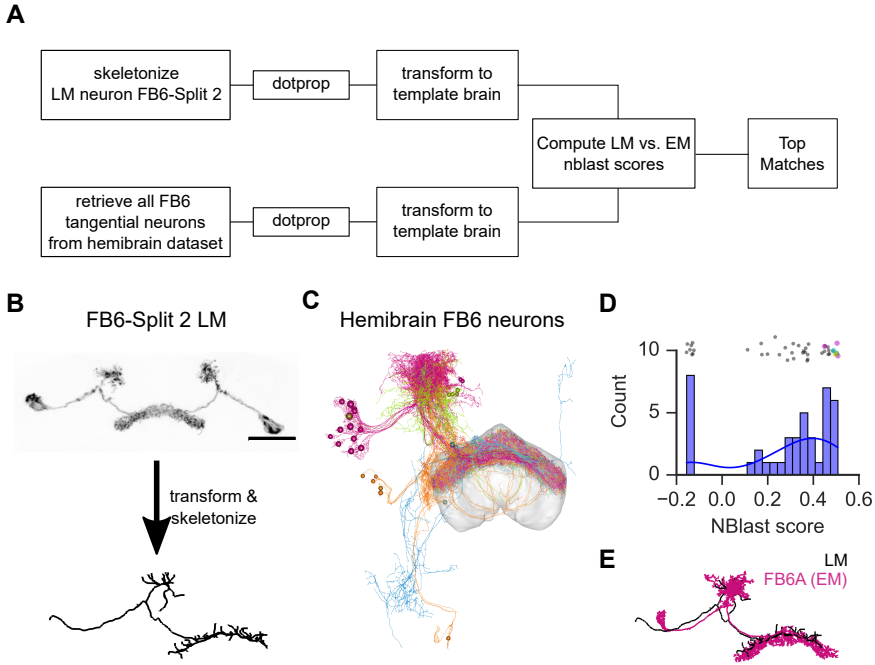


Figure 4.6: FB6 split-GAL4 lines label a single pair of FB6A neurons. (A) Using a computational pipeline, we compare LM imaging data to characterized cell types in the EM hemibrain dataset. (B) LM MCFO images are transformed and skeletonized in order to compare them to all (C) FB layer 6 neurons in the EM hemibrain dataset. (D) Matching scores using NBLAST (Costa et al., 2016) validated the identification of FB6A neurons in our FB6-Split GAL4 lines.

and the lack of specific nutrients have a profound impact on the behavior of animals (Münch et al., 2020). Accordingly, previous studies have shown that the sleep state as well as energy state of the animal affects activity in the CX, including the FB (Musso et al., 2021; Sareen et al., 2021). As we had shown that the AA state of the animal alters foraging parameters (Corrales-Carvajal et al. (2016) and Fig. 3.1), we therefore set out to test if activity in FB6A neurons is altered by protein deprivation.

We applied electrophysiological whole-cell patch-clamp recordings located at the soma of a single FB6A neuron (Fig. 4.7A). We showed that the

firing rate in the soma of FB6A have a higher frequency in fully fed compared to protein-deprived flies (Fig. 4.7B-D). Furthermore, the sub-threshold properties of FB6A resting potential also exhibited larger hyperpolarization in fully fed flies than in deprived ones (Fig. 4.7E).

4.2.5 Activity in dFB neurons is required for adapting patch engagement policies based on temporal estimates of patch density

Classical literature on optimal foraging theory (Charnov, 1976; Pyke et al., 1977) postulates that animals have evolved to adapt their foraging decisions on resource density to optimize long-term outcomes. Consequently, we asked whether dFB neurons as a key substrate for regulating the transition from exploration to exploitation, i.e. the decision to stop at food patches, could potentially also play an important link in the translation from resource density estimation to optimal behavioral policies following the MVT (Charnov, 1976). To test this hypothesis, we pooled data from the silencing screen and retests for two dFB GAL4 lines (75A02- and 84C10-GAL4), as well as control lines (Empty-GAL4). We binned the data into different travel time segments (see Materials & Methods for details on defining and computing travel times). For the probability of stopping at yeast patches, we showed that the Empty-GAL4 control flies exhibit a monotonously increasing relationship, as predicted by MVT (Charnov, 1976) and wild-type data (see Section 3.4.3). Strikingly, this relationship is abolished for both the 75A02- and 84C10-GAL4 silenced flies (Fig. 4.8A), which indicates that these flies are not able to adapt their foraging decision-making to the estimated resource density. However, this is specific to the decision to stop at a food patch, whereas patch leaving decisions exhibit monotonously increasing exploitation (staying longer) with respect to the travel time for the control flies as well as the dFB-silenced flies. Taken together, these data suggest that the dFB neurons are important in translating resource density estimated by the travel time between food patches into appropriate behavioral policies for the transition from exploration to exploitation as predicted

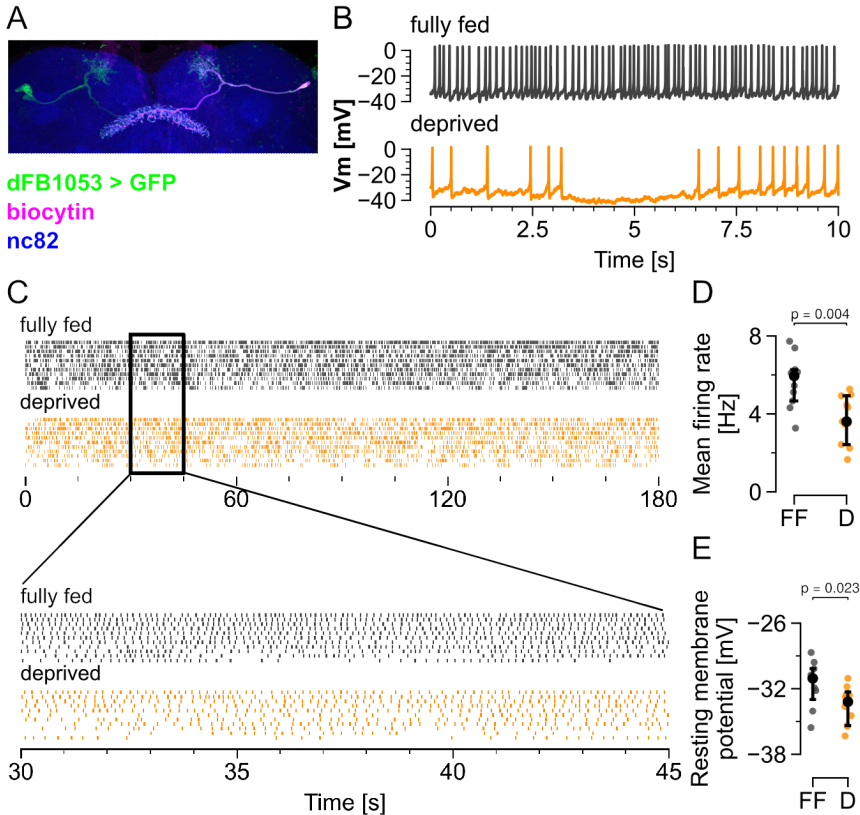


Figure 4.7: Firing rate and excitability is increased in fully fed compared to deprived flies. (A) GFP-targeted whole-cell patch-clamp recordings in FB6A neuron. Upon entry, biocytin injection indicates cell identity of recorded cell. (B) Example trace of membrane potentials with spikes from whole-cell patch-clamp recordings for fully fed (black) and protein-deprived (orange) conditions. (C) Raster plot of three-minute long recordings for all tested fully fed (black) and protein-deprived (orange) flies. Inset of 15-seconds long excerpt raster plot taken at 30 to 45 seconds. (D) Mean firing rate (in Hz) and (E) resting membrane potential (in mV) for fully fed (FF) and protein-deprived (D) flies. P-values were computed using Wilcoxon ranksum test.

by the MVT. Furthermore, the fact that this translation only affects patch engagement decisions, but not patch leaving hints at the possibility that the

dFB neurons more likely receive information about resource density rather than performing itself the computation for this estimation.

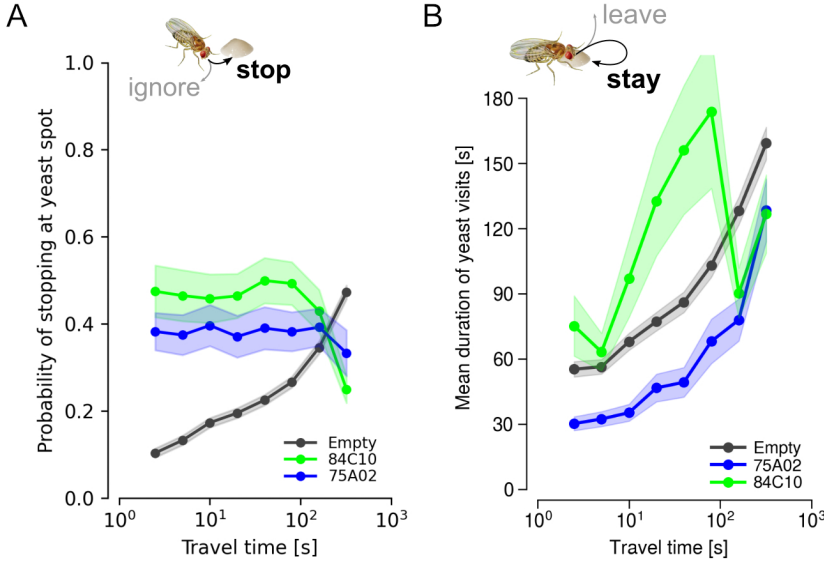


Figure 4.8: Silencing dFB neurons expressed by 84C10- and 75A02-GAL4 disrupts travel time-dependent increase in exploitation for stopping, but not staying. (A) Probability of stopping at yeast patches as function of travel time (in s). (B) Mean duration of yeast visits (in s) as function of travel time (in s). See Section 4.4 Materials & Methods for details on defining and computing travel times. Markers indicate mean values per bin and shaded areas indicate mean $\pm$ SEM.

