

Molecular and neuronal mechanisms underlying protein appetite

Ana Patrícia Tomé Francisco



Dissertation presented to obtain the **Ph.D degree in Neuroscience**
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Instituto de Tecnologia Química e Biológica António Xavier | Universidade NOVA de Lisboa

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MOLECULAR AND NEURONAL MECHANISMS
UNDERLYING PROTEIN APPETITE

ANA PATRÍCIA TOMÉ FRANCISCO

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MOLECULAR AND NEURONAL MECHANISMS UNDERLYING PROTEIN APPETITE

This doctoral thesis was written by Ana Patrícia Tomé Francisco and it is the result of her own work. Any work done in collaboration is explicitly described 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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SUMMARY

A balanced diet is key for an animal's health and well-being. Choices such as what to eat or how much food to ingest have a great impact on the overall fitness of animals. However, the molecular and neuronal mechanisms that influence these decisions are poorly understood. This is particularly true for dietary proteins, which are important determinants of lifespan and reproduction. I combined behavioral, anatomical and bioinformatic analysis with nutritional, microbial, molecular and neuronal manipulations to identify novel mechanisms underlying protein appetite.

First, I showed that in *Drosophila melanogaster*, essential amino acids (eAAs) and the concerted action of the commensal bacteria *Acetobacter pomorum* and *Lactobacilli* are critical modulators of food choice. Using a chemically defined diet, I demonstrated that the absence of any single eAA from the diet is sufficient to elicit specific appetites for AA-rich food. Furthermore, commensal bacteria buffer the animal from the lack of dietary eAAs: both increased yeast appetite and decreased reproduction induced by eAA deprivation are rescued by the presence of commensals. Surprisingly, these effects did not seem to be due to changes in AA levels, suggesting that gut bacteria act through a different mechanism to change behavior and reproduction.

Second, I explored which molecular and neuronal mechanisms could be underlying the strong effect of eAA deprivation on protein feeding. I used a transcriptional approach to understand how the activity of genes in the nervous system is modulated by diet. Using this approach, I identified *marmite*, a gene predicted to encode a secreted peptide with an unknown function in *Drosophila*. To characterize whether *marmite* is involved in feeding decisions, I used quantitative feeding analysis,

together with CRISPR-Cas9-based genome engineering techniques. We show that both *marmite* mutants and neuronal knockdown of *marmite* specifically increased the intake of proteinaceous food, whereas neuronal *marmite* overexpression reduced protein appetite. Moreover, activation of *marmite*-expressing neurons is sufficient to reduce yeast feeding in a *marmite*-dependent manner, suggesting that Marmite is a protein-specific satiety signal. Thus, I have identified a novel neuropeptide that plays a key role in nutritional homeostasis and feeding decisions in *Drosophila melanogaster*.

Finally, sequence and phylogenetic analysis revealed that Marmite is similar to the vertebrate neuropeptide B (NPB) and neuropeptide W (NPW), which have been proposed to affect food intake. Neuronal expression of either of the genes encoding the human NPB and NPW peptides led to a decrease in yeast intake, similar to *marmite* expression in flies. To further investigate this functional conservation, I expressed *marmite* and *NPB* genes in a mouse model and evaluated the effect on food intake. Mice expressing either *marmite* or *NPB* increased food intake, indicating the same biological function.

The results presented in this thesis highlight the importance of the nutritional state in modulating physiology and behavior. I identified a new neuropeptide mediating food intake in *Drosophila*, which is evolutionarily and functionally conserved. This thesis opens a new avenue of research to further investigate the role of this new family of neuropeptides mediating nutritional homeostasis and feeding decisions across different taxa.

Keywords: feeding behavior, amino acid state, commensal bacteria, neuropeptide, *Drosophila melanogaster*, functional conservation

TÍTULO

MECANISMOS MOLECULARES E NEURONAIS SUBJACENTES AO APETITE PARA PROTEÍNA

SUMÁRIO

Uma alimentação equilibrada é fundamental para a saúde e bem-estar animal. Escolhas como o que comer ou quanto ingerir têm um grande impacto no fitness dos animais. No entanto, os mecanismos moleculares e neuronais que influenciam estas decisões são pouco compreendidos. Isto é particularmente verdade no caso das proteínas, que são importantes determinantes de longevidade e reprodução. Nesta tese, combinei análises comportamentais, anatômicas e bioinformáticas com manipulações nutricionais, microbiológicas, moleculares e neuronais para identificar novos mecanismos subjacentes ao apetite proteico.

Primeiro, mostrei que em *Drosophila melanogaster*, aminoácidos essenciais (AAEs) e a ação conjunta das bactérias comensais *Acetobacter pomorum* e *Lactobacilli* são potentes moduladores da escolha alimentar. Usando uma dieta quimicamente definida, demonstrei que a ausência de um único AAE da dieta é suficiente para provocar apetites específicos por alimentos ricos em AAs. Além disso, as bactérias comensais protegem o animal da falta de AAEs na dieta: tanto o aumento do apetite por levedura quanto a diminuição da reprodução induzida pela privação de AAEs são resgatados pela presença destas bactérias. Surpreendentemente, estes efeitos não parecem ser devidos a alterações nos níveis de AAs, sugerindo

que as bactérias intestinais agem por meio de um mecanismo diferente para alterar o comportamento e a reprodução.

Em segundo lugar, explorei que mecanismos moleculares e neuronais poderiam estar subjacentes ao forte efeito da privação de AAEs na ingestão de alimentos ricos em proteínas. Usei uma análise do transcriptoma para entender como a atividade dos genes no sistema nervoso é modulada pela dieta. Com esta abordagem, identifiquei *marmite*, um gene que parece codificar um péptido secretado com uma função desconhecida em *Drosophila*. Para caracterizar se *marmite* está envolvido em decisões alimentares, usei uma metodologia quantitativa para avaliar o consumo de alimentos, juntamente com técnicas de engenharia genética baseadas em CRISPR-Cas9. A mutação de *marmite* bem como o bloqueio neuronal de *marmite* aumentaram especificamente a alimentação de levedura enquanto a sobrepressão de *marmite* reduziu o apetite por levedura. Além disso, a ativação de neurónios que expressam *marmite* é suficiente para reduzir a alimentação de levedura de uma forma dependente de *marmite*, sugerindo que Marmite atua como um sinal de saciedade específico para proteína. Assim, identifiquei um novo neuropéptido que desempenha um papel fundamental na homeostasia nutricional e nas decisões alimentares em *Drosophila*.

Finalmente, a análise filogenética revelou que Marmite é semelhante ao neuropéptido B (NPB) e neuropéptido W (NPW) em vertebrados, que foram previamente descritos como estando envolvidos na ingestão de alimentos. A sobreexpressão neuronal de qualquer um dos genes que codificam a forma humana de NPB e NPW levou a uma diminuição na ingestão de levedura, semelhante à sobreexpressão de *marmite* em moscas. Para investigar ainda mais esta conservação funcional, expressei os genes de *marmite* e *NPB* em ratinhos e avaliei o

efeito na ingestão de alimentos. Tanto ratinhos que expressam *marmite* como *NPB* aumentaram a ingestão de comida, indicando que estes dois neuropeptídeos têm a mesma função biológica.

Os resultados apresentados nesta tese destacam a importância do estado nutricional na modulação da fisiologia e do comportamento. Identifiquei um novo neuropeptídeo que medeia a ingestão de alimentos em *Drosophila*, e que é evolutiva e funcionalmente conservado. Esta tese abre assim um novo caminho de pesquisa para continuar a investigar o papel desta nova família de neuropeptídeos, envolvidos na homeostasia nutricional e nas decisões alimentares em diferentes taxonomias.

Palavras-chave: comportamento alimentar, níveis de aminoácidos, bactérias comensais, neuropeptídeo, conservação funcional, *Drosophila melanogaster*.

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LIST OF ABBREVIATIONS

AA	amino acid	DSK	drososulfakinin
AAE	aminoácidos essenciais	eAA	essential amino acid
AAV	adeno-associated virus	FC	fold change
AKH	adipokenetic hormone	FIT	female-specific independent of <i>transformer</i>
<i>Ap</i>	<i>Acetobacter</i> <i>pomorum</i>	flyPAD	fly proboscis activity detector
Arg	arginine	GCN2	general control nonderepressible 2
AstA	allatostatin A	GFP	green fluorescence protein
AstC	allatostatin C	GPCR	G protein-coupled receptor
BCAA	branched-chain amino acid	GRNs	gustatory receptor neurons
C- terminal	carboxyl-terminus	His	histidine
CAFE	capillary feeder	HM	holidic medium
CCK	cholecystokinin	Ile	isoleucine
CNS	central nervous system	IPCs	insulin-producing cells
Crz	corazonin	Kir 2.1	inwardly-rectifying potassium channel 2.1
DA	dopamine	<i>Lb</i>	<i>Lactobacillus brevis</i>
Dh31	diuretic hormone 31	Leu	leucine
Dh44	diuretic hormone 44		
DILPs	<i>Drosophila</i> insulin- like peptides		

LH	lateral horn	PI	pars intercerebralis
Lk	leucokinin	Pro	proline
<i>Lp</i>	<i>Lactobacillus plantarum</i>	RNAi	ribonucleic acid interference
Lys	lysine	RNAseq	RNA sequencing
Met	methionine	SEM	standard error of the mean
MIP	myoinhibitory peptide	SEZ	subesophageal zone
mmt	marmite	SMP	superior medial protocerebrum
N-terminal	Amino-terminal	sNPF	short neuropeptide F
neAA	nonessential amino acid	SP	signal peptide
NPB	neuropeptide B	Thr	threonine
NPBWR1	neuropeptides B and W receptor 1	Trp	tryptophan
NPBWR2	neuropeptides B and W receptor 2	TrpA1	transient receptor potential A1
NPF	neuropeptide F	Tyr	tyrosine
NPW	neuropeptide W	Upd1/2	unpaired 1/2
NPY	neuropeptide Y	Val	valine
P:C	protein:carbohydrate	VMH	ventromedial hypothalamus
PBS	phosphate-buffered saline	VNC	ventral nerve cord
PBST	phosphate-buffered saline with Tween 20	YBM	yeast-based medium
Phe	phenylalanine	YPI	yeast preference index

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OVERVIEW

This thesis is divided into five chapters. Chapter 1 provides a general introduction to the topics that will be discussed throughout the thesis. It outlines general concepts and knowledge about nutritional homeostasis, nutrient-specific appetites, neuropeptides and neuronal circuits involved in feeding regulation, the effect of host-microbiota interactions and the use of the model organism *Drosophila melanogaster* to study feeding decisions.