4.3 Discussion

In this chapter, we analyzed the dataset acquired from performing a large-scale silencing screen described in Chapter 3 with a specific focus on the parameter of the *probability of stopping at yeast patch*, which relates to the decision parameter as the fly transitions from exploration to exploitation of a given nutrient substrate. We discovered a neuronal substrate that controls this transition from exploration to exploitation as a decision policy when

encountering a food patch, thereby regulating the exploration-exploitation tradeoff for this decision. We showed that silencing a population of neurons in the dorsal fan-shaped body leads to an increased exploitative drive to stop at food patches while neither affecting other foraging decision, such as patch leaving, nor the overall food intake, suggesting that these neurons do not encode the general internal state of nutrient deprivation. We further show that a single pair of neurons, identified as FB6A, is required to decrease the likelihood of transitioning from exploration to exploitation. Using whole-cell patch-clamp recordings, we showed that the FB6A neurons exhibit modulations in excitability and firing rate upon protein deprivation with lower spiking activity, as predicted from our silencing experiments.

4.4 Materials & Methods

4.4.1 Fly husbandry

Flies were reared on yeast-based medium (per liter of water: 8 g agar [NZYTech, Portugal], 80 g barley malt syrup [Próvida, Portugal], 22 g sugar beet syrup [Grafschafter, Germany], 80 g cornflour [Próvida, Portugal], 10 g soya flour [A. Centazi, Portugal], 18 g instant yeast [Saf-instant, Lesaffre, France], 8 ml propionic acid [Argos], and 12 ml nipagin [Tegospet, Dutscher, UK] [15% in 96% ethanol] supplemented with instant yeast granules on the surface [Saf-instant, Lesaffre, France]) and kept at 25°C, 70% Relative Humidity (RH) in a 12h/12h light-dark cycle.

4.4.2 Internal state manipulations & holidic media

Yeast deprivation was induced by transferring 2-6 days old flies to vials containing a half paper tissue soaked with 5 ml of 100 mM sucrose solution for 10 days. Fully fed flies were age-matched. All flies were transferred to vials with the respective fresh medium every two days. For the mated conditions 10 female flies were kept together with 5 male flies. For virgin conditions, 10 virgin flies without males were kept per vial.

4.4.3 Fly stocks & genetics

The list of all stocks and genotypes used in this study are shown in Table A.1. We obtained stocks from Bloomington Drosophila Stock Center (NIH P4oODo18537), HHMI Janelia Research Campus, Vienna Drosophila Research Center, and Kyoto Stock Center.

4.4.4 Foraging assays

The standard foraging assay is performed in an arena with 5 μ l patches of 10% yeast and 100 mM sucrose, respectively, distributed in a radial pattern (see Fig. 3.1A, Fig. 3.2 and below in subsection Image-based tracking setup). The hardware component blueprints are stored in a GitHub repository (LINK). Per setup, we recorded fly trajectories of four individual flies in separated arenas for one hour. We built 12 of these setups which were connected to four PCs for simultaneous data acquisition. We used custom-written software in Bonsai (Lopes et al., 2015) for the data acquisition (see details below in the subsection Tracking algorithm).

4.4.5 flyPAD assays

flyPAD assays were performed based on a protocol previously described (Itskov et al., 2014). Flies were given a choice between 10% yeast and 20 mM sucrose, both in a 1% agarose solution. Flies were individually transferred to flyPAD arenas by mouth aspiration and allowed to feed for 1 h at 25°C, 70% RH. flyPAD data were acquired using the Bonsai framework (Lopes et al., 2015), and analyzed in MATLAB [R2013b 8.2.0.701 64-bit, Mathworks Inc.] using custom-written software, as described before (Itskov et al., 2014).

4.4.6 Image-based tracking setup

We built a high-throughput tracking setup that can record four flies in individual arenas using a custom-built acrylic arena holder. Arenas were placed above an IR backlight for video recording using a FLIR Flea3 camera [Teledyne FLIR LLC, USA; model FL3-U3-32S2M-CS] acquiring videos at 30

frames per second and a resolution of $1408 \text{ px} \times 1408 \text{ px}$. This resolution resulted in an average real-world pixel resolution of 7.8 px per mm.

4.4.7 Tracking algorithm

We used a custom, real-time tracking algorithm using the Bonsai framework (Lopes et al., 2015). Fly centroids and trajectories were extracted using a background subtraction method, and for each recorded frame we measured the x- and y-position of the centroid, major and minor axis as well as the angle of the centroid. We also developed custom-written Python scripts to determine the head position of the fly in an offline algorithm. This algorithm compares the pixel intensity of both extreme points of the centroid to determine the head, which is darker than the tail position due to the transparency of the wings. We then propagated the head position by applying a proximity rule (Gomez-Marín et al., 2011), in which the position closer to the previous head position is assigned as head position. This rule becomes invalid when flies jump, therefore we detected jumps as segments to reapply the pixel intensity comparison. We further validated correct head position assignment, by comparing the movement vector to the heading vector, which should largely align because flies mostly walk forwards. Using this head detection method, we validated the accuracy by manual visual inspection to be correct in more than 98% of frames (1000 frames randomly sampled).

4.4.8 Behavioral classification

From the head and body trajectories, we calculated the displacements between each of the consecutive frames. By dividing these displacements by the time interval between the frames, we obtain the translational speeds of head and centroid positions. We subsequently filtered the speed time series using a Gaussian kernel with a window length of 36 frames and a standard deviation of 3.6. Similarly, we calculated the rotational speed from displacements in the angle of the centroid to head relative to the reference frame. Based on these kinematic variables, we used threshold values to

classify behavioral segments. "Walking" segments are defined by instances, in which the fly's head speed is above 2 mm/s, while "Jumps" are above 20 mm/s. "Turns" are defined by instances, in which the fly's rotational speed is larger than 125 °/s. "Resting" segments are instances, in which the fly's head speed is lower than 0.2 mm/s. "Micromovements" are instances, which have not been so far classified otherwise, and as such the head speed falls into the range of 0.2 to 2 mm/s. Micromovements which are performed closer than 2.5 mm to a yeast patch are considered "yeast micromovements", while those closer than 2.5 mm to a sucrose patch are called "sucrose micromovements". We filtered out spurious high body speeds that rarely occur during micromovements.

4.4.9 Patch encounters and visits

"Encounters" are segments, in which the fly is closer than 3 mm to a given food patch, regardless of whether feeding occurs or not. "Visits" are all micromovements that occur on the same food patch without the fly leaving a distance larger than 5 mm from the patch. The "probability of stopping at a food patch" is calculated by dividing the total number of visits by the total number of encounters. Since each encounter can at most have one visit, the resulting ratio is between 0 and 1, and therefore can be described as a probability. For patch leaving decisions, we calculated the "mean duration of visits" for all patches of each substrate. The "ratio of returns to the same food patch" is calculated by dividing the number of returns to the same patch divided by the total number of subsequent visits of the same substrate.

4.4.10 Travel time analysis

We calculated the travel time for each resource encounter as the time duration from the end of the last encounter of a resource of the same substrate, regardless of whether a visit occurs, to the beginning of the current encounter. We binned the observed travel times to obtain the probability of stopping. Bins were determined by a $\log_{10}$ scale and overlapping to increase sampling across all bins. The final lineplots are plotted with markers

indicating mean values for the respective bin (placed at the left side of the bin) as well as shaded areas indicating the SEM around the mean.

4.4.11 Immunohistochemistry, image acquisition, and analysis

For visualizing *GAL4* expression patterns, males from each *GAL4* line were crossed to females homozygous for the *UAS-CD8::GFP* or similar GFP-tagged reporters. Mated female flies with both the *Gal4* and the UAS reporter were kept on standard diet for 3 days prior to dissections. Samples were dissected in 4°C PBS, then transferred to formaldehyde solution (4% paraformaldehyde in PBS + 10% Triton-X) and incubated for 20–30 min at room temperature. Samples were washed three times in PBST (0.5% Triton-X in PBS) and then blocked in normal goat serum 10% in PBST for 60 min at room temperature. Primary antibodies used were: Rabbit anti-GFP (TP401, Torrey Pines Biolabs) at 1:6000; Mouse anti-GFP (A11120, Invitrogen) 1:500; Rabbit anti-Mmt (Eurogentec, generated in this study, see below); and Mouse anti-NC82 (nc82 was deposited to the DSHB by Buchner, E., Developmental Studies Hybridoma Bank) at 1:10. All primary antibodies were prepared in 5% normal goat serum in PBST and incubations were performed for 3 days at 4°C with shaking. They were then washed in PBST 2–3 times for 10–15 min at room temperature. The secondary antibodies used were Anti-Mouse 488 (A11029, Invitrogen), Anti-mouse A594 (A11032, Invitrogen), Anti-rabbit A488 (A11008, Invitrogen), Anti-Rabbit A594 (A11037, Invitrogen) at 1:500 in 5% normal goat serum in PBST and samples were then incubated for 3 days at 4°C with shaking. They were again washed in PBST 2–3 times for 10–15 min at room temperature. Samples were mounted in Vectashield (H-1000, Vector Laboratories). Images were captured on a Zeiss LSM 980 confocal microscope using a 20x or 40x objective using Zeiss ZEN Software, and Fiji (Schindelin et al., 2012) was used for post-processing and image analysis.

4.4.12 Alignment and registration to a standardized brain template

Confocal stacks were aligned to a standard reference template (JFRC2, see details¹, Jenett et al. (2012)) using a custom-written script in Advanced Normalization Tools software (ANTs) (Avants et al., 2014). The script is stored on a GitHub repository LINK.

4.4.13 GAL4 lines

GAL4 lines were constructed and characterized at the Janelia Research Campus as described in (Jenett et al., 2012). We selected GAL4 lines based on anatomical neuropil targeting and sparseness based on publicly available confocal imaging data². All GAL4 drivers tested in this study are listed in Table A.1. For testing the GAL4 lines, we used an Empty-Gal4 driver line (BDSC #68384) as control.