Chapters 2, 3 and 4 comprise the main results of my research. Chapter 2 focuses on the role of essential amino acids (eAAs) and commensal bacteria in controlling food choice and reproduction. In Chapter 3, I describe the function of a previously uncharacterized gene, that I named *marmite*. I provide anatomical, transcriptional and functional data supporting the idea that this gene encodes a new neuropeptide involved in protein intake and homeostasis in *Drosophila*. In Chapter 4, I explore the evolutionary history of this new *Drosophila* neuropeptide and find that Marmite defines a new family of neuropeptides, including the vertebrate neuropeptides B and W. Moreover, using flies and mice, I demonstrate that these neuropeptides share not only sequence similarities but also functional conservation, playing a role in feeding regulation.

Finally, in Chapter 5, I provide an overall discussion of my results, aiming to incorporate them in the context of what is currently known in the field and how they can be further investigated in the future.

Para o Didi

CHAPTER 1

GENERAL INTRODUCTION

Animal behavior is a fascinating phenomenon in the natural world. It ranges from simple actions, to maintain the most basic animal functions, to complex behaviors requiring intricate brain computations. Animals display a repertoire of different behaviors from which they have to selectively choose from. But how does this occur? Animals have to dynamically choose their actions taking into consideration not only the external environment, but also their internal context, or state. This *internal state* can be defined as the combination of different molecular and cellular processes within the organism that define its current state. It can comprise different states, such as nutritional or reproductive, that are interconnected.

The nervous system is the ultimate responsible for generating behavioral outputs. It has to integrate external and internal information in order to produce an adequate response. But what is an adequate response? Animals perform physiological and behavioral adaptations to maintain relatively constant and optimal organismal conditions, or *homeostasis*. Animal life is not possible without a constant supply of nutrients to ensure survival and overall health. Therefore, the regulation of nutrient intake is a key part of an animal's behavior. But how does the nervous system do it? What are the molecular and neuronal mechanisms underlying feeding behavior and how is it modulated by the animal's internal states?

In this introduction, I will first address the phenomenon of nutritional homeostasis and specific appetites. Secondly, I will focus on neuronal sensing pathways informing the brain of the animal's nutrient state. Thirdly, I will describe neuronal populations regulating feeding behavior in the fruit fly *Drosophila melanogaster* and the importance of neuropeptide signaling. Next, I will highlight the microbiota as a modulator of behavior and physiology. Finally, I will introduce the study of feeding behavior in flies as a read out for our questions.

1.1 - NUTRITIONAL HOMEOSTASIS

Nutrition is essential for health and well-being of all animals. Therefore, a main function of the nervous system is to detect food sources from the environment, integrate that information with the nutritional needs of the organism and produce appropriate feeding decisions to achieve a balanced nutritional state. Decisions such as when to eat, or which or how much food to ingest have a great impact on the overall fitness and reproductive success in organisms across the animal kingdom. A balanced intake of macronutrients, such as proteins, lipids and carbohydrates, is particularly important to maintain nutritional homeostasis. Any deviation due to a lack, imbalance or overconsumption of specific nutrients can have a negative impact on fitness. For example, while energy is vital for life, excessive intake of calories has a major impact in human health, due to its link to obesity and other metabolic disorders (Caballero, 2007). Similarly, protein intake is especially important for building and maintenance of body tissues, but its overconsumption is known to reduce lifespan and negatively impact health (M. E. Levine et al., 2014; Piper et al., 2011; Solon-Biet et al., 2014). Therefore, animals, including humans, must use different physiological and behavioral strategies to tightly regulate the intake of nutrients in order to maintain nutrient homeostasis.

Nutrients are not separately ingested but mixed within food, and dietary macronutrient composition has been shown to affect fitness in species ranging from *Drosophila* to mammals (Simpson et al., 2015). In flies, increasing dietary protein:carbohydrate (P:C) ratio decreases lifespan, while increasing reproductive output (K. P. Lee et al., 2008). Consistent with this, the known benefits of caloric restriction have been specifically linked to a decrease in amino acid intake (Grandison et al.,

2009). Similarly, in mice, reducing protein in the diet optimized longevity and health (Solon-Biet et al., 2014). Thus, regulating food intake towards a nutritional balance that would optimize life history traits could confer an evolutionary advantage. As such, one could expect that animals have evolved strategies to be able to regulate not only their food intake as a whole, but also the intake of specific nutrients (Figure 1.1).

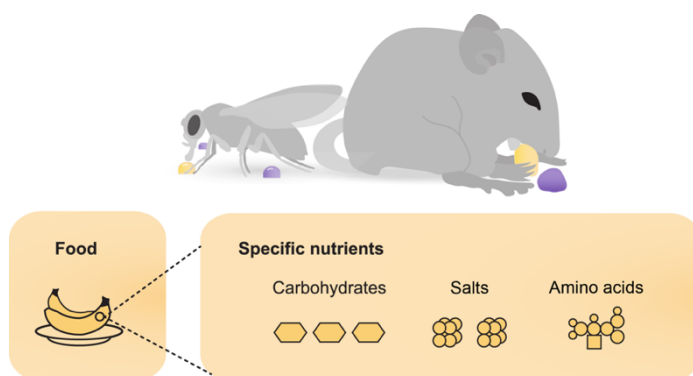


Figure 1.1 - Food is composed of specific nutrients. Adapted from Münch et al. (2020).

1.1.1 - NUTRIENT-SPECIFIC APPETITES

From insects to mammals, multiple studies have reported the presence of nutrient-specific appetites either to compensate for the lack of a nutrient or in anticipation for a future physiological need: female rats, for example, increase their intake of sodium during pregnancy due to the high demands of this nutrient, while only increasing calcium intake later to support lactation (Richter & Barelare, 1938). In *Drosophila*, mating leads to a feed-forward signal to increase salt appetite (Walker et al., 2015). Proteins are composed of amino acids (AAs) and these are very

important macronutrients, essential for growth and maintenance of tissues. Female flies also increase their protein appetite after mating in anticipation for egg production (Walker et al., 2015). Additionally, deficits in dietary protein also lead to an innate compensatory appetite for protein-rich foods (Ribeiro & Dickson, 2010). For most *Drosophila* species, the main source of protein in the wild is yeast (Phaff et al., 1956) and flies compensate for yeast deprivation by changing their feeding preferences from a sucrose to a yeast source (Ribeiro & Dickson, 2010; Vargas et al., 2010). Like insects, the ability to regulate the intake of protein has also been demonstrated in rodents (Leib & Knight, 2015) and even humans (Griffioen-Roose et al., 2012). However, despite the behavioral evidence for nutrient-specific appetites, the molecular and neuronal mechanisms by which the levels of different nutrients drive behavioral changes are still not fully understood.

1.2. – NEURONAL NUTRIENT SENSING

To behaviorally respond to an environmental cue such as food, an important information for the nervous system to consider is the nutritional state of the animal, through sensing of macro- and micronutrients. The mechanisms by which neuronal nutrient sensing occurs can be broadly divided into three categories: direct and indirect mechanisms within the nervous system and via inter-organ communication (Figure 1.2). In the following sections I will provide some examples for each category.

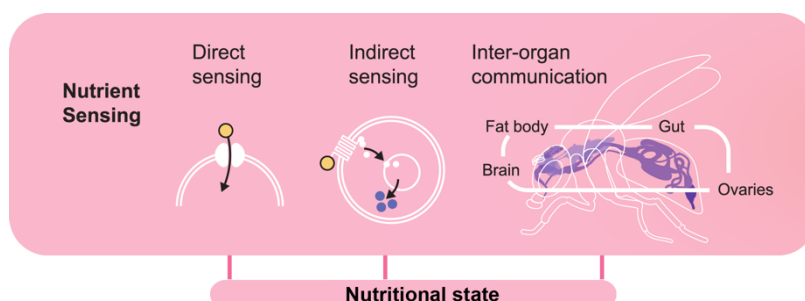


Figure 1.2 - Different types of nutrient sensing informing the nervous system about the nutrient state of the animal. Adapted from Münch et al. (2020).

1.2.1 - DIRECT SENSING

Direct sensing in the central nervous system includes sensors, such as receptors and transporters, expressed in neurons that directly respond to nutrients and lead to the activation of the neurons or intracellular signaling cascades (Münch et al., 2020). The TOR pathway is the canonical pathway for AA sensing (Hara et al., 1998) and, in *Drosophila*, its neuronal expression has been linked to food selection (Ribeiro & Dickson, 2010; Vargas et al., 2010). Another example is the gustatory receptor GR43a, which is expressed in a subset of central brain neurons to detect fructose

from the hemolymph (Miyamoto et al., 2012). The neurons expressing GR43a receptors act as fructose sensors mediating feeding in a nutritional-state dependent manner. Another case that could also be considered direct sensing of nutritive sugars corresponds to the neurons producing the diuretic hormone 44 (Dh44) (Dus et al., 2015). This small subset of neurons in the pars intercerebralis (PI) are specifically activated by nutritive sugars in the hemolymph via a glucose transporter, leading to Dh44 release. Dh44 binding to different receptors in the nervous system and in the gut lead to increased proboscis extension response and excretion, respectively (Dus et al., 2015). Recently, this same neuronal population has also been reported to act as a sensor for specific amino acids, through a putative transporter (Yang et al., 2018).

1.2.2 - INDIRECT SENSING

Indirect sensing mechanisms also occur in the nervous system, but rely not on direct sensing of nutrients but on the consequences of their availability. A good example is the GCN2 pathway, a canonical nutrient sensing pathway (Dever & Hinnebusch, 2005). In nutrient balanced conditions, transfer RNAs (tRNAs) are bound to amino acids; when a tRNA is not bound to amino acids, it instead binds to GCN2. Accumulation of GCN2-bound tRNAs activates the GCN2 signaling cascade. In *Drosophila* larvae, GCN2 is proposed to act in dopaminergic neurons to promote the rejection of amino acid-imbalanced diets (Bjordan et al., 2014). In mice, while classic studies reported the neuronal functions of GCN2 (Hao, 2005; Maurin et al., 2005), the involvement of neuronal GCN2 in coordinating amino acid feeding in rodents has been recently challenged (Leib & Knight, 2015). These studies highlight how little it is

still known about amino acid sensing in neurons, as well as the challenges in pinpointing the molecular basis of neuronal nutrient sensing and how it is connected to behavior.

1.2.3 - INTER-ORGAN COMMUNICATION

Nutrient sensing mechanisms via *inter-organ communication* rely on sensing in a specific organ, which sends a signal conveying the nutritional information over short or long ranges to an effector organ, like the brain. The levels of many hormones, such as cholecystokinin (CCK), insulin, leptin, and ghrelin are modulated by nutrient availability (Andermann & Lowell, 2017; Beutler & Knight, 2018). Yet, while some are involved in altering feeding, the behavioral impact of many of these factors remains poorly studied. It is especially unclear whether these nutrient state signals regulate nutrient-specific appetites. Interestingly though, some hormonal signals have been shown to respond to the levels of specific nutrients. One example is the fibroblast growth factor 21 (FGF21) (Hill et al., 2019). This hormone is produced primarily by the liver and signals to various tissues by endocrine signaling which has significant impact on metabolism. Importantly, FGF21 expression responds to dietary conditions, and when injected intracerebrally, it specifically increases protein preference in mice. In flies, the fat body is functionally analog to the liver and adipose tissue of mammals. A peptide specifically produced in the head fat body, called female-specific independent of transformer (FIT) has been described as a protein sensor (Sun et al., 2017). Protein consumption increases *fit* expression and the peptide then acts via the insulin pathway to suppress protein intake. However, the function of this peptide might not be specific to protein, as

a recent study shows that the germline alters sugar appetite by regulating the expression of FIT, suggesting a role in carbohydrate homeostasis as well (Carvalho-Santos et al., 2020). The gut is the entry point for ingested nutrients in the body, making it a good candidate for nutrient sensing. Accordingly, gut-derived peptides have been described to act as nutrient sensors. One example is the Diuretic Hormone 31 (Dh31) which has been recently suggested as mediating the switch between feeding and courtship (H.-H. Lin et al., 2022). The presence of amino acids in the gut promotes the release of Dh31, which in turn acts on Dh31 receptor neurons in the brain to promote feeding suppression (H.-H. Lin et al., 2022). Another recent example of gut-brain nutrient sensing is the peptide CNMamide (CNMa), expressed in enterocytes in the anterior gut (B. Kim et al., 2021). The authors proposed that these cells sense amino acid levels, either from the diet or derived from the microbiome and relay this information to the brain via CNMa, establishing a microbiome-gut-brain axis to mediate a compensatory appetite upon amino acid deprivation. Even though the exact mechanisms by which gut-derived peptides and peptide hormones are not fully uncovered, they represent good examples of signaling molecules mediating nutritional state information.

These different types of sensing mechanisms ultimately inform the brain of the nutritional state of the animal. However, it is still largely unclear how this molecular nutrient state information is coordinated and translated into changes in the activity of specific neurons and then into further changes in the overall circuitry to induce appropriate behavioral outputs. Moreover, it is also not clear whether internal sensing of different nutrients converges on and activates a common feeding circuit or whether there are dedicated nutrient-specific circuits acting in parallel.

1.3 – NEURONAL REGULATION OF FEEDING

The brain is responsible for the combination and processing of information from external stimuli and from the internal state of the animal. From sensory detection, to higher-order centers and motor commands, internal state information can be integrated at different levels to modulate these sensory-motor transformations and adapt them to the current state of the organism (Münch et al., 2020). Feeding behavior is no exception and nutritional state information can be integrated at different stages (Figure 1.3). In this section, I will describe some examples of neuronal populations which have been described to be involved in the regulation of feeding in *Drosophila melanogaster*.

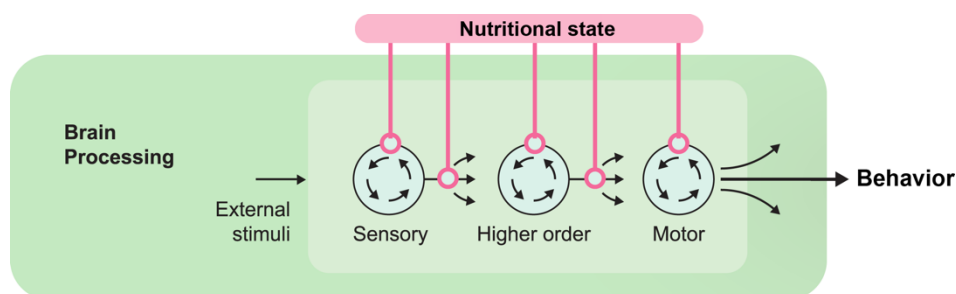


Figure 1.3 - Nutritional state information is integrated at different levels of brain processing to generate behavior. Adapted from Münch et al. (2020).

Sensory modalities like taste and olfaction play a key role in determining which food sources animals approach and consume (Corrales-Carvajal et al., 2016; Scott, 2018). Sensory processing is therefore a crucial step in the regulation of feeding decisions. Nutritional state has been shown to modulate different aspects of chemosensory processing, for example, by enabling a more efficient detection of food cues or by modulating their saliency (Inagaki et al., 2014; LeDue et al., 2016).

Indeed, starvation lowers the detection thresholds for attractive, food-related cues, like sugars, detected by sugar-sensing Gustatory Receptor Neurons (GRNs), while at the same time, increasing the tolerance for aversive cues, such as bitter compounds in bitter-responsive GRNs (Inagaki et al., 2012, 2014; LeDue et al., 2016). Moreover, changes in sensory processing can be nutrient-specific. A recent study identified a population of IR76b-expressing GRNs required for yeast intake and whose activity is enhanced by amino acid deprivation (Steck et al., 2018). The response of sweet GRNs, however, is not changed upon amino acid deficiency. Furthermore, the authors mapped specific subsets of neurons within this population to different behavioral parameters, such as initiation and sustaining of feeding, unravelling important neuronal populations in promoting protein homeostasis (Steck et al., 2018).

Beyond sensory neurons, several populations of interneurons have been associated with modulation of feeding behavior. The Sub esophageal zone (SEZ) is described as the taste and feeding center in the insect brain, as it comprises projections from GRNs and harbors cell bodies of motor neurons controlling the proboscis during feeding, along with several interneurons (Scott, 2018). Different studies have described neuronal populations specially involved in sucrose appetite (S. M. Kim et al., 2017; S. Lin et al., 2019). For example, starvation increases sensitivity to sugar, at least in part, via increased dopamine (DA) release onto *Gr5a*-expressing sugar-sensing GRNs (Inagaki et al., 2012; Marella et al., 2012). Indeed, activation of a single pair of DAergic neurons termed TH-VUM is sufficient to increase proboscis extension responses towards sugar, but not water or bitter compounds (Marella et al., 2012). Moreover, the activity of this pair of neurons is modulated by nutritional state. Another example is a subset of cholinergic interneurons, IN1, in the SEZ, which receives input

from sweet-sensing pharyngeal GRNs, and are important for sustaining sugar feeding in starved flies (Yapici et al., 2016). Four interoceptive neurons (ISNs) in the SEZ also mediate sugar intake by oppositely regulating sugar and water consumption in flies (Jourjine et al., 2016).

The above examples highlight the advanced knowledge of the neuronal circuits underlying sugar intake. However, only recently the interneuronal circuits specifically mediating protein feeding started to be uncovered. Recently, a group of neurons was shown to mediate protein homeostasis (Münch et al., 2022). This study used pan-neuronal volumetric activity imaging in the *Drosophila* brain to identify a new region in the SEZ, called *borboleta*, which is modulated by protein state. Additionally, the functional validation of this modulation showed that activation of neurons in this region is sufficient to promote food choice towards a protein-rich food (Münch et al., 2022).

Also, outside the SEZ, diverse sets of neurons have been implicated in feeding regulation. The activity of a small group of dopaminergic neurons, named DA-WED, has been proposed to increase upon protein deprivation (Q. Liu et al., 2017). Activation of these neurons leads to a gain in yeast appetite whereas silencing suppresses the yeast appetite elicited by protein deprivation. In parallel, DA-WED neurons also seem to modulate sucrose consumption in an opposite way. Glutamine deprivation seems to be particularly necessary to increase DA-WED activity, but the mechanisms by which these neurons sense the need for amino acids are still unknown.

A small group of neurons named Taotie neurons form a cluster in the PI, a region known to also harbor neurosecretory cells, such as insulin producing cells (IPCs) (Zhan et al., 2016). The activity of these neurons reflects the nutritional state of the animal. Besides, their activation leads

to food consumption in fed flies and suppresses secretion of the *Drosophila* insulin-like peptides (DILPs), promoting hunger behaviors, at least partially by inhibiting the satiety effect of DILPs (Zhan et al., 2016).

Another component of nutrient selection and hunger response in flies is mediated by a set of neurons innervating the ellipsoid body (EB), expressing SLC5A11, a sodium/solute co-transporter-like (*cupcake*) (Dus et al., 2013; Park et al., 2016). SLC5A11 loss-of-function or silencing of EB neurons affect nutrient selection (Dus et al., 2013). Also, the activity of these neurons is modulated by feeding state, and they are both necessary and sufficient for several hunger-driven behaviors, including food odor attraction, nutritive sugar preference and food intake (Park et al., 2016).

Downstream of neurons modulating behavioral responses according to the nutritional state, are motor control neurons that implement these responses as a series of distinct motor modules (Shiu et al., 2022; Sterne et al., 2021). These are often called command neurons. In *Drosophila*, a single pair of neurons was reported as feeding command neurons, termed FDG (Flood et al., 2013). Activation of these neurons is sufficient to initiate the feeding motor program and they are also necessary for normal feeding, indicating a role in the feeding motor hierarchy.

Brain processing and integration of internal state and external information is a crucial step for animals to adapt their behavior to their current or future needs (Münch et al., 2020; Walker et al., 2015, 2017). As described above, several neuronal populations modulating feeding have already been identified, promoting either satiety or hunger-driven behaviors. Additionally, neuropeptides, an important signaling class of molecules, play a major role in the regulation of feeding at a global scale and will be discussed in the next section.

1.4 – NEUROPEPTIDES AND FEEDING BEHAVIOR

The fitness and survival of an animal depend on its ability to adapt its physiological and behavioral processes to the dynamic environmental conditions. This requires flexible adjustments to be able to integrate external environmental cues with the internal state and needs of the animal. The nervous and endocrine systems are crucial mediators of this adaptation, using signals with different spatial and temporal scales of action. While synaptic transmission is fast, at a slower timescale, signaling is usually performed by neuropeptides or peptide hormones (Nässel & Zandawala, 2019, 2022).

1.4.1 - NEUROPEPTIDE BIOSYNTHESIS

Neuropeptides and peptide hormones are produced in neurons or neurosecretory cells of the nervous system or in specific peripheral sites. The synthesis often occurs in neurons with large cell bodies and long axon ramifications (Nässel & Zandawala, 2019). Moreover, most neuropeptides are produced in a small number of cells, sometimes only a few pairs, such as SIFamide in the *Drosophila* brain (Martelli et al., 2017).

Following the transcriptional activation of genes encoding large precursor peptides (prepropeptides), enzymatic cleavage can give rise to more than one peptide from the same precursor (Nässel & Zandawala, 2019). The prepropeptides comprise specific features, necessary for neuropeptide production. First, they have a signal peptide (SP) in their amino (N)-terminal that directs the trafficking of the protein to the secretory pathway. Second, they possess the progenitors of the mature peptides as well as spacer peptides, which correspond to fragments with

unknown biological function. Finally, prepropeptides can harbor several motifs of basic amino acids (arginine, histidine or lysine), which are target sites for enzymatic cleavage by proprotein convertases. After proteolytic cleavage, peptides are often subject to post-translational modifications, such as amidation, glycosylation or formation of disulfide bridges. Mature peptides are packed into dense-core vesicles and transported to axon terminals for storage and release (Figure 1.4).