4.4.14 Split-GAL4 lines

Split-GAL4 lines were constructed and characterized by the Rubin lab as in previous work (Pfeiffer et al., 2010). Briefly, we tested candidate AD and DBD hemidriver pairs (Dionne et al., 2018; Tirian & Dickson, 2017) for expression in cell types of interest and assembled successful combinations into stable fly strains that were then used for subsequent experiments. Split-GAL4 lines generated in this study and images of their expression patterns are available online (<https://www.janelia.org/split-GAL>). All Split-GAL4 drivers created in this study are listed in Table A.1. For testing the Split-GAL4 lines, we used an Empty Split-Gal4 driver line (BDSC #79603) as control.

¹JFRC2 link: <http://natverse.org/nat.flybrains/reference/JFRC2.html>

²Flylight link: <https://flweb.janelia.org/cgi-bin/flew.cgi>

4.4.15 Cell-type morphological analysis

First, we converted the aligned confocal stack into a skeleton using “3D Visualization-Assisted Analysis” software suite (Vaa3D)³ (Peng et al., 2010). Second, this skeleton is transformed into the Janelia/Google hemibrain reference space (Scheffer et al., 2020) and converted it into a vectorized point cloud using the Natverse package⁴ (Bates et al., 2020) in R (R Foundation for Statistical Computing, n.d.). Then, we used the algorithm NBLAST (Costa et al., 2016) to calculate similarity scores between the skeletonized LM neuron and available layer 6 FB neurons from the hemibrain dataset (Hulse et al., 2021; Scheffer et al., 2020). All custom-written scripts are stored in a GitHub repository (LINK).

4.4.16 Optogenetic assays

We performed optogenetic assays using 10 W high-power LEDs [Luxon] at ... Fully-fed flies were reared on Vienna standard food with 0.4 mM/l all-trans retinal [...]. Yeast-deprived flies were transferred after 3-7 days to vials containing a quarter paper tissue soaked with 5 ml of 100 mM sucrose solution and 0.4 mM/l all-trans retinal [Sigma-Aldrich]. Closed-loop optogenetic assays were performed with two stimulation conditions: either the LED turns on when the fly approaches the food patch (approx. 10 mm distance), or the LED turns on after the fly has initiated feeding by using a shorter activation radius (approx. 2 mm) and a delay of 3 s to ensure that the fly engages in feeding. We wrote custom software in the Bonsai framework to automatically control LED stimulation and switch between these two conditions probabilistically. Open-loop controlled optogenetic assays were performed using a stimulation protocol of repeated 1 s-long stimulations (constant; power density) followed by 20 s without stimulation in the foraging assay (see above). Similar to the closed-loop protocol, this

³Vaa3D link: <https://alleninstitute.org/what-we-do/brain-science/research/products-tools/vaa3d/>

⁴Natverse link: <https://natverse.org/>

open-loop protocol was controlled by custom-written software in Bonsai. Both workflows are stored in a GitHub repository ([LINK](#)).

4.4.17 Whole-cell patch-clamp recordings

All the recordings were obtained once from individual preparations (one recording per fly). To reduce variation of firing rate, actual recordings always started approx. 5 min after break-in and continued for a certain fixed time. In the raster plot, individual data from each group are arranged to match the recording date. Similarly, the raw membrane potential traces were from three independent recordings with each column matched. There was no current injection from a recording pipette during recording. Resting membrane potential was estimated by smoothing the membrane potentials (Butterworth filter at 5 Hz).

4.4.18 Statistical analysis

For all statistical analyses, if not otherwise stated, we use non-parametric, two-sided hypothesis testing (i.e., Wilcoxon-Mann-Whitney ranksum tests). Specifically, we used the SciPy (v.1.8.0, Virtanen et al. (2020)) implementation of the function `stats.ranksum`.

4.4.19 Data and code availability

We stored all source data and Python/R custom-written visualization scripts in a GitHub repository accompanying the publication ([LINK](#)). Hardware and software files for the performing data acquisition and analysis can be found in a GitHub repository ([LINK](#)). Further raw data is available upon reasonable request.

4.5 Acknowledgments

We thank Gwyneth Card, Richard Benton, and Adam Claridge-Chang for providing fly strains. We thank Tanya Wolff for anatomical information

and confocal imaging of Split-GAL4 lines created in the Rubin lab. Lines obtained from the Bloomington Drosophila Stock Center (NIH P40ODO18537) were used in this study. We thank Gonalo Lopes, Joo Frazo, Niccol Bonacchi, Filipe Carvalho and Artur Silva for help in software and hardware design. We thank all members of the Behavior and Metabolism laboratory for helpful discussions and comments on the manuscript. This project was supported by the Portuguese Foundation for Science and Technology (FCT) postdoctoral fellowship SFRH/BPD/79325/2011 to PMI and doctoral fellowship PD/BD/114273/2016 to DG; the Human Frontier Science Program Project Grant RGP0022/2012; the BIAL Foundation Grants (283/14 and 279/16). Research at the Centre for the Unknown is supported by the Champalimaud Foundation.

DISCUSSION

In this thesis, I have investigated the underlying neural circuits for nutrient exploration and decision-making in *Drosophila melanogaster*. First, I presented an optogenetic experimental setup called optoPAD, which we built in order to create closed-loop optogenetic paradigms to study neurons involved in food choice. Second, I developed an optimized high-throughput video tracking setup based on Corrales-Carvajal et al. (2016). Using this setup, we performed a large-scale neurogenetic silencing screen of more than 500 different GAL4 driver lines¹ to test the effect of silencing activity in different neuronal subsets onto foraging parameters. Third, we identified that a specific population of neurons in the dFB is important to control the transition from exploration to exploitation. Fourth, we measured the membrane potential of a single pair of FB6A neurons and showed that the firing rate of FB6A neurons is modulated by protein state. Finally, I showed that the activity in dFB neurons does not only integrate internal states but is also required for adapting resource density of food patches to foraging

¹More than 15000 flies tested

decisions. Overall, these results argue for a modular organization of different aspects of foraging behaviors controlled in distributed sets of neurons across the brain. As such, different internal states can act at distinct levels of sensory processing (Münch et al., 2022) and higher-order cognitive processing to produce synergistic effects on foraging and feeding behaviors. As such, these findings not only have implications for our understanding of nutritional regulation and foraging behavior, but also more generally how higher-order brain areas are integrating different internal and external signals to dynamically generate appropriate behavioral policies.

In this section, I will discuss some of the insights and implications from this work; explore how these expand our understanding of controlling foraging and higher-order cognitive processing; and propose directions for future investigation, both for this work and for the field as a whole. I will then provide another perspective on normative goals of foraging, which incorporates feedforward signals that are predictive of nutritional requirements, and describe how the data presented here fit into this framework.

5.1 Creating open-source high-throughput experimental setups to study complex behaviors

In this thesis, I presented two experimental setups for high-throughput behavioral assaying of *Drosophila* in order to study feeding and foraging decisions: the *optoPAD* and the *MoHiVE* tracking setups. We optimized the setups for scalability, robustness and modularity. Scalability allows for simple extension of building multiple copies of the setups to be run in parallel, which increases the high-throughput capacity of the setup. The *optoPAD* setup has 32 individual arenas with individually controlled LEDs, and we built 12 *MoHiVE* tracking setups (each with 4 individual arenas) that could easily be run by one person at once. As such, we are able to test 32 flies per hour in the *optoPAD*, and 48 flies per hour in the *MoHiVE* —a high number required for large-scale neurogenetic screening. Robustness is meant as reliability and easy-to-use, which allowed us to operate the setups with many

different (differently skilled) users achieving comparable results. This is due to robust materials for the hardware (see Methods for details) and an intuitive graphical user interface using the Bonsai framework (Lopes et al., 2015). Furthermore, additional analysis and visualization scripts have been custom written in Python, which require minimal user input to analyze high-dimensional behavioral data and plot results. Finally, both setups are highly modular: the *optoPAD* is equipped with 4 colored RGBA LEDs which allows for modular optogenetic activation or inactivation depending on the required wavelength of the genetically expressed opsins (i.e. CsChrimson activation at 625 nm, GtACR1 inactivation at 523 nm). The *MoHiVE* tracking setup can exchange between four smaller arenas with 50 mm diameter and one larger arena with 170 mm diameter, which enabled us to study how nutrient patch density affects foraging decisions. We built these modular parts using acrylic glass and a laser cutting system. Methods for maximizing throughput in behavioral assays for large-scale phenotyping have been employed in many studies not only in flies (Alisch et al., 2018; Branson et al., 2009; Geissmann et al., 2017; Robie et al., 2017), but also other model organisms like nematodes *C. elegans* (Barlow et al., 2022; Yemini et al., 2013) and zebrafish *D. rerio* (Bruni et al., 2016; Franco-Restrepo et al., 2019). These approaches often aim to reduce space and costs, while increasing parallelism as well as ease-of-handling (i.e., introducing the animal to the experimental assay, and time taken between two experimental runs). While it is obvious that the data acquisition should have enough resolution to observe and quantify behaviors, high-throughput ethomics approach balance the speed-accuracy trade-off, in which a reduced resolution may improve the throughput. Another possible approach employed by some laboratories is a two-stage strategy, where high-throughput behavioral assays are used in conjunction with more high-resolution, but low-throughput assays that allow for a more detailed dissection of underlying neuronal circuit dynamics. In fact, our results in Chapter 4 exemplified this two-stage strategy, in which our large-scale foraging screen identified a neuronal substrate that subsequently has been dissected more closely with further experimentation of behavior and physiology, using optogenetics, two-photon imaging, and

electrophysiology. Taken together, we have created two open-source high-throughput experimental setups to study complex behaviors, which allows us to study neuronal substrates of naturalistic behaviors in high throughput and detail. Future improvements will increase the resolution and throughput even further. New imaging and embedded computing modules allow for relatively low-cost and low-energy video acquisition. Advances in machine learning and computer vision can further improve the automated behavioral analysis ranging from accurate 3D tracking of individual limbs and body parts (Gosztolai et al., 2021; Mathis et al., 2018; Pereira et al., 2022), including the proboscis for directly quantifying feeding microstructure. Additionally, latent states such as exploratory and exploitative phases could be identified using unsupervised methods employed on time series data (Berman, 2018; Calhoun et al., 2019; Costa et al., 2019; Costa et al., 2021).