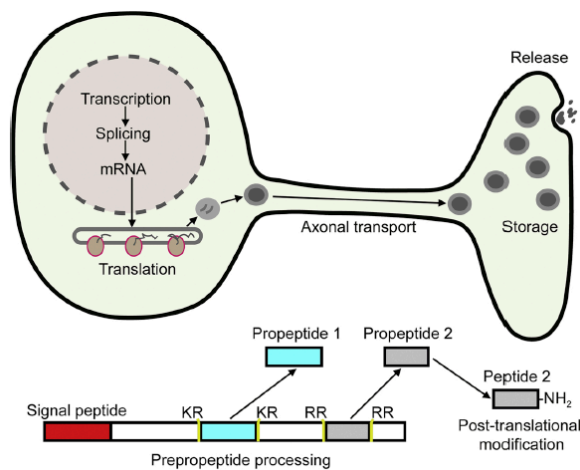


Figure 1.4 - Neuropeptide synthesis and processing. Adapted from Nässel & Zandawala (2019).

1.4.2 - ORIGIN AND EVOLUTION OF NEUROPEPTIDES

Neuropeptides, an important class of signaling molecules, are present in different phyla in the animal kingdom (Nässel & Zandawala, 2019). However, defining the origins and evolutionary relationships of neuropeptides from different species has been challenging (Elphick et al., 2018). Recently, though, this task has become easier with the advent of the genome era, with the sequencing of *C. elegans*, *D. melanogaster* and human genomes, as well as several other species. This genome information

allowed scientists to investigate the presence and evolutionarily relationships between simple and more complex systems in a more comprehensive way (Elphick et al., 2018). For example, genomic and transcriptomic analysis of species of sponges exposed several predicted neuropeptides, with dibasic residues and amidation sites (L. Schoofs et al., 2017). Nonetheless, even though neuropeptides seem to be found very early in animal evolution, the majority of bilaterian neuropeptides and their receptors have not been found in non-bilaterian species (Jékely, 2013). Despite the importance of genomic and sequence information, due to their small size and the conservation of only a few critical residues in most cases, establishing evolutionary relationships based on sequence queries is still challenging (Elphick et al., 2018). Even so, several examples of neuropeptide conservation have been discovered. These include functionally conserved neuropeptides between insects and vertebrates, playing a role in diverse aspects of the animal's life, from physiology to behavior, including feeding (Nässel & Zandawala, 2019).

1.4.3 - *DROSOPHILA* NEUROPEPTIDES INVOLVED IN FEEDING BEHAVIOR

Animals fluctuate between different levels of nutrients in their bodies that drive opposite behaviors. The lack of nutrients or energy generates a *hunger* state in the animal, leading it to adapt its behavior to prioritize food search and consumption. Conversely, food ingestion leads to a state of *satiety*, leading to meal cessation and reduced foraging behavior (Corrales-Carvajal et al., 2016; Itskov et al., 2014). A diverse range of neuronal signals induced by nutritional state have been reported and they are often divided into satiety or hunger signals. However, for

some of these signals, a clear categorization as hunger or satiety signal is not so easy to establish. Nevertheless, in this section, I will focus on described satiety and hunger neuropeptide signaling in fruit flies.

In *Drosophila*, several neuropeptides have been proposed to act as *satiety* signals, i.e. reducing food consumption. One example is *hugin*, thought to be the fly homologue of the mammalian Neuromedin U (Melcher et al., 2006). High levels of *hugin* mRNA correlate with low food intake in larvae and activation of *hugin* neurons decreases yeast intake, while silencing increases feeding in both larvae and adults (Melcher & Pankratz, 2005; A. Schoofs et al., 2014).

Myoinhibitory peptides (MIPs) have also been described as satiety signals in the adult fly brain, with sequence similarities to the mammalian Galanin, previously implicated in body weight and feeding regulation (Blackburn et al., 1995; Gundlach, 2002; Min et al., 2016). Both *mip* mutant flies and silencing of MIP neurons lead to an increase in feeding motivation and consumption, whereas activation of MIP neurons reduces food intake in flies (Min et al., 2016). Galanin receptors are also reported to be homologs of the *Drosophila* Allatostatin A (*AstA*) receptors (Birgül et al., 1999). Indeed, activation of neurons expressing *AstA* inhibits food intake (Hergarden et al., 2012), suggesting a role in promoting satiety. This function seems to be nutrient-specific - unlike control flies which prefer sucrose, activation of *AstA*-expressing neurons shifts this preference to yeast, suggesting that *AstA* might be a satiety signal for sugar but not protein (Hentze et al., 2015). Moreover, *AstA* seems to play a role in the balance between the DILPs and the glucagon-like peptide adipokenetic hormone (*Akh*) (Hentze et al., 2015).

DILPs are fly equivalents of insulin. The *Drosophila* genome possesses eight DILP genes, with different patterns of expression. DILPs 2, 3 and 5 are co-expressed in Insulin Producing Cells (IPCs) in the PI of the adult and larval brains (Garelli et al., 2012; Ikeya et al., 2002). DILPs have been implicated in hunger-driven behaviors (Loh et al., 2017; Tsao et al., 2018; Yu et al., 2016). Nutrient state regulates DILP secretion from the IPCs, where deprivation leads to DILPs accumulation, while food intake leads to secretion and increased levels in circulation (Rajan & Perrimon, 2012).

Unpaired 2 (Upd2), produced by fat cells in the head is the fly homologue of leptin, while Unpaired 1 (Upd1), also a leptin-like peptide, is produced in a small subset of neurons. Both have been reported to function as satiety signals and neuronal knockdown of *upd1* increases food attraction and intake (Beshel et al., 2017; Rajan & Perrimon, 2012). Moreover, Upd1 signaling interferes with neuropeptide F (NPF), the fly homologue of the orexigenic mammalian neuropeptide Y (NPY) (Beshel et al., 2017), which will be described in more detail later in this section.

Drosulfakinins (DSKs) are a class of neuropeptides expressed in the central brain, including expression in the IPCs. DSK has a mammalian homologue, cholecystokinin (CCK), which is also involved in feeding regulation. IPC-specific *dsk* knockdown increases food intake supporting its effect as a satiety signal (Söderberg et al., 2012).

In sum, a variety of satiety signals produced in the brain have already been described to play a role in food intake in *Drosophila*. However, how the brain integrates all these and other unknown satiety signals in order to orchestrate a combined behavioral response is still unclear.

To maintain nutrient homeostasis, animals also display a variety of *hunger* signals to promote food seeking and consumption upon fasting or poor nutritional contexts. SIFamide, for example, is produced in only four neurons in the pars intercerebralis with projections throughout the central nervous system (CNS) and these neurons are activated by starvation (Martelli et al., 2017; Terhzaz et al., 2007). Artificial activation of SIFamide neurons promotes appetitive behavior in satiated flies as well as an increase in food intake in a SIFamide dependent manner, indicating that SIFamide acts as a hunger signal. However, neuronal silencing of SIFamide neurons did not decrease feeding in starved flies, suggesting that other, redundant signals might be promoting food intake. Indeed, activation of the satiety MIP-expressing neurons inhibits SIFamide neurons, suggesting that other downstream targets of MIP neurons could regulate feeding suppression (Martelli et al., 2017). Activation of Hugin neurons has also been described to increase activity of SIFamide neurons, but the functional role of this change remains unclear (Martelli et al., 2017).

Neuropeptide F (NPF) is a homologue of the mammalian neuropeptide Y (NPY), which is a known orexigenic signal (Brown et al., 1999; Horio & Liberles, 2021). NPF is expressed in the brain but also in endocrine cells in the gut. Neurons producing NPF have been shown to promote feeding in flies as well as increased taste responsiveness, food odor attraction and hunger-dependent food memory (Beshel & Zhong, 2013; Chung et al., 2017; Hergarden et al., 2012; Inagaki et al., 2014; Krashes et al., 2009). NPF signaling seems to act in parallel with the satiety factor AstA, since coactivation of both set of expressing neurons leads to an intermediate phenotype (Hergarden et al., 2012). As mentioned above, NPF neurons are also inhibited by the satiety factor Upd1 via its receptor

domeless (Beshel et al., 2017). Therefore, NPF neurons seem to integrate information from other signaling pathways to generate a hunger signal.

It is also worth mentioning that recently, gut-derived NPF has been shown to have a different role in feeding (Malita et al., 2022; Yoshinari et al., 2021). Gut-NPF is sugar-responsive and loss of gut NPF increases food intake, challenging the view of NPF as a hunger signal. Gut-NPF signaling acts on AKH-producing cells in the corpora cardiaca as well as in insulin producing cells to regulate lipid and carbohydrate metabolism (Yoshinari et al., 2021). Another study also reported a role of gut-NPF in the regulation of sugar intake, acting via AKH to promote sugar satiety, while increasing protein consumption, therefore also modulating food choice (Malita et al., 2022). Also in the gut, Allatostatin C (AstC) is released from enteroendocrine cells upon nutrient stress and acts on AKH-producing cells to mediate increase in food intake (O. Kubrak et al., 2022).

Another NPY-related peptide, the short neuropeptide F (sNPF), although not evolutionarily related to NPF, also mediates food intake (Nässel & Wegener, 2011). Gain- and loss-of-function experiments targeting sNPF promote or suppress feeding in adult flies, respectively, suggesting a role in hunger signaling (K.-S. Lee et al., 2004). This could be partly due to its regulation of bitter sensitivity during starvation (Inagaki et al., 2014).

A well-known signal involved in physiological and metabolic regulation is the adipokinetic hormone (AKH), a glucagon-like peptide in insects. AKH is expressed in the corpora cardiaca and starvation induces its release to the hemolymph (S. K. Kim & Rulifson, 2004). AKH and DILPs are key regulators of energy homeostasis and their roles are mutually inhibitory (Buch et al., 2008). Food intake is also modulated by AKH, either by being the downstream target of AstC or NPF signaling (O.

Kubrak et al., 2022; Malita et al., 2022) or by acting on neuronal populations involved in feeding regulation: AKH signaling activates four interoceptive SEZ neurons (ISNs) to regulate sugar intake and also reduces bitter sensitivity in adult flies (Inagaki et al., 2014; Jourjine et al., 2016).

Another peptide involved in feeding regulation is Corazonin (Crz), evolutionarily related to AKH (S. Lin et al., 2019). Activation of *crz*-expressing neurons increases food intake, while *crz* knockdown in the same neurons reduces feeding, suggesting that Crz promotes hunger-driven behaviors (Hergarden et al., 2012; O. I. Kubrak et al., 2016). Additionally, Crz has also been implicated in other physiological roles, including modulation of osmotic stress and homeostasis (Zandawala et al., 2021).