5.2 Studying the neuronal elements of naturalistic behaviors and cognition

In this thesis, we aimed to study how naturalistic behaviors and cognitive functions arise from neuronal subsets in the fly brain. Animals evolved in highly complex and uncertain environments, producing a wide array of behaviors, including navigation, foraging, prey capture, and conspecific interactions, which vary over timescales ranging from milliseconds to days. Importantly, the problem of finding appropriate food resources and deciding how long to exploit found resources is ethologically relevant and key to the survival in all animals. Traditionally, these behaviors were the focus of studies in ecology and ethology, while systems neuroscience focused largely on highly controlled, repeatable behavioral assays that act rather on short timescales and in highly artificial laboratory environments. Recent advances in machine learning, miniaturization, and computation allow for studying freely moving animals in more natural conditions in high spatial and temporal resolution (Alisch et al., 2018; Geissmann et al., 2017; Mathis et al., 2018), while simultaneously applying systems neurosciences

approaches: performing neuronal, sensory or motor perturbations (Cruz et al., 2021; Jazayeri & Afraz, 2017), modeling behavioral strategies (Adden et al., 2022; Wosniack et al., 2022), and recording from large numbers of neurons while animals are freely moving (Marques et al., 2020). This thesis embodies this approach by building an experimental framework for studying feeding and foraging decisions in a high-throughput manner and pairing this methodology with a large-scale neuronal inactivation screen. Not only did we uncover several general observations about how the brain regulates exploitation and exploration for nutrient patches, but also showed that specific neuromodulatory systems and neurons are required for decreasing overall exploitative drive which effectively diminishes food intake. Moreover, we identified a specific neuronal substrate in a central region of the fly brain, which is important for controlling the transition from exploration to exploitation. While similar approaches have been used to map general behaviors (Robie et al., 2017) as well as functions of specific descending interneurons (Cande et al., 2018), we are the first to employ such a strategy in the context of nutrient foraging.

5.3 The central complex as part of a context-dependent, deep actor-critic system

The large-scale neurogenetic silencing screen presented in this thesis revealed neurons in a central insect brain area called CX, which regulate specific aspects of the flies' decision-making when encountering a food patch. In this section, I conclude our findings from this work and present implications that suggest the central complex as a multi-layered recurrent neural network for adaptive, context-dependent action selection and spatio-temporal processing (Hulse et al., 2021). This has not only implications on a computational and algorithmic level in terms of what and how complex and highly cognitive tasks can be solved by insects, but also extends to homologous computations done in certain areas of the vertebrate brain. As such, understanding how insect brains provide mechanistic solutions to these

complex, cognitive problems may not only be of interest for studying how adaptive behaviors are generated by insect, or more broadly invertebrate brains, but can potentially also be related to parts of the vertebrate brain, including humans (Barron & Klein, 2016; Chittka, 2017). Furthermore, it also lends bio-inspired solutions to robotic systems as many of them are tasked with similar computational problems, i.e. navigating towards goal locations (Arena et al., 2011; Arena et al., 2009; de Croon et al., 2022; Goldschmidt et al., 2017; Kaushik & Olsson, 2020; Stone et al., 2017).

Suggesting context-dependent action selection as one of the key computational functions of the CX also offers wider implications for theoretical modeling and neuro-inspired robotics. For example, tangential neurons in the FB offer the computational advantage or potential for gating mechanisms by means of its homogenous distribution of synaptic connections across the different columns of the FB (Dan et al., 2021; Hulse et al., 2021). These neurons could send behavior-contextual signals, such as efference copy signals related to the behavioral state, and other internal motivation signals, such as hunger or arousal, which then potentiate downstream goal representation and/or goal-directed actions. In other modelling literature, such tangentially-gated signalling has been used to model the distinct motivational states of outward and inward routes in central-place-foraging insects (Cruse & Wehner, 2011; Goldschmidt et al., 2017). Indeed, experiments in foraging ants have shown that the memory retrieval of routes—and therefore goal directions—is dependent on the motivational state of the animal (Collett et al., 2006; Wehner et al., 2006). Here we show that the activity in tangential FB neurons of the type FB6A are modulated by the protein state. As such, directional information of the food spot (e.g., olfactory, gustatory or visual cues) could be gated in a way to facilitate the behavioral policy of ignoring the food patch to continue exploration. However, further experiments are required to show whether these neurons directly integrate internal states or whether these changes are derived from indirect effects of the protein deprivation. Taken together, our study reveals a neuronal substrate involved in higher-order foraging decisions by integrating internal state information. This is a first step towards a mechanistic

understanding of how cognitive tasks are solved computationally in the small brains of insects. Nonetheless, a full understanding of how the central complex integrates different sensory modalities together with internal states to generate behavioral policies has yet to be fully elucidated. Future work will go further to study CX function in the context of freely behaving or moving flies with simultaneous neuronal recordings (Haber Kern et al., 2019) of calcium or voltage indicators and manipulations that either activate or silence specific subpopulations of neurons in the CX.

5.4 Outstanding questions

It is obvious, while this thesis has provided some answers to the questions and aims I outlined in the Chapter 1, there are still outstanding open questions.

5.4.1 How are foraging behaviors algorithmically organized in the fly brain?

While the results obtained from this study have led to substantial insights into how foraging behaviors and decision-making are organized and regulated, a holistic picture of how foraging behaviors are organized within the fly brain is still missing. We have shown that a population of neurons in the dorsal FB specifically regulates the transition from exploration to exploitation when encountering a food patch. We further showed that these neurons are not only modulated by internal state, but are also required to facilitate travel time-dependent regulation of the decision to stop at food patches as predicted by optimal foraging strategies. Our large-scale silencing screen also identified neuronal subsets that generally increase exploitative behaviors including total duration of feeding. These included IPCs, Lk, AstA, that have previously shown to act as satiety signals (Lin et al., 2019). Such global neuromodulatory signalling could act upon many different regions in the brain, which could argue for a modular organization of different foraging behaviors (e.g., decision to stop, decision to leave, local search, etc.). Such

modularity is further confounded by our preliminary results of lines that specifically affect the decision to leave a food patch. Interestingly, we were unable to identify neuronal subsets that specifically regulate exploratory behaviors, such as the ratio of returns or the directedness of patch-to-patch transitions. As such, this could be explained by the complexity of exploration vs. "discrete" decision-making requiring a larger, more distributed network of neurons which were not sufficiently targeted by our screening approach which focused on sparser expressions. Another reason could be that redundancy and parallel circuitry facilitates exploratory behaviors, because the ability to explore environments for food is so fundamental to the survival of the organism that evolutionary pressures have led to multiple control systems that act synergistically and redundantly when one of them fails. Future work will extend the foundation laid by the approach employed here in this thesis by carefully designing testable hypotheses for experimental interrogations.

Causal relationships between neuronal activity and foraging behaviors can be directly proven by using an experimental setup that uses a freely moving fly on an air-suspended ball, such that a two-photon microscope or electrodes could simultaneously record neuronal activity in targeted cells. These setups are often built with a visual virtual reality (Haberkern et al., 2019) or directed olfactory plumes (Matheson et al., 2022). As such, to facilitate naturalistic foraging behaviors, the setup would require an apparatus to dynamically provide food. A recent study has built this by adding a robotic arm that contains a food delivery system, which has been developed to allow for long-term recordings over hours or even days (Flores-Valle & Seelig, 2022). A different study used sucrose delivery to study dopaminergic reinforcement pathways in the MBs (Zolin et al., 2021). Such an assay could be further extended by adding the possibility to apply acute optogenetic activation or silencing of targeted neurons (Matheson et al., 2022). Such elaborate experimental setups will enable scientists to set the stage for investigating the fly brain circuitry underlying adaptive foraging in complex multisensory environments.

5.4.2 How does the central complex facilitate foraging behaviors?

This thesis provides evidence for tangential dorsal fan-shaped body (FB) neurons importance for regulating the transition from exploration to exploitation. We showed that the activity in these neurons does not act as a direct motor program command, but rather sets a behavioral policy. But how such policy is then processed by downstream circuits to form the actual behavioral action remains open. However, literature on the central complex anatomy and function may provide hypotheses for testing in future work. For example, certain columnar neurons in the FB integrate information from heading and linear speed to compute a vector that represents the travel direction (Lu et al., 2022; Lyu et al., 2022). Furthermore, another set of columnar neurons show activity that corresponds to directional odor information (Matheson et al., 2022). Both examples also connect the spatial representation of such vectors or goals to potential premotor outputs that could support directional steering towards or away from it (Hulse et al., 2021; Rayshubskiy et al., 2020). As such, tangential neurons in the FB, like the ones shown in this thesis, could provide a gating mechanism that instructs downstream circuits to either steer towards or away from goals, e.g. food patches. A similar mechanism has been proposed for goal-directed flying in an operant conditioning paradigm (Dan et al., 2021). Future work is required to test this hypothesis, involving experiments in a freely behaving context with simultaneous neuronal recordings and perturbations (Haber Kern et al., 2019).