Leucokinin (Lk) is an insect neuropeptide involved in several physiological processes and behaviors (S. Lin et al., 2019). Its receptor is analog to the tachykinin receptors in mammals, but a Lk homologue has not yet been identified. Besides roles in osmotic regulation and sleep, Lk has also been reported to be involved in feeding regulation, where *Lk* and *Lkr* mutants decrease feeding (Yurgel et al., 2019; Zandawala et al., 2018). Lk is expressed in different sets of neurons in the Lateral horn (LH), SEZ and abdominal ganglia, which seem to have different physiological functions (Yurgel et al., 2019; Zandawala et al., 2018). Another study reported that both activation or silencing of *Lk*-expressing neurons reduced food intake (Y. Liu et al., 2015). Thus, Lk seems to play a complex role and further studies are needed to elucidate the mechanisms by which Lk signaling affects feeding and other behaviors.

As described, several peptides mediating satiety and hunger information have been already identified in flies and other species.

However, despite the described roles, for many of these peptides it is still unclear whether their effects mediate general feeding or are nutrient-specific. Moreover, the big challenge for the nutritional neuroscience field is to understand how all these different signals are integrated at the circuit and organism level. Do they act in parallel or in convergent pathways? Mapping the intricate network of signals and their neuronal subsets is a key step to elucidate how animals integrate and balance different types of information in order to generate a concerted behavioral action to meet their nutritional needs. Additionally, the vast repertoire of conserved neuropeptides allows the use of simpler organisms to advance the mechanistic understanding of the machineries, pathways and circuits underlying the neuropeptide regulation of feeding.

1.4.4 – VERTEBRATE NEUROPEPTIDES B AND W

As previously mentioned, neuropeptides and hormones are ancient molecules and have also been described in vertebrates as regulators of energy homeostasis and feeding behavior (Beutler & Knight, 2018). Some of these share sequence and functional similarities across different species and are thought to be evolutionarily conserved (Nässel & Zandawala, 2019). One example is the already mentioned NPY in mammals and NPF in *Drosophila*, which are involved in feeding regulation. Leptin, glucagon and galanin are other known examples of feeding-related peptides in vertebrates with orthologues in insects (Nässel & Zandawala, 2019). However, for the propose of this thesis I will specifically focus on neuropeptide B (NPB) and neuropeptide W (NPW). These neuropeptides are less well-studied but thought to be involved in the regulation of several

metabolic and physiological processes, including feeding (Chottova Dvorakova, 2018; Sakurai, 2013). Both neuropeptides were initially identified as ligands for the G protein-coupled receptors (GPCRs) GPR7 and GPR8, later renamed NPBWR1 and NPBWR2, respectively. NPB was identified and purified from extracts of bovine hypothalamus (Brezillon et al., 2003; Fujii et al., 2002; Tanaka et al., 2003), whereas NPW was identified from extracts of both bovine and porcine hypothalamus (Brezillon et al., 2003; Shimomura et al., 2002; Tanaka et al., 2003). NPW has two isoforms, NPW23 and NPW30, depending on the number of AAs, and its name derives from the tryptophan (W) residues in the beginning and at the end of the described mature peptide (Shimomura et al., 2002). NPB has either 23 (NPB23) or 29 (NPB29) amino acids in humans, whereas in non-humans only NPB29 has been described (Chottova Dvorakova, 2018). Its name was chosen due to the post-translational bromination in the first tryptophan residue (Fujii et al., 2002). Both neuropeptides are found in several vertebrate species including human, rodents, chickens and pig with high AA sequence similarities (Figure 1.5) (Chottova Dvorakova, 2018).

human NPB	WYKPAAGHSSYSVGRAGLLSGLRRSPYA
bovine NPB	WYKPTAGQGYYSVGRAGLLSGFHRSPYA
rat NPB	WYKPAAGSHHYSVGRAGLLSSFHREBPT
mouse NPB	WYKPAAGPHHYSVGRASGLLSSFHREBPT
xenopus NPB	WYKQSTGPSYYSVGRASGLLSGIRRSPTDI
zebrafish NPB	WYKQSTGPSYYSVGRASGLLSGIRRSPTV
human NPW	WYKHVASPRYHTVGRAGLLMGLRRSPYLW
porcine NPW	WYKHTASPRYHTVGRAGLLMGLRRSPYMW
rat NPW	WYKHVASPRYHTVGRASGLLMLRRSPYLW
mouse NPW	WYKHVASPRYHTVGRASGLLMLRRSPYQW

Figure 1.5 - Amino acid sequences of NPB and NPW. Black coloring indicates amino acid identities between NPB and NPW. Grey coloring indicates conserved amino acids within NPB or NPW. Adapted from Hondo et al. (2008).

Several methods to determine mRNA and peptide distribution of NPB and NPW in different organs and tissues have been used. These included RT-PCR analysis, *in situ* hybridization, immunohistochemistry, electron microscopy and radioimmunoassay (Chottova Dvorakova, 2018; Sakurai, 2013). Both NPB and NPW are mainly produced in the brain, but are also found in several peripheral organs. Within the brain, the hippocampus, hypothalamus and the amygdala are the regions with higher expression (Chottova Dvorakova, 2018; Date et al., 2010; Fujii et al., 2002; Schulz et al., 2007; Takenoya et al., 2010). The presence of these neuropeptides in the hypothalamus, namely in the arcuate nucleus and ventromedial hypothalamus (VMH), is particularly interesting as these regions are known to be involved in feeding regulation (Andermann & Lowell, 2017; Timper & Brüning, 2017).

As described above, NPB and NPW are the ligands of two receptors, NPBWR1 and NPBWR2, which were first identified by cloning opioid-somatostatin-like receptor genes from human genomic DNA (O'Dowd et al., 1995). NPBWR1 recognizes both peptides with a moderate preference for NPB, whereas NPBWR2 is slightly selective for NPW (Tanaka et al., 2003). As their ligands, both receptors are found in the central nervous system as well as in peripheral tissues (Brezillon et al., 2003; Chottova Dvorakova, 2018; Sakurai, 2013). NPBWR1 mRNA was found in several brain areas including the VMH, as seen for NPB (Schulz et al., 2007). Interestingly, NPBWR2 is not found in rodents, even though is present in other mammals, which limits the information about its tissue distribution (D. K. Lee et al., 1999).

Both NPB and NPW have been reported to affect feeding behavior, but their roles seem complex, with studies showing either an increase or a reduction in food intake. Intracerebroventricular (i.c.v.) injection of the

mature peptide of NPB in *ad libitum* fed rats increased food intake over a period of 4h (Samson et al., 2004). Other studies, however, showed a reduction in food intake after NPB i.c.v. injection (Aikawa et al., 2008; Tanaka et al., 2003). Moreover, both NPBWR1 and NPB knockout mice were reported to develop adult onset obesity (Ishii et al., 2003; Kelly et al., 2005). For NPW, the reports investigating food intake are similar, with some studies indicating that NPW promotes feeding (Baker et al., 2003; A. S. Levine et al., 2005; Shimomura et al., 2002), whereas others report the opposite (Mondal et al., 2003; Naso et al., 2014). Interestingly, plasma NPW and gastric *NPW* expression seem to respond to nutrient availability (Camino et al., 2008; Li et al., 2013, 2014; Mondal et al., 2006). In rats, plasma NPW levels of the gastric antrum decreased upon fasting and increased upon refeeding (Mondal et al., 2006). *NPW* mRNA from the stomach mucosa in rats and mice has also been reported to decrease after fasting (Camino et al., 2008; Li et al., 2013). Moreover, glucose and mostly protein consumption, but not lipids, increase *NPW* mRNA expression (Li et al., 2014), indicating a possible nutrient-specific satiety role for NPW.

Overall, the discrepancy in reports studying the effects of NPB/W in food intake suggests that NPB/W signaling is a complex process. Different variables such as the exact nutritional state of the animals, the nutrient content of the food or even their microbial composition could be modulating their effect on feeding. Further studies are needed in order to elucidate the mechanisms underlying the effects of these neuropeptides in feeding regulation.

1.5 - HOST-MICROBIOME INTERACTIONS IN BEHAVIOR AND PHYSIOLOGY

Microorganisms have long populated the earth and have lived in a community with multicellular organisms. The word *microbiota* is the collective term for the millions of microorganisms that live in and on an organism, such as humans (Cryan et al., 2019). In the last decades there has been a growing interest in host-microbiota interactions and the particular effect of these microorganisms in human health and disease (Clemente et al., 2012).

Although bacteria and other microorganisms could be pathogenic, many of the microbiota have co-evolved to have a mutualistic or commensal relationship with their host. These beneficial interactions lead to strong effects on the host, from physiology to metabolism, including obesity (Ley et al., 2006; Subramanian et al., 2015; Turnbaugh et al., 2006). Recent studies in both *Drosophila* and mice have also highlighted the importance of commensal bacteria in controlling growth and development, especially in poor nutritional contexts (Schwarzer et al., 2016; Shin et al., 2011; Storelli et al., 2011).

Besides effects on physiology and metabolism, an increasing number of studies have proposed that commensal bacteria, particularly gut bacteria, can also have a profound effect on brain function and behavior, establishing an important new field of research, the microbiota-gut-brain axis (Cryan et al., 2019). Remarkably, the impact of gut bacteria is not restricted to a specific set of functions or behaviors, but there are several examples ranging from anxiety (Clarke et al., 2013; Heijtz et al., 2011; Neufeld et al., 2011), autism spectrum disorders (Sgritta et al., 2019; Sharon et al., 2019), stroke (Singh et al., 2016) and neurodevelopment

disorders (Hsiao et al., 2013). Other examples of commensal bacteria affecting bulk food intake (Vijay-Kumar et al., 2010), locomotion (Schretter et al., 2018) and social behavior (Sgritta et al., 2019; Sharon et al., 2010) have also been reported.

From a gut microbiota point of view, host diet is not only important for its own growth but also for its biosynthetic capacities (Sandhu et al., 2017). Dietary conditions are known to be a major modulator of gut microbiota composition. For example, both high fat and high fiber diets have been shown to affect microbial composition in mice (Sonnenburg et al., 2016; Turnbaugh et al., 2006). Thus, by changing microbial composition, diet can also indirectly affect host physiology and brain function. Furthermore, the gut microbiota alter the nutritional environment of the host in different ways (Ezra-Nevo et al., 2020). First, bacteria can synthesize and provide nutrients to the host that are not available from the diet. A classic example is the symbiotic relationship between aphids and the intracellular bacteria of the *Buchnera* genus (Douglas, 1998). The presence of *Buchnera*, which provides essential amino acids to the host, allows these insects to thrive while feeding on sap, which contains very small amounts of amino acids. In both *Drosophila* and humans, gut bacteria have also been proposed to provide eAAs (B. Kim et al., 2021; Metges, 2000). Second, microbiota can increase the digestion of proteins, for example, therefore improving the uptake of amino acids (Erkosar et al., 2015; Yamada et al., 2015). Third, gut bacteria can metabolize nutrients from the host diet that are unusable by the host, making them available (Ezra-Nevo et al., 2020). Finally, bacterial communities can also compete with the host for the same dietary nutrients, especially in poor nutritional contexts, therefore reducing the amount available for the host and leading to a deficit (Huang & Douglas, 2015). Another important mechanism, by

which microbiota can affect host physiology, brain function and behavior is by using the dietary nutrients as precursors to synthesize specific metabolites or signaling molecules. The neurotransmitter GABA (γ -aminobutyric acid) was found to be produced by *Bacteroides sp*, however, its functional relevance to the host neuronal function remains to be elucidated (Strandwitz et al., 2019). All the described phenomena support the idea that the interaction of diet with the microbiota is a key component of their function and is likely to play an important role in the mechanisms by which gut microbes affect physiology and brain function (Figure 1.6) (Ezra-Nevo et al., 2020).

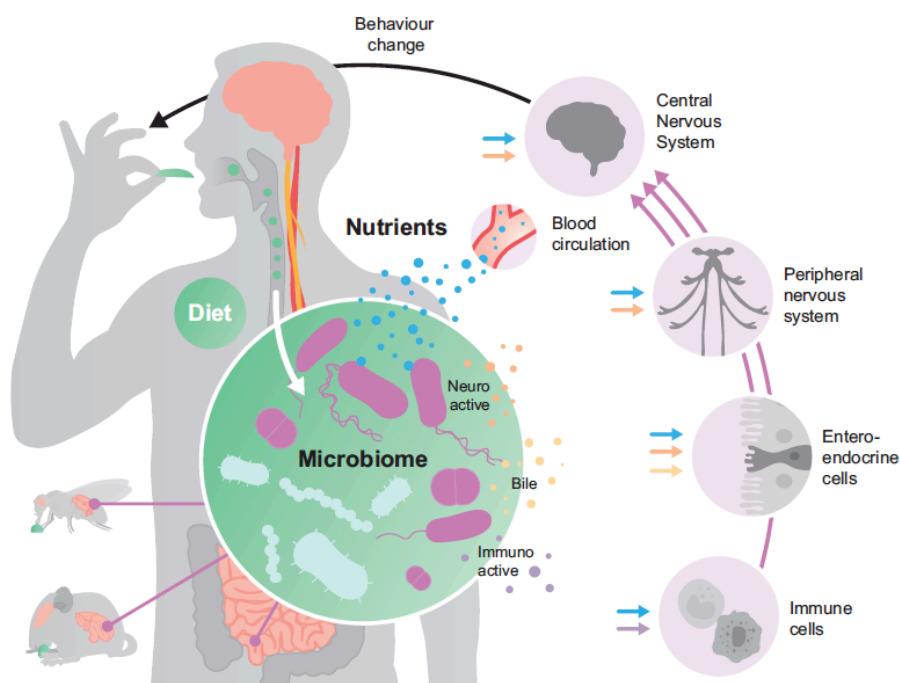


Figure 1.6 - Diet-microbiome-host interactions action on the gut-brain axis and behavior. Adapted from Ezra-Nevo et al. (2020).

In vertebrates, including humans, studying the effects of gut microbiota in physiology and brain function is particularly challenging due to the vast diversity and complexity of the microbial composition, leading to conclusions mostly inferred from correlational rather than causal relationships (Cryan et al., 2019). The use of gnotobiotic animal models, with a defined microbial composition is crucial to establish a causal contribution of specific microbes to specific effects on the host and the mechanisms underlying them. In this context, simpler organisms, such as *Drosophila*, became important models to study host-microbial interactions (Douglas, 2019). They have a reduced number of gut microbiota species and their powerful genetic and behavioral toolkits make them ideal candidates to dissect the cellular and molecular mechanisms underlying how microbes modulate host functions (Ezra-Nevo et al., 2020). Furthermore, given the importance of the host diet on microbial composition and activity, the use of chemically defined diets (Piper et al., 2014, 2017) provides an important tool to dissect the individual contribution of specific nutrients in diet-microbial-host interactions.

1.6 – HOW TO STUDY FEEDING BEHAVIOR IN *DROSOPHILA*

Fruit flies, like other animals, need to ingest food in order to fulfil their energy requirements and maintain nutritional homeostasis. Therefore, the study of their feeding behavior is a key aspect to understand how the nutritional state of the animals is regulated and translated into motor commands to generate behavior. Specifically, when studying nutrient-specific appetites, experimental designs need to not only have a precise behavioral readout to quantify the different aspects of the feeding motor program, but also a tight control of the nutrient content of the food.

Innately, the behavior of an animal is heavily dependent on its feeding state. Consequently, the nutrient content of the diet in which animals were kept is a key variable in almost all studies. Most laboratories use a so-called standard diet. However, the exact sources and quantities of the ingredients as well as the method for its preparation often diverge and are not clearly stated. The variability and inconsistency in diets used in different laboratories is particularly problematic in the context of studies that require specific nutrient manipulations in their protocols. One way to overcome these difficulties is to use holidic diets. These chemical synthetic diets are made entirely from purified ingredients in defined concentrations and allow for the addition or removal of specific nutrients. One example is the diet developed by Piper and colleagues which is adequate to support reproduction and lifespan of flies (Piper et al., 2014). Besides, these holidic diets can be constantly improved. In this sense, matching the AA content of the diet to the predicted exome of the flies showed improved growth and reproduction, without affecting lifespan (Piper et al., 2017). Moreover, an important aspect to consider is to

separate the experiments into two phases: one in which the diet is changed to induce a nutrient-specific deficit in the animal; and a second one, in which different diets are presented to the animal in a test or preference assay to reveal possible homeostatic alterations in feeding preference. Therefore, the use of holidic diets provides a framework to precisely manipulate the content of foods and it is an invaluable tool in the field of nutritional neuroscience.

To reliably study behavior, it is crucial to analyze it in a detailed and highly quantitative way. One definition of feeding behavior is a sequence of hierarchically organized behavioral submodules (Itskov et al., 2014; Pool & Scott, 2014). These modules range from the foraging or exploration of possible food sources to the organization of consummatory behavior, or food intake (Figure 1.7). Foraging can be studied in laboratory conditions by video-based methods that track the fly's movement in an arena with different food patches or local search assays (Corrales-Carvajal et al., 2016; Dethier, 1957; Kim & Dickinson, 2017). This allows for the quantification of food-seeking behaviors, ranging from global exploration to local exploitation of resources.

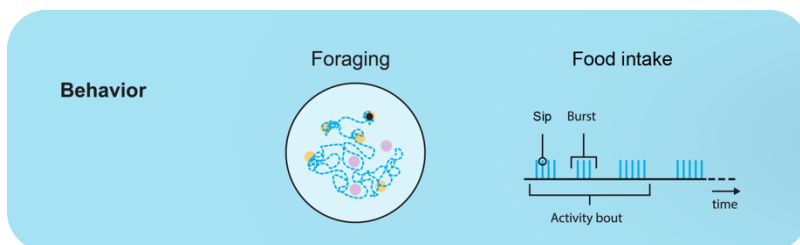


Figure 1.7 - Foraging and food intake microstructure in flies. Adapted from Münch et al. (2020).

In flies, due to their small size and to the little amount of food ingested, measuring food intake in a quantitative way has been challenging. Nevertheless, different methods have been developed over the years to measure food intake in *Drosophila* (Figure 1.8) (Itskov & Ribeiro, 2013). A classic approach is the two-color food choice assay (Dus et al., 2011; Ribeiro & Dickson, 2010; Tanimura et al., 1982). This is a simple method where two food sources are mixed with different non-absorbable dyes and the flies are left to feed for a defined period of time. To have a quantitative readout of food intake, the content of the digestive tract can be measured using a spectrophotometer. Moreover, to test for food preference, a qualitative assessment can be achieved *post hoc* by visually scoring the color of the flies' abdomen (Figure 1.8a). A major disadvantage of this method is that it lacks information about the feeding dynamics and does not take into consideration the food excreted by the flies. A similar method using radioactively labelled food source was also developed to measure food intake, however it only allows for an indirect comparison of food preferences, being most useful to measure absolute food intake (Carvalho et al., 2006; Vargas et al., 2010).

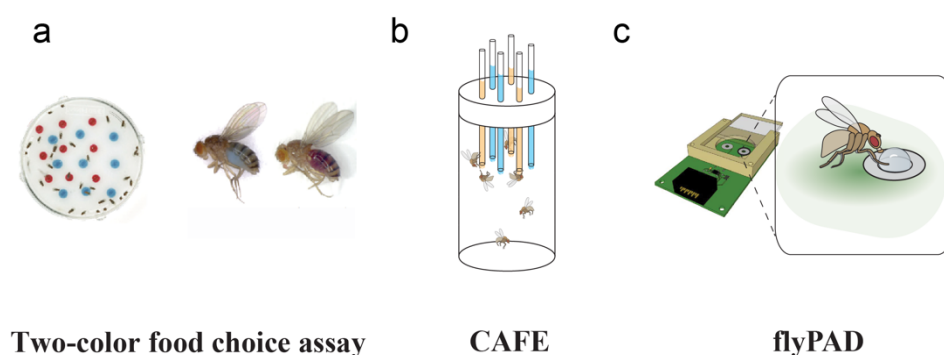


Figure 1.8 - Methods for studying feeding behavior in flies. (a) Two-color food choice preference assay (adapted from Ribeiro & Dickson, 2010). (b) CAFE assay. (c) flyPAD setup.

Another method that allows for a temporal analysis of feeding is the CAPillary FEeding assay, CAFE (Ja et al., 2007). The flies feed from graduated capillaries filled with liquid food and the food intake is measured by changes in the liquid levels within the capillaries (Figure 1.8b). This method allows monitoring of feeding for long periods of time and different food sources, using multiple capillaries. However, it is a very laborious method and the flies need to eat in an upside-down position, which is not naturalistic and could affect their feeding habits.

Recently, a new type of approaches using capacitance or voltage-based methods were developed. Both the fly Proboscis and Activity Detector (flyPAD) and Fly Liquid-Food Interaction Counter (FLIC) use capacitance/voltage-based measurements to detect the physical interaction of individual flies with different food sources (Itskov et al., 2014; Ro et al., 2014). Single flies are put into arenas with two food wells and the interaction of the proboscis with each food source is quantified. Specifically, the flyPAD technology demonstrated that the physical interactions of the proboscis with the food, called sips, are highly stereotypical and correlated with food intake (Figure 1.7 and 1.8c). Thus, the flyPAD allows the quantification of food intake from two independent sources and the dynamic measurement of feeding (Itskov et al., 2014). Recently, the flypad technology was improved to combine optogenetic stimulation, created the optoPAD (Moreira et al., 2019).