Besides locomotor programs, activity in the dFB could also act on other behavioral modules such as feeding. Indeed, downstream circuits of FB6A connect to a cell type named FS₄ (Hulse et al., 2021), which has projections in the flange, a neuropil located close to the esophagus and the subesophageal zone and, as such, potentially involved in controlling feeding behavior (Hartenstein et al., 2018; Ito et al., 2014). Moreover, a neuron projecting to FB as well as the lateral accessory lobes has been shown to be required and sufficient for yeast feeding (Liu et al., 2017). Nonetheless,

downstream effectors of computations and behavioral functions of higher-order neuropils like the central complex and the mushroom bodies are difficult to decipher, because there are many different, parallel output pathways (Hulse et al., 2021; Li et al., 2020). Taken together, it remains unknown how signals from the central complex affect downstream processing circuitry to effectively drive different behaviors, but the work presented in this thesis provides an interesting entry point into further investigation into solving this puzzle.

5.5 Concluding remarks: Nutrient allostasis as a normative goal for brain computations to maximize foraging outcomes

In the concluding remarks, I would like to take a broader view on the emerging picture from this study. The central idea of the brain as a predictive system dates back to Helmholtz (Von Helmholtz, 1867), who postulated that the brain constantly generates sensory data to match incoming stimuli with internal models of the environment and our bodies. While it has been shown that the brain can infer the statistical structure of the external environment (Srinivasan et al., 1982; Warrant, 2016) and predict sensory consequences of chosen motor actions (Fujiwara et al., 2022; Fujiwara et al., 2017; Kim, Fenk, et al., 2017), there is growing evidence for the predictions of internal (nutritional) states to guide homeostatic regulation in an allostatic, feed-forward controlling way (Reed et al., 2022; Sterling, 2018; Walker et al., 2017). The emerging picture is that insects and mammals employ anticipatory mechanisms that go beyond the current view of homeostasis being achieved through reactive negative feedback (Berrios et al., 2021; Walker et al., 2015; Zimmerman et al., 2016). A mechanistic understanding of how the brain controls nutrient intake in general and specifically foraging decisions must therefore take into account such feed-forward allostasis. As such, cost-benefit models must not only infer current but also future needs of the animal. Furthermore, the relationship between risks and outcomes is

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often defined as a trade-off between time spent travelling (energy loss) and energy gain. Not only is it evident that even when stationary, animals have a base level of metabolic cost, but also the requiring multiple macronutrients not only to simply derive energy paints a more complex picture of how animals must regulate their nutrient intake (Corrales-Carvajal et al., 2016). This process involves integrating information about multidimensional nutrient and other metabolic states —like mating and sleep (Corrales-Carvajal et al., 2016; Glass et al., 2011). Such complexity of a high-dimensional internal state space could either create the need for a fixed hierarchy of needs (Maslow, 1943) or competition of different drives and appetites (Sutton & Krashes, 2020).

Furthermore, intero- and exteroceptive predictive modeling as core computational elements in foraging decision-making provide a powerful algorithmic framework for balancing complex exploration-exploitation trade-offs. The brain rewards and reinforces better-than-predicted foraging outcomes to match physiological needs, thereby encouraging the organism to learn effective regulatory foraging behaviors. Such integrated reinforcement of intero- and exteroceptive signals likely involves neuromodulatory systems, such as DA neurons (Grove et al., 2022). Computationally, this creates a causal loop between maximizing foraging outcomes via exploration, and optimizing nutrient intake. This means exploratory behaviors enable the animal to create opportunity by encountering nutrient patches, which may be exploited to reach optimal intake, which in turn leads to an increase in future exploratory capacity (i.e., how far to venture out from safe locations or general risk taking). This is best exemplified by the observation of increasing searching area relative to the cumulative intake during local search movements in many different animal species (Bell, 1985; Bond, 1980; Claver & Ambrose, 2003; Corfas et al., 2019). As such, future work in theoretical modeling of foraging behaviors must take into account the closed-loop interactions between internal and external state predictions and their integrated regulation (Keramati & Gutkin, 2014; Schulkin & Sterling, 2019).

The brain, by prioritizing behaviors and dynamically adjusting the intake of energy and nutrients, reduces costly errors and exploits more opportunities (future long-term exploitation via exploration). Hence, although such higher-order computations come with significant metabolic and neuronal allocation costs, allostatic control schemes for nutrient intake and foraging pay off in the long term and therefore provide a proto-cognitive blueprint and core principles in organismal neural design.

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This thesis contains a total number of 341 references.



SUPPLEMENTARY INFORMATION ABOUT LARGE-SCALE NEUROGENETIC SCREENING

This appendix provides supplementary information about the large-scale neurogenetic screening to identify neuronal subsets involved in foraging.

Fly stocks used in screening:

Stock ID	GAL4/SplitGAL4	Description	Stock No.	Stock Center
9997	w1118	control line		
9998	Empty Split	control line	79603	Bloomington
9999	Empty	control line	68384	Bloomington
0001	50H05		38764	Bloomington
0002	AstA		51979	Bloomington
0003	NPF			
0004	Or83b/ORCO			
0005	DH44		51987	Bloomington

APPENDIX A. SUPPLEMENTARY INFORMATION ABOUT
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0006	DH44-R1			
0007	DH44-R2			
0008	67Eo3	pegs	39441	Bloomington
0009	57Fo3	"pegs / IN1; (Yapici et al., 2016)"	46386	Bloomington
0010	83Fo1	"IN1 (broader); (Yapici et al., 2016)"		
0011	Ir56d	possibly involved in AA detection		
0012	3oAo8	"TPN1; (Kim et al., 2017)"	49513	Bloomington
0013	VT057358	TPN2	203226	VDRC
0015	11Ho9	TPN3	48478	Bloomington
0016	NP1562	"2nd order taste neuron; (Kain and Dahanukar, 2015); sGPN"	112700	Kyoto
0017	SS00730	"DNao2 (stochasticity: 1/4, bg: C)"		Janelia
0018	SS00731	"DNao1 (stochasticity: 0/4, bg: C)"		Janelia
0019	SS01545	"DNdo2 (stochasticity: 0/4, bg: A)"		Janelia
0020	SS01570	"DNdo2 (stochasticity: 0/4, bg: A)"		Janelia
0021	SS02259	"DNg13 (stochasticity: 0/4, bg: C)"		Janelia
0022	SS02393	"DNao8 (stochasticity: 0/3, bg: A)"		Janelia

0023	SSo2395	"DNco2 (stochasticity: o/4, bg: B)"		Janelia
0024	SSo2542	"DNbo1 (stochasticity: o/3, bg: B)"		Janelia
0025	SSo2634	"DNgo2 (stochasticity: o/1, bg: A)"		Janelia
0026	SSo4161	"DNdo2 (stochasticity: o/4, bg: A)"		Janelia
0027	81E10	Fdg neuron	48367	Bloomington
0028	NP883	Fdg neuron	103803	Kyoto
0031	E564	PERin		
0033	34Go2	ISN	49797	Bloomington
0035	38Ho2	Cupcake neuron	47352	Bloomington
0039	2oDo6	sNPF neurons	48892	Bloomington
0040	2oF11	sNPF neurons	49852	Bloomington
0041	VTo10252	sNPF neurons	202258	VDRC
0043	MBo21B	OA-VPM ₄	68297	Bloomington
0047	38Ao6	OA-VL	49977	Bloomington
0048	Crz	Corazonin expressing neurons	51976	Bloomington
0049	Leuc	Leucokinin expressing neurons		
0050	Lkr	Leucokinin receptor expressing neurons		
0051	MIP	myoinhibitory peptide expressing neurons	51983	Bloomington
0052	Taotie	Taotie neurons	57616	Bloomington

APPENDIX A. SUPPLEMENTARY INFORMATION ABOUT
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0053	WED1	Wedge 1 neuron		
0055	70G12	Downstream of wedge neurons	39552	Bloomington
0056	75B10	Downstream of wedge neurons	39884	Bloomington
0057	72B03	Downstream of wedge neurons	39610	Bloomington
0058	32A06	Downstream of wedge neurons	49885	Bloomington
0059	HugS3	Subset of Hugin expressing neurons	58769	Bloomington
0060	19G02	Contains compass neurons (E-PG)	48860	Bloomington
0061	sNPF			
0062	Lk			
0063	Tk			
0064	98			
0065	SIFa			
0066	E49; TM2/TM6b			
0067	21Fo1	putative SAG downstream neuron	49862	Bloomington
0068	Or42a (III)	palp OSN (pb1A)	9969	Bloomington
0069	Or71a (II)	palp OSN (pb1B)	23121	Bloomington
0070	Or85e (I)	palp OSN (pb2A)	23134	Bloomington
0071	Or33c (I)	palp OSN (pb2A)	9965	Bloomington
0072	Or46a (III).9.10A	palp OSN (pb2B)	9979	Bloomington
0073	Or59c (III)	palp OSN (pb3A)	23899	Bloomington
0074	Or85d (II)	palp OSN (pb3B)	23915	Bloomington
0077	51Do6	scaf neurons	46019	Bloomington

0079	87Go7	P-F3N2d neuron (CX functional paper); CPU4	40502	Bloomington
0081	67Do9	P-F3N2v neurons (CX functional paper); CPU4c	49618	Bloomington
0082	84Ho5	PF-LCre neurons (output to LAL); CPU1a	40405	Bloomington
0083	55Go8	Delta7 neurons (CX functional paper); CX bottleneck	50422	Bloomington
0084	47Go8	CX functional paper	50328	Bloomington
0085	49Ho5	CX functional paper	38714	Bloomington
0086	75Ho4	"CX functional paper, EB & SMP"	39909	Bloomington
0087	32A11	CX functional paper	62831	Bloomington
0088	18Ao5	CX functional paper	48794	Bloomington
0089	72Ho6	CX functional paper	39801	Bloomington
0090	SSo2615	CX functional paper		Janelia
0091	26Bo7	CX functional paper	49160	Bloomington
0092	31A11	CX functional paper	49660	Bloomington