The recent improvements in nutritional manipulations and behavioral methods together with the fly's vast genetic toolkit and fast life cycle allow for large genetic, neuronal and behavioral high-throughput screens (Flood et al., 2013; Liu et al., 2020). Together, this makes the fruit flies great model organisms to disentangle the molecular, cellular and neuronal mechanisms underlying food intake and nutritional homeostasis.

1.7 - AIMS OF THE THESIS

Nutritional homeostasis is key for the animal's fitness and survival. Recently, some of the mechanisms underlying general food intake have been unraveled, but how nutrient-specific appetites are controlled by the brain is less understood. This is particularly true for dietary amino acids. The doctoral research presented in the chapters 2, 3 and 4 of this thesis aims to: 1) identify the modulators of protein appetite and 2) pinpoint new molecular and neuronal mechanisms regulating this behavior.

CHAPTER 2

*COMMENSAL BACTERIA AND
ESSENTIAL AMINO ACIDS CONTROL
FOOD CHOICE BEHAVIOR AND
REPRODUCTION*

Note: The material in this chapter has been published in the peer-reviewed journal PLoS Biology:

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* These authors contributed equally to this work.

Author Contributions: APF, RLG, ZCS and GTF performed experiments and analyzed data. MA, CB and APE performed experiments. PMI and MDWP provided resources. CR acquired funding, provided supervision, project administration, visualization and wrote the original draft. APF, RLG, ZCS, GTF, PMI and CR reviewed and edited the manuscript. Conceptualization by ZCS, RLG and CR.

2.1 - SUMMARY

Choosing the right nutrients to consume is essential to health and wellbeing across species. However, the factors that influence these decisions are poorly understood. This is particularly true for dietary proteins, which are important determinants of lifespan and reproduction. In this chapter, we show that in *Drosophila melanogaster*, essential amino acids (eAAs) and the concerted action of the commensal bacteria *Acetobacter pomorum* and *Lactobacilli* are critical modulators of food choice. Using a chemically defined diet, we show that the absence of any single eAA from the diet is sufficient to elicit specific appetites for AA-rich food. Furthermore, commensal bacteria buffer the animal from the lack of dietary eAAs: both increased yeast appetite and decreased reproduction induced by eAA deprivation are rescued by the presence of commensals. Surprisingly, these effects do not seem to be due to changes in AA titers, suggesting that gut bacteria act through a different mechanism to change behavior and reproduction. Thus, eAAs and commensal bacteria are potent modulators of feeding decisions and reproductive output. This demonstrates how the interaction of specific nutrients with the microbiome can shape behavioral decisions and life history traits.

2.2 - INTRODUCTION

The appropriate intake of nutrients has a major beneficial impact on health and lifespan (Simpson et al., 2015; Simpson & Raubenheimer, 2012). The level of dietary protein intake has emerged as a key determinant of overall mortality, fecundity, and lifespan in species ranging from humans (M. E. Levine et al., 2014) to mice (Solon-Biet et al., 2014) and *Drosophila* (Grandison et al., 2009; K. P. Lee et al., 2008; Piper et al., 2011, 2014). Accordingly, animals, including humans, are able to direct food choice in order to tightly control protein intake (Gosby et al., 2011; Griffioen-Roose et al., 2012; Itskov & Ribeiro, 2013; Ribeiro & Dickson, 2010; Simpson et al., 2015; Simpson & Raubenheimer, 2012). Despite the striking physiological and behavioral impact of nutritional proteins, how animals direct feeding decisions to ensure protein homeostasis is not understood. A major obstacle in identifying the rules governing food choice is the nutritional complexity of natural foods, which hinders the discovery of the nutritional variables controlling feeding decisions.

In *Drosophila melanogaster*, yeast is thought to cover the protein as well as most other noncaloric nutritional requirements (Piper et al., 2014). In adult females, yeast appetite is driven by two main internal states: mating and lack of yeast (Itskov & Ribeiro, 2013; Ribeiro & Dickson, 2010; Vargas et al., 2010; Walker et al., 2015). The molecular and circuit mechanisms leading to an increase in yeast appetite upon mating have been extensively characterized. During copulation, the male-derived Sex Peptide is transferred to the female and acts on the neuronal Sex Peptide Receptor, leading to the silencing of a postmating neuronal circuit, consisting of SPSN/SAG/octopamine components, which projects to the central brain to change feeding preference from sugar to yeast

(Corrales-Carvajal et al., 2016b; Ribeiro & Dickson, 2010; Walker et al., 2015). Besides mating, the other known determinant of protein intake is removal of yeast from the diet, which leads to a strong compensatory appetite for yeast (Ribeiro & Dickson, 2010). The mechanisms underlying this homeostatic change in appetite are less well understood. This is partially due to the fact that yeast is a complex food containing different nutrients, including amino acids (AAs), carbohydrates, vitamins, and sterols (Bos et al., 1976; US Department of Agriculture, Agricultural Research Service, Nutrient Data Laboratory). However, it is still unknown which nutrient(s), when absent, trigger flies to ingest yeast. Identifying the mechanisms controlling protein homeostasis in *Drosophila* requires untangling this nutritional complexity.

The interaction of microbiota with ingested nutrients has emerged as a major determinant of health and disease, including obesity (Clemente et al., 2012; Kau et al., 2011; Ley et al., 2006; Sharon et al., 2014; Subramanian et al., 2015; Turnbaugh et al., 2006). Commensal bacteria have also been proposed to affect a wide array of brain functions (Cryan & Dinan, 2012; Ezenwa et al., 2012; Mayer et al., 2014; Sampson & Mazmanian, 2015; Wong et al., 2015). Despite being an intense field of research, the importance of microbe–nutrient interactions in influencing behavior remains poorly understood. In vertebrates, this task is especially challenging given the complexity of their microbiota and the large set of nutritional parameters that could influence their function. Furthermore, in the context of nutrition, research on microbiota has mainly focused on their role in carbohydrate homeostasis (Huang & Douglas, 2015; Turnbaugh et al., 2006). More recently, however, the importance of commensal bacteria in controlling growth (Schwarzer et al., 2016; Shin et al., 2011; Storelli et al., 2011) and in protecting children from malnutrition symptoms (Smith

et al., 2013) indicate that the microbiome could also play a pivotal role in protein homeostasis. However, the importance of commensals in protein homeostasis and in directing food choices has not been directly addressed.

2.3 - RESULTS

2.3.1 - Essential amino acids, but not other nutrients, control yeast and amino acid preference

Yeast deprivation leads to a strong compensatory appetite for yeast (Ribeiro & Dickson, 2010) (Figure 2.1b). Given the complexity of this resource, it has not been possible to identify the nutrients that, when absent, trigger flies to ingest yeast. To answer this question, we decided to manipulate each nutrient present in yeast independently using a chemically defined (holidic) diet (Piper et al., 2014) (Figure 2.1a) and study their effects on feeding decisions using a two-color food choice assay (Ribeiro & Dickson, 2010; Tanimura et al., 1982). The holidic medium is able to suppress yeast appetite to the same extent as a yeast-based medium (Figure 2.1b and 2.1c), supporting the idea that it provides the necessary nutrients to support adult behavior (Piper et al., 2014). Removal of AAs from the holidic medium induced a potent yeast appetite, indistinguishable from that observed upon yeast deprivation (Figure 2.1b and 2.1c). Removing folic acid, metals, nucleic acids, lipids, sterols, or vitamins, however, did not lead to a significant increase in yeast appetite (Figure 2.1c). This effect stands in strong contrast to the clear effects on lifespan and egg production of removing these nutrients (Piper et al., 2014).

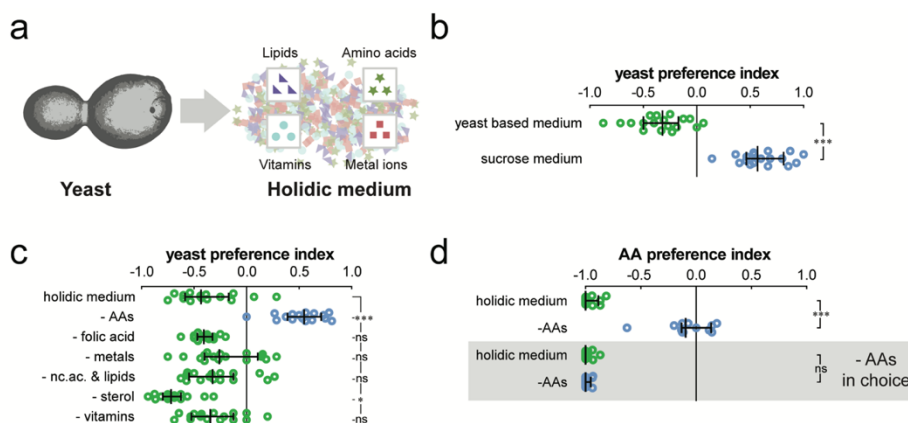


Figure 2.1 - Flies specifically increase yeast and amino acid preference upon AA deprivation. (a) The holidic diet allows the analysis of the impact of specific nutrients contained in yeast. (b) Yeast preference of flies kept on a yeast-based medium and medium without yeast (sucrose medium). (c) Yeast preference of flies kept on holidic medium and holidic medium lacking different specific nutrients. (d) AA preference of flies kept on full holidic medium and holidic medium lacking all AAs. $n = 12-18$. In this and the following Figures, circles represent yeast or AA preference in single assays, with a line representing the median and whiskers representing the interquartile range. Green signifies diets with full AA content and blue signifies diets lacking one or more AAs.

To identify the nutrients that flies select when deprived of AAs, we used the holidic diet in the choice paradigm. We gave the flies a choice between a holidic base diet containing sucrose without AAs and one containing AAs but no carbohydrates. AA deprivation shifted flies' preference from the sucrose-containing option towards the AA-containing option (Figure 2.1d). This shift in preference was specific to AAs since it was abolished by removal of AAs from the choice medium, whereas removing any other class of nutrients left the shift intact (Figure 2.1d and Figure 2.2). These results suggest that similarly to yeast deprivation

(Toshima & Tanimura, 2012), upon AA deprivation, flies specifically select a diet containing AAs.