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0093	76E11	CX functional pa- per	39933	Bloomington
0096	12Go4	CX functional pa- per	48523	Bloomington
0097	50C10	SEZ expression (Braincode)	38741	Bloomington
0098	20Ao7	SEZ expression (Braincode)	49287	Bloomington
0100	93Fo1	SEZ expression (Braincode)	47230	Bloomington
0102	93Bo7	SEZ expression (Braincode)	40641	Bloomington
0103	91Ho5	SEZ expression (Braincode)	40594	Bloomington
0104	70Ao4	SEZ expression (Braincode)	46627	Bloomington
0105	43Go4	SEZ expression (Braincode)	49556	Bloomington
0106	83H11	SEZ expression (Braincode)	40373	Bloomington
0108	52H12	SEZ expression (Braincode)	38856	Bloomington
0109	47Fo9	SEZ expression (Braincode)	50322	Bloomington
0111	32Ho3	SEZ expression (Braincode)	49733	Bloomington
0112	82Eo8	SEZ expression (Braincode)	40150	Bloomington
0113	56Do7	SEZ expression (Braincode)	39149	Bloomington

0120	VT050300	SEZ expression (Braincode)	204723	VDRC
0123	48Bo6		50349	Bloomington
0124	49Do8		38681	Bloomington
0125	49Do9		38682	Bloomington
0126	49Fo9	interesting expres- sion projectinng to SLP	38699	Bloomington
0127	49Go2		38703	Bloomington
0128	50C12	motor	38743	Bloomington
0129	50Eo7	SEZ descending	38749	Bloomington
0130	50E11	SEZ descending	38750	Bloomington
0131	50F11	ascending	38756	Bloomington
0132	51Fo9		38792	Bloomington
0133	52F11	AMMC?	49579	Bloomington
0135	53Ao6	SAG?	38860	Bloomington
0136	53A10		49582	Bloomington
0139	53E11		50438	Bloomington
0140	53Ho7		49587	Bloomington
0141	54Ao9	SAG?	50460	Bloomington
0144	54E11		48203	Bloomington
0146	54G12		41280	Bloomington
0147	54Ho1		47650	Bloomington
0149	55A10		39099	Bloomington
0150	55Co2		39104	Bloomington
0152	56Fo6	looks like motor neurons	39159	Bloomington
0153	58Go3	MBE as well	39193	Bloomington
0154	58Ho1	MN1 proboscis re- traction	39197	Bloomington
0155	59Ao6		39205	Bloomington

APPENDIX A. SUPPLEMENTARY INFORMATION ABOUT
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o156	59Do8		48220	Bloomington
o157	59Eo6		48222	Bloomington
o158	60B12		39239	Bloomington
o160	60Do5	interesing AMMC SEZ connection	39247	Bloomington
o161	60D10	motor ?	41284	Bloomington
o162	60Eo2	mechanosensory?	39250	Bloomington
o163	60Fo9		39255	Bloomington
o165	60G10	SEZ and Lateral dorsal neurons	39261	Bloomington
o166	60Ho3	mechanosensory input?	39263	Bloomington
o167	64Fo1		39307	Bloomington
o168	64Go5		39316	Bloomington
o169	65F12		39367	Bloomington
o170	65Ho7	AMMC	39381	Bloomington
o171	65Ho9	Dirty SEZ descend- ing	47389	Bloomington
o172	66Ao3	AMMC	49615	Bloomington
o173	66Bo5	Dirty SEZ descend- ing	39389	Bloomington
o175	67B11	ascending SEZ	39405	Bloomington
o177	67Fo1	AMMC SEZ	39447	Bloomington
o178	68Ao6		39449	Bloomington
o179	68Bo2	SEZ	39454	Bloomington
o182	69Eo6	motor and SLP?	39493	Bloomington
o183	69Fo2	SEZ	39494	Bloomington
o184	69Fo6	clean SEZ	39497	Bloomington
o187	70Bo3	SEZ input/output busy	39512	Bloomington
o188	70Bo8	AMMC	39515	Bloomington

0189	70Co7	SEZ	39523	Bloomington
0190	70Go1	very intresting SEZ and AMMC projec- tions	39546	Bloomington
0191	70Ho2	AMMC and visual?	48299	Bloomington
0192	70Ho6	AMMC	39555	Bloomington
0193	71Bo8	AMMC	39566	Bloomington
0195	72Do8	SEZ and AMMC	39771	Bloomington
0196	72G12	SMP and strong AMMC	39796	Bloomington
0198	73G10	Ascending and de- scending SEZ	39830	Bloomington
0199	74C10	AMMC	39848	Bloomington
0200	74Eo9	AMMC	39860	Bloomington
0203	75Bo9	ascending SEZ and EB dirty	39883	Bloomington
0204	75C10	SEZ and visual?	48322	Bloomington
0205	75Do2	"SEZ, AL and Sparse EB"	46935	Bloomington
0206	75Do3	AOTU?	39891	Bloomington
0207	75Do6	SEZ projections	39892	Bloomington
0208	75Fo2	"SEZ and AMMC, Saddle"	39898	Bloomington
0209	76B12	AMMC	39922	Bloomington
0210	76Co4	AMMC and inter- esting SP neuron	48621	Bloomington
0211	76Go1	very busy AMMC and SEZ sensory	39938	Bloomington
0212	77Bo7	AMMC and de- scending SEZ	48336	Bloomington
0213	77C10	SEZ descending	39958	Bloomington

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0214	77E11		39971	Bloomington
0215	78A03		48341	Bloomington
0216	78A12	SEZ	39987	Bloomington
0217	78E05	SEZ descending	39998	Bloomington
0218	78E06	SEZ descending	39999	Bloomington
0219	78E09		40000	Bloomington
0220	23E05		49029	Bloomington
0221	18H11		48832	Bloomington
0222	43D05		41259	Bloomington
0223	64A10		47700	Bloomington
0224	44H10		41267	Bloomington
0225	14F03		48648	Bloomington
0226	20G07		48613	Bloomington
0228	10H05		48276	Bloomington
0229	10B11		48247	Bloomington
0230	11A09		47847	Bloomington
0231	10E02		48262	Bloomington
0232	10E07		48440	Bloomington
0233	11C07		48448	Bloomington
0234	11F03		48464	Bloomington
0235	12A11		48485	Bloomington
0236	12A12		48486	Bloomington
0237	12B01		48487	Bloomington
0239	12D09		48503	Bloomington
0240	12D12		48506	Bloomington
0241	12E04		48508	Bloomington
0242	12E08		48509	Bloomington
0243	12G01		48520	Bloomington
0244	12G08		47855	Bloomington
0245	12G09		48525	Bloomington
0247	13A08		48539	Bloomington

0248	13Bo8		48546	Bloomington
0249	13Go4		48582	Bloomington
0250	13Go6		48584	Bloomington
0251	13Ho4		48589	Bloomington
0252	14A12		48596	Bloomington
0253	14Co7		48605	Bloomington
0254	14Eo5		48642	Bloomington
0255	14Eo6		48643	Bloomington
0256	14Go3		48657	Bloomington
0258	14Go8		48661	Bloomington
0259	15Ao1		48670	Bloomington
0260	15Ao5		48672	Bloomington
0261	15Bo7		48678	Bloomington
0262	15C11		48684	Bloomington
0263	15D10		48689	Bloomington
0264	15Go8		48702	Bloomington
0265	16Co5		48718	Bloomington
0266	16Co9		48720	Bloomington
0267	16Do1	P-FN neurons	48722	Bloomington
0268	16Do6		48724	Bloomington
0269	16E11		48729	Bloomington
0270	16F12		48739	Bloomington
0271	18Ao3		48792	Bloomington
0272	18Bo5		48799	Bloomington
0273	18Go1		48823	Bloomington
0274	18Ho3		48829	Bloomington
0275	19Co6		48843	Bloomington
0276	19Eo2		49833	Bloomington
0277	19Eo6		48850	Bloomington
0278	19Fo5		48855	Bloomington
0279	19F12		48858	Bloomington

APPENDIX A. SUPPLEMENTARY INFORMATION ABOUT
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0280	19Go3		49837	Bloomington
0281	19Go5		49839	Bloomington
0282	19Ho6		49840	Bloomington
0283	20Ao3		48871	Bloomington
0284	20Do1		48889	Bloomington
0285	20Fo6		47892	Bloomington
0286	20Ho8		48915	Bloomington
0287	21Fo3		49863	Bloomington
0288	21Go1		48951	Bloomington
0289	21Ho9		49867	Bloomington
0290	21H11		48960	Bloomington
0291	22Do4		48981	Bloomington
0292	22E10		49302	Bloomington
0293	22Fo2		48039	Bloomington
0294	23B11		49019	Bloomington
0295	24Co7		49074	Bloomington
0296	24Eo5		49081	Bloomington
0297	24Ho3		49098	Bloomington
0298	24Ho8		49100	Bloomington
0299	24H12		49101	Bloomington
0300	25Ao1		49102	Bloomington
0301	25Bo7		49112	Bloomington
0302	25Co1		49115	Bloomington
0303	25Do1		49122	Bloomington
0304	25Eo4		49125	Bloomington
0305	25E10	MB?	49320	Bloomington
0306	25Fo4		49128	Bloomington
0308	25Ho3		49143	Bloomington
0309	26Bo4		49158	Bloomington
0310	26B11		49164	Bloomington
0311	26Co6		49169	Bloomington

0313	27Co9		49217	Bloomington
0315	27Fo2		48072	Bloomington
0316	28Bo5		49447	Bloomington
0317	28Eo1		49457	Bloomington
0318	29Ao1		48086	Bloomington
0319	29A11		49881	Bloomington
0320	29Co7		49340	Bloomington
0321	29Co8		49480	Bloomington
0322	29D11		49484	Bloomington
0323	29Go8		49500	Bloomington
0324	30Ao2		49510	Bloomington
0325	30A12		49516	Bloomington
0326	30Co3		49526	Bloomington
0327	30D11	visual?	48098	Bloomington
0328	30Eo3		49633	Bloomington
0329	30Eo9		49637	Bloomington
0330	30Go8		48101	Bloomington
0331	31Bo1	visual	49662	Bloomington
0332	31Bo7		49664	Bloomington
0333	31Co3	visual?	48103	Bloomington
0334	31G11		49689	Bloomington
0335	32Bo3		49886	Bloomington
0336	32Do2		49709	Bloomington
0337	32Fo1		49359	Bloomington
0338	32Ho8		49735	Bloomington
0339	33Ao1		49737	Bloomington
0340	33Eo4		49752	Bloomington
0341	33Eo6		48114	Bloomington
0342	33G12		49758	Bloomington
0343	34Bo2		49768	Bloomington
0344	34B11		49774	Bloomington

APPENDIX A. SUPPLEMENTARY INFORMATION ABOUT
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o345	34Eo7		49790	Bloomington
o346	34Fo6		48125	Bloomington
o347	34G10		49802	Bloomington
o348	35Ao7		49813	Bloomington
o349	35C10		49902	Bloomington
o351	37G11		49539	Bloomington
o352	37G12		49967	Bloomington
o353	38Bo6		49986	Bloomington
o354	38Eo8	nice SEZ expres- sion too	50008	Bloomington
o355	38Fo8	nice SEZ expres- sion too	50012	Bloomington
o356	39Bo9		50036	Bloomington
o357	39C11		50042	Bloomington
o358	40Do5		50084	Bloomington
o360	41Ao5		48144	Bloomington
o361	41Ao8		50108	Bloomington
o362	41B12	SEZ expression too	49547	Bloomington
o363	42Ao6		41245	Bloomington
o364	42Fo6		41253	Bloomington
o365	42Ho1		48150	Bloomington
o366	43Bo6		50177	Bloomington
o367	43Do1	very interesting anatomy connect- ing to SEZ	64345	Bloomington
o368	43Do9		49553	Bloomington
o369	45C10		50226	Bloomington
o370	45Do4		48160	Bloomington
o371	45F12		50240	Bloomington
o372	46Do2		50263	Bloomington
o373	46Eo3		41270	Bloomington

o374	46Go6	"VIEW, very interesting projections in SEZ as well"	41271	Bloomington
o375	46Ho3		50279	Bloomington
o376	46Ho4		50280	Bloomington
o377	47Do8		50305	Bloomington
o378	47Fo7		50320	Bloomington
o379	47F12		50324	Bloomington
o380	48Fo9		50377	Bloomington
o381	48Ho4	sensory input to SEZ	50392	Bloomington
o382	48H11		50396	Bloomington
o383	49A11		50405	Bloomington
o384	49Bo7	MB expression	50410	Bloomington
o385	49Co4		50415	Bloomington
o386	49Co6	MB expresssion	50417	Bloomington
o387	49Do2		38678	Bloomington
o388	49Do4		38679	Bloomington
o389	49E12		38693	Bloomington
o391	49H12		48178	Bloomington
o392	50Ao1		38717	Bloomington
o393	50A10		49392	Bloomington
o394	50Bo6	SMP and SEZ	38728	Bloomington
o395	50Do4		38744	Bloomington
o397	50Ho7		38765	Bloomington
o398	51Bo7		46013	Bloomington
o399	51Bo9	SEZ	48184	Bloomington
o401	51Eo5		38782	Bloomington
o402	51Go3		38796	Bloomington
o403	52Bo2		38814	Bloomington
o404	52Do3	SAG?	38826	Bloomington

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0405	52Fo8		49578	Bloomington
0407	52H10		38854	Bloomington
0409	53Bo3		49583	Bloomington
0410	53Bo8		38865	Bloomington
0411	53Co1		38868	Bloomington
0412	53Co2		38869	Bloomington
0414	53Eo1		50431	Bloomington
0415	53Fo3		48197	Bloomington
0417	54Ao8	mushroom body ex- trinsic	50459	Bloomington
0418	54E12		39077	Bloomington
0420	54Go7		39087	Bloomington
0421	54Ho4		39090	Bloomington
0422	55Bo8		48207	Bloomington
0423	55D12		39430	Bloomington
0424	56Co8		39144	Bloomington
0425	56Co9		39145	Bloomington
0426	56Eo7		39153	Bloomington
0427	56Eo9		47949	Bloomington
0428	56Ho9		39166	Bloomington
0429	58Do7	SEZ MN? and AL with EB	39179	Bloomington
0430	58Fo7	Strange SEZ projec- tion calyx projec- tion	39188	Bloomington
0431	58F11	EB SEZ and others	48214	Bloomington
0432	58G11	FB and SEZ	39195	Bloomington
0433	58Ho5	sleep drive	39198	Bloomington
0434	58H10		39201	Bloomington
0435	59B10	Sensory input?	39209	Bloomington
0436	60B11	dirty	39238	Bloomington

o438	64A11	PB SP and sensory input to SEZ	39289	Bloomington
o439	64Co4	mechanosensory input?	39296	Bloomington
o440	64Do9	FB a bit dirty	49603	Bloomington
o441	64Ho4		39323	Bloomington
o442	65Ao6	PB	39330	Bloomington
o443	65A11	with AMMC	39333	Bloomington
o444	65Bo4		39336	Bloomington
o445	65Co3	very nice FB	41290	Bloomington
o446	65Go7	EB with descending PI	39372	Bloomington
o447	65Go9		39374	Bloomington
o448	67Ao6	busy CX expression with AL	39397	Bloomington
o449	68A10	SEZ and busy FB	39451	Bloomington
o450	68Bo6		39458	Bloomington
o451	68C10	EB and ascending SAG?	39470	Bloomington
o452	69Ho4	visual?	46622	Bloomington
o453	70Ao2	FB	39506	Bloomington
o455	70Bo4	EB and very busy SEZ and VNC	39513	Bloomington
o456	72Ao4		46665	Bloomington
o457	72Bo7		39764	Bloomington
o458	72Do6	"EB, AL and SEZ descending"	39769	Bloomington
o459	72Go1	Dirty EB	39788	Bloomington
o460	72Go3	dirty FB	48309	Bloomington
o461	72Go6	"FB dirty, sleep drive"	39792	Bloomington

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o462	72Go7	FB dirty	49622	Bloomington
o463	73Ao6		39805	Bloomington
o464	73Bo5	EB	48312	Bloomington
o465	74Co8	PB dirty	46711	Bloomington
o466	75Ao2	dirty FB	39878	Bloomington

o467	75H10	"SEZ, PB and EB "	39913	Bloomington
o468	76Ao3	SLP	39917	Bloomington
o469	76Bo6	AOTU?	48327	Bloomington
o470	76B11	very dirty but inter- esting superior neu- ron	39921	Bloomington
o471	76Co5	EB and SEZ descending or ascending to SEZ	39924	Bloomington
o472	76Co6	wide SP projec- tions	39925	Bloomington
o473	76Co7	FB and SP with SEZ	39926	Bloomington
o474	76Go9	PB and EB very dirty	47755	Bloomington
o475	76Ho1	optic?	39941	Bloomington
o476	78Bo6	EB but rest is very busy	48343	Bloomington
o478	49B11		50411	Bloomington
o479	81Eo1		40114	Bloomington
o480	47Go7	LAL	50327	Bloomington
o481	51Fo5	Dorsal neurons + SEZ	38789	Bloomington
o482	85Ho6	SEZ + CX	40440	Bloomington
o483	83Bo1	"Prow, Saddle, SEZ, descending"	40349	Bloomington

o484	81C12	SEZ taste receptors (which?)	48364	Bloomington
o485	65Go8	Interesting projections to SEZ	39373	Bloomington
o487	42Bo2	"SEZ, Saddle, ascending"	50145	Bloomington
o488	16Ho4	"SEZ, AL, AMMC, Saddle"	48745	Bloomington
o489	SS47449	"SEZ neuron, but dirty"		Janelia
o490	SS47467	Noduli?		Janelia
o491	SS50447	"CX, PB"		Janelia
o492	SS50464	"EB, SLP??"		Janelia
o493	SS50477	SEZ neuron??		Janelia
o494	SS50487	CX		Janelia
o495	SS50517	"FB,SLP?"		Janelia
o496	SS50574	PB and EB ring		Janelia
o497	SS50576	Gall??		Janelia
o498	SS50594	Gall??		Janelia
o499	SS00117	PB projection but only lateral		Janelia
o500	SS01078	"Input from IPS, SPS; output to Neck"		Janelia
o501	78Fo7	PEG downstream	47409	Bloomington
o502	78Go3	Dirty PEG downstream	40011	Bloomington
o503	78Go9	SEZ and CX	40015	Bloomington
o504	79A11	SEZ and AMMC	40025	Bloomington
o505	79Co8	SEZ descending	40032	Bloomington
o506	79C11	SEZ ascending	40033	Bloomington

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0507	8oHo5	motor neuron?	40095	Bloomington
0508	8oHo4	both ascending and descending populations	47076	Bloomington
0509	81Ao6		40099	Bloomington
0510	81Ao7		40100	Bloomington
0512	81B12	motor neuron	40107	Bloomington
0514	81Go5		48368	Bloomington
0516	82Bo3	SEZ plus AOTU	40132	Bloomington
0518	82B12	Ascending to SEZ	47962	Bloomington
0519	82Eo1	AMMC	40147	Bloomington
0520	82E12	AMMC	40153	Bloomington
0524	84Bo9	SEZ the rest is busy	40381	Bloomington
0527	86Go2	AMMC?	40465	Bloomington
0528	86G12	SEZ	47977	Bloomington
0529	86Ho2	SEZ descending very busy	49422	Bloomington
0530	86H12	SEZ descending and MB	48387	Bloomington
0531	87Ao1	SEZ ascending	40472	Bloomington
0533	87Co8		40483	Bloomington
0534	8oCo7	EB	40074	Bloomington
0535	8oDo7	EB	40078	Bloomington
0536	8oHo2	Descending and FB	40093	Bloomington
0537	81Fo1		40120	Bloomington
0538	82H12	EB	40346	Bloomington
0539	83A10	EB	48371	Bloomington
0540	83Bo5		40352	Bloomington
0541	84C10	FB clean	48378	Bloomington
0542	84D12	FB clean	40394	Bloomington
0543	84Eo2	SLP	46776	Bloomington

0544	84Ho9	EB dirty	47803	Bloomington
0545	85Co3	SLP with SAG ascending	47971	Bloomington
0546	85Fo3	Dirty with SEZ and AMMC	40431	Bloomington
0547	85Go9		69300	Bloomington
0548	85G10	FB with weak SEZ	48384	Bloomington
0552	81Eo8	PEG downstream	40117	Bloomington
0557	87Fo8		40495	Bloomington
0559	88Ao4		40510	Bloomington
0560	88B12	AMMC	46851	Bloomington
0561	88Co2		40519	Bloomington
0562	88Do1		48395	Bloomington
0563	88Eo9	AMMC	46857	Bloomington
0564	88Fo6	AMMC and EB	40527	Bloomington
0565	88Fo8	PEG downstream	47982	Bloomington
0568	89Co2	EB	48404	Bloomington
0569	89Eo1	PEG downstream	46885	Bloomington
0570	92Go5	EB very interesting expression	48416	Bloomington
0571	92Ho1	AMMC and SMP	40631	Bloomington
0572	94A10	motor	47246	Bloomington
0573	9Eo5	PEG downstream	47427	Bloomington
0574	10Go1	SLP	48270	Bloomington
0576	34Eo5	Pars Intercerebralis	49789	Bloomington
0577	64D11	Pars Intercerebralis	41289	Bloomington
0578	19Bo9	Pars Intercerebralis	48840	Bloomington
0579	60C12	Pars Intercerebralis	39244	Bloomington
0580	26Bo1	Pars Intercerebralis	49156	Bloomington
0581	22D11	Pars Intercerebralis	48982	Bloomington
0582	19G10	Pars Intercerebralis	47887	Bloomington

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o583	43Fo7	Pars Intercerebralis	49554	Bloomington
o584	92B11	"Pars Intercerebralis, Prow, Flange, SEZ"	40605	Bloomington
o585	54Bo5	Pars Intercerebralis	50465	Bloomington
o586	56Go9	Pars Intercerebralis	39163	Bloomington
o587	43G11	Pars Intercerebralis	47930	Bloomington
o588	65C11	Pars Intercerebralis	39347	Bloomington
o589	48F11	Pars Intercerebralis	50379	Bloomington
o590	12Go2	Pars Intercerebralis	48521	Bloomington
o591	10H10	Pars Intercerebralis	48445	Bloomington
o592	45Eo3	Pars Intercerebralis	50229	Bloomington
o594	35C12	PEG downstream	49904	Bloomington
o596	47Ao8	Pars Intercerebralis	50288	Bloomington
o597	51F10	Pars Intercerebralis	38793	Bloomington
o598	72F12	Pars Intercerebralis	39787	Bloomington
o599	33Eo1	Pars Intercerebralis	49750	Bloomington
o600	22H10	Pars Intercerebralis	49005	Bloomington
o601	38Go8	Pars Intercerebralis	50020	Bloomington
o602	19A11	Pars Intercerebralis	47880	Bloomington
o603	45Bo8	PEG downstream	50222	Bloomington
o604	25Go4	PEG downstream	49316	Bloomington
o605	13Fo8	PEG downstream	48576	Bloomington
o606	MB010B	broad KC		Janelia Split
o607	MB152B	broad KC		Janelia Split
o608	MB364B	Broad KC except a'b'		Janelia Split
o609	MB417B	Broad KC except gamma		Janelia Split
o610	MB607B	KC gamma d		Janelia Split

o611	MB009B	KC gamma d and main		Janelia Split
o612	MB461B	KC a'b'		Janelia Split
o613	MB418B	KC a'b' m		Janelia Split
o614	MB371B	KC ab p	68383	Bloomington
o615	MB008B	KC ab all		Janelia Split
o616	MB477B	KC ab s		Janelia Split
o617	MB594B	KC ab core		Janelia Split
o618	MB013B	SIFamide		Janelia Split
o619	MB005B	KC a'b'		Janelia Split
o620	MB131B	KC gamma		Janelia Split
o621	Or42a (II)	palp OSN (pb1A)	9970	Bloomington
o622	Or71a (III)	palp OSN (pb1B)	23122	Bloomington
o623	Or85e (III)	palp OSN (pb2A)	23293	Bloomington
o624	Or33c (III)	palp OSN (pb2A)	9966	Bloomington
o625	Or46a (III).9.4	palp OSN (pb2B)	9980	Bloomington
o626	Or59c (I)	palp OSN (pb3A)	23900	Bloomington
o627	Or85d (III)	palp OSN (pb3B)	24148	Bloomington
o628	Or33c (II).G	palp OSN (pb2A)	23290	Bloomington
o629	Or33c (II).C	palp OSN (pb2A)	23893	Bloomington
o630	Or46a (II)	palp OSN (pb2B)	23291	Bloomington
o631	MB072C		2135120	Janelia
o632	SS24671		3025869	Janelia
o633	SS25950	LH2316	3024932	Janelia
o634	SS24820	LH2392	3024051	Janelia
o635	SS22763		3024053	Janelia
o636	SS22675		3024058	Janelia
o637	SS03797		3024018	Janelia
o638	SS03237		3020181	Janelia
o639	SS03791		3032163	Janelia
o640	SS03801		3032166	Janelia

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o641	SSo4962		3032173	Janelia
o642	SS15297		3032177	Janelia
o643	SS16786		3032185	Janelia
o644	SS16574		3032196	Janelia
o645	SS16337		3032188	Janelia
o646	SS16986		3032194	Janelia
o647	SS52244			
o648	SSo2255			
o649	SS52245			
o650	SSooo78			
o651	SS52577			
o652	SS54295			
o653	SS27853			
o654	SSo2191			
o655	SSo2195			
o656	SSooo90			
o657	SSoo408			
o658	SSooo98			
o659	SSo2254			
o660	SS52590			
o661	SS54549			
o662	SSo2239			
o663	SS52578			
o664	SS52266			
o665	SS52257			
o666	SS52313			
o667	SS52267			
o668	SSo4778			
o669	SS25983			
o670	SSo2198			
o671	SSo4773			

o672	SSo4912			
o673	SS46517			
o674	SS46524			
o675	SS47398			
o676	SS47432			
o677	SS47384			
o678	SS47392			
o679	SS46525			
o680	SS50498			
o681	SS50502			
o682	SS50510			
o683	SSo2718			
o684	SS50420			
o685	SS50417			
o686	Trh			
o687	Ddc			
o688	TH.1	from Anderson		
o689	TH.2	from Wu		

Table A.1: Fly stocks used in screening

APPENDIX A. SUPPLEMENTARY INFORMATION ABOUT
LARGE-SCALE NEUROGENETIC SCREENING

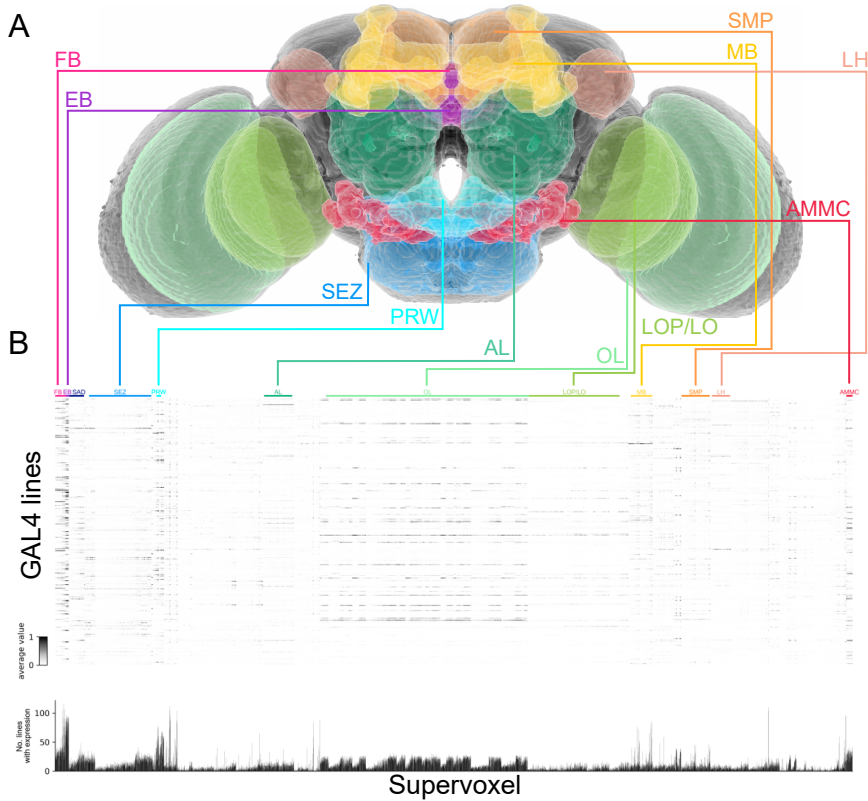


Figure A.1: Expression data of Janelia Generation 1 GAL4 lines used in the foraging screen. (A) Colored regions of anatomical neuropils which have an increased number of lines with GAL4 expression. The neuropils are FB, EB, SEZ, Prow (PRW), Antennal Lobes (AL), Optic Lobes (OL), Lobular Complex (LOP/LO), MB, Superior Medial Protocebebrum (SMP), Lateral Horns (LH), Antennal Mechanosensory and Motor Center (AMMC). (B) Average value of GAL4 expression of each GAL4 driver line in a supervoxel region (as defined in Robie et al. (2017)).



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Identifying neural circuits for nutrient exploration and decision-making in *Drosophila melanogaster*

Dennis Goldschmidt