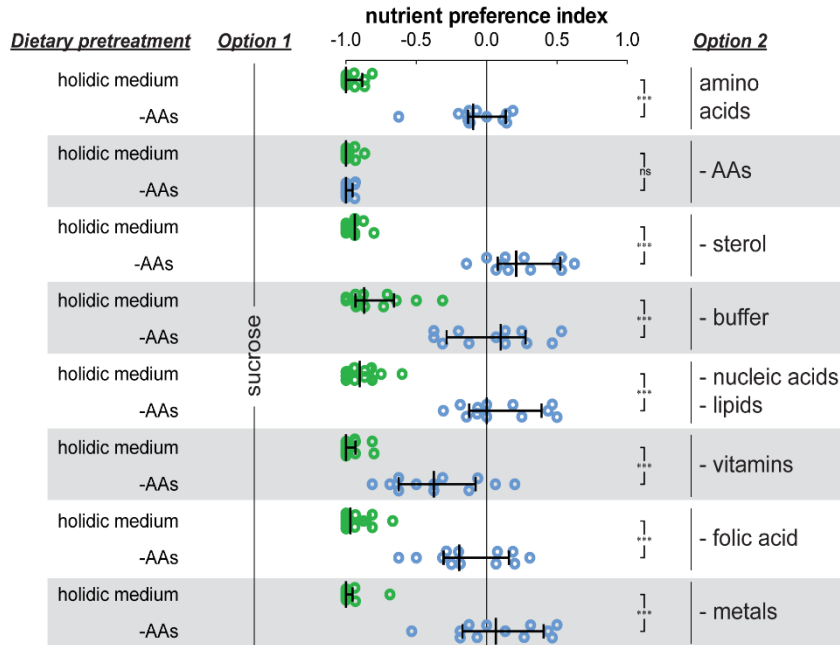


Figure. 2.2 - Deprivation of amino acids specifically increases amino acid appetite.

Feeding assay in which flies were given the choice between the holidic medium lacking AAs (sucrose option) and the holidic medium lacking sucrose and one of the different nutrient classes. Animals were either kept on full holidic medium or holidic medium lacking AAs. $n = 12-15$.

In our paradigm, the decision to switch from eating sucrose to yeast or AAs is therefore guided by the absence of AAs, while the absence of other physiologically important dietary nutrients does not lead to an increase in yeast or AA appetite.

AAs can be broadly classified as either essential (eAAs) or nonessential (neAAs). neAAs can be synthesized by the animal, allowing animals to be largely independent from dietary uptake of these important

building blocks (Bender, 2012). It is currently unclear whether animals sense these two types of AAs differently and if they have different effects on nutrient choice (C. L. Simpson et al., 1991; Wu et al., 2012). We tested this by manipulating each type of AAs independently. Removal of all eAAs from the diet induced a yeast (Figure 2.3a) and AA appetite (Figure 2.3b) that were indistinguishable from that observed upon removal of all AAs. The complete removal of neAAs, however, had no effect on nutrient choice (Figure 2.3a and 2.3b). Given that we adjust the total level of AAs to maintain a constant amount of nitrogen in the diet, these results also show that it is the identity of the AAs and not the nitrogen level in the diet that leads to changes in food choice.

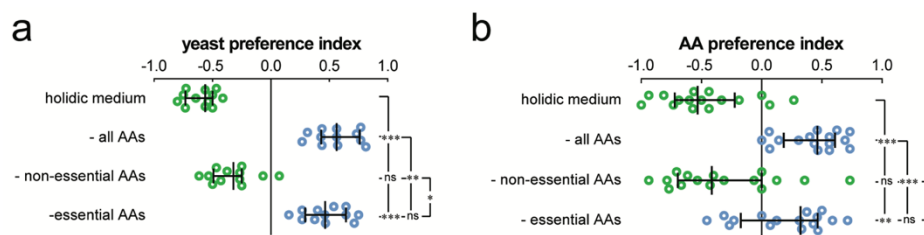


Figure 2.3 - Deprivation of eAAs but not neAAs increases yeast and amino acid preference. Yeast (a) and AA (b) preference of flies kept on complete holidic medium or holidic medium lacking all AAs, all neAAs, or all eAAs. $n = 12-18$.

Intriguingly, AA deprivation induced a preference for both eAAs and neAAs, suggesting that the phagostimulatory power of AAs is not correlated with their nutritional importance, as indicated by previous studies (Toshima & Tanimura, 2012) (Figure 2.4). Taken together, these data strongly indicate that eAAs are specific mediators of protein and AA appetite and highlight the ability of animals to efficiently buffer the absence of neAAs.

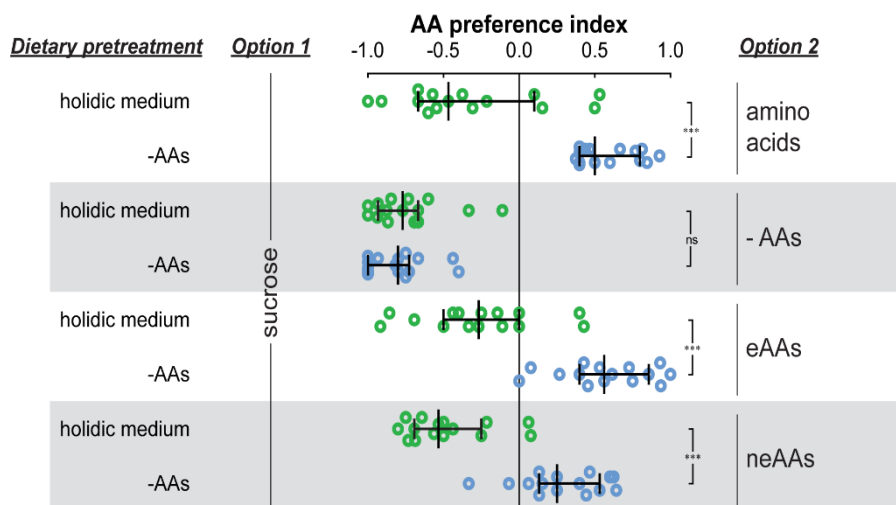


Figure. 2.4 - AA deprivation induces a preference for both eAAs and neAAs. Feeding assay in which flies were given the choice between two options: 1) the holidic medium lacking amino acids (sucrose) and 2) the holidic medium lacking sucrose, lacking sucrose and all AAs, lacking sucrose and neAAs, or lacking sucrose and eAAs. $n = 12-15$.

2.3.2 - The absence of any single essential amino acid can induce a potent yeast appetite

Behavioral (C. L. Simpson et al., 1991), physiological (Grandison et al., 2009), and molecular studies (Hara et al., 1998) have suggested that different single AAs can vary widely in their potency to suppress protein appetite and to activate nutrient-sensitive pathways. We therefore took advantage of the unique possibility to manipulate single dietary AAs afforded by the holidic diet to remove every eAA individually from the diet and test the effect on food choice. Strikingly, removal of any eAA was sufficient to induce a clear increase in yeast choice (Figure 2.5a). The extent to which they did so did not differ, suggesting that each eAA has a similar impact on food choice. Furthermore, we quantified the effect of

removing specific AAs from the diet on the intake of sucrose and yeast extract using a method to quantify food intake (Ja et al., 2007) (the capillary feeder (CAFE) assay; Figure 2.5b). Consistent with our results using the two-color assay, removal of either all AAs or single eAAs (arginine or valine) led to a specific increase in yeast extract intake without affecting carbohydrate intake (Figure 2.5b). In agreement with previous reports (Corrales-Carvajal et al., 2016), these data indicate that the changes in food choice induced by AA deprivation in the two-color choice assay are due to an increase in yeast appetite and not to a decrease in sucrose intake. They further indicate that single eAAs are potent and specific nutritional modifiers of protein intake, highlighting their unique importance in controlling food choice.

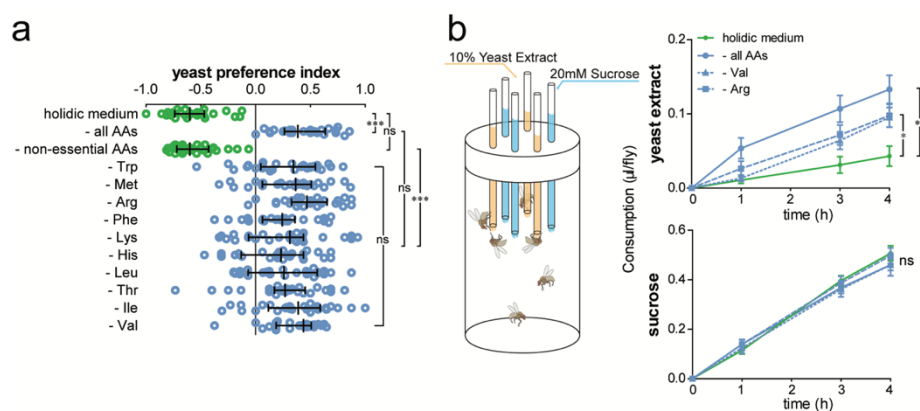


Figure 2.5 - Flies specifically increase yeast appetite upon single essential amino acid (eAA) deprivation. (a) Feeding preference of flies kept on holidic medium or holidic medium lacking all AAs, all neAAs, or single eAAs in the context of no neAAs. $n = 26$. (b) Cumulative intake measurement of yeast extract and sucrose using the CAFE assay. Flies were prefed a holidic diet containing either all AAs, no AAs, all AAs except Val, or all AAs except Arg. Significance was tested for the intake volume at 4 h. Dots represent the mean $\pm$ SEM, $n = 10$.

2.3.3 - Loss of peripheral synthesis of neAAs increases yeast appetite

Animals can synthesize neAAs in order to compensate for their absence from the diet. For example, tyrosine (Tyr) can be synthesized from phenylalanine (Phe) through the action of phenylalanine hydroxylase, which in *Drosophila* is encoded by the *Henna* gene (Figure 2.6a) (Silva et al., 1992). In humans, mutations in phenylalanine hydroxylase cause phenylketonuria, the most common metabolic disease (Fölling, 1934; Mitchell et al., 2011). Patients with phenylketonuria suffer from elevated Phe and low Tyr titers, leading to severe complications including neurological and behavioral symptoms (de Groot et al., 2010). Strict adherence to a diet low in Phe and high in Tyr allows patients to lead an asymptomatic life, highlighting the impact of dietary AAs on human health (MacDonald et al., 2011).

We mimicked the genetic lesion leading to phenylketonuria by knocking down the *Henna* gene ubiquitously (Supplementary Figure 2.1), thus transforming Tyr from a neAA to an eAA. This allowed us to test if the capacity to homeostatically trigger changes in food choice is related to the specific identities of the ten eAAs or if it can be driven by low levels of any AA. While removal of dietary neAAs in control animals did not lead to the induction of a yeast appetite, the same dietary manipulation in *Henna* knockdown animals led to a strong yeast appetite (Figure 2.6b and Fig 2.6c). This increased yeast appetite was indistinguishable from that observed upon removal of all AAs. Supplementing the diet lacking neAAs with Tyr suppressed the preference of flies for yeast in a dose-dependent manner, indicating that the phenotype was specifically due to an acute lack of Tyr and not to other detrimental effects of our genetic manipulation (Figure 2.6b). Importantly, the addition of proline, a neAA which is not

synthesized by phenylalanine hydroxylase, did not suppress the *Henna* phenotype, further emphasizing the specificity of the metabolic manipulation (Figure 2.6c). These results strongly suggest that flies can detect the absence of any limiting AA independent of their specific identity (eAA versus neAA).

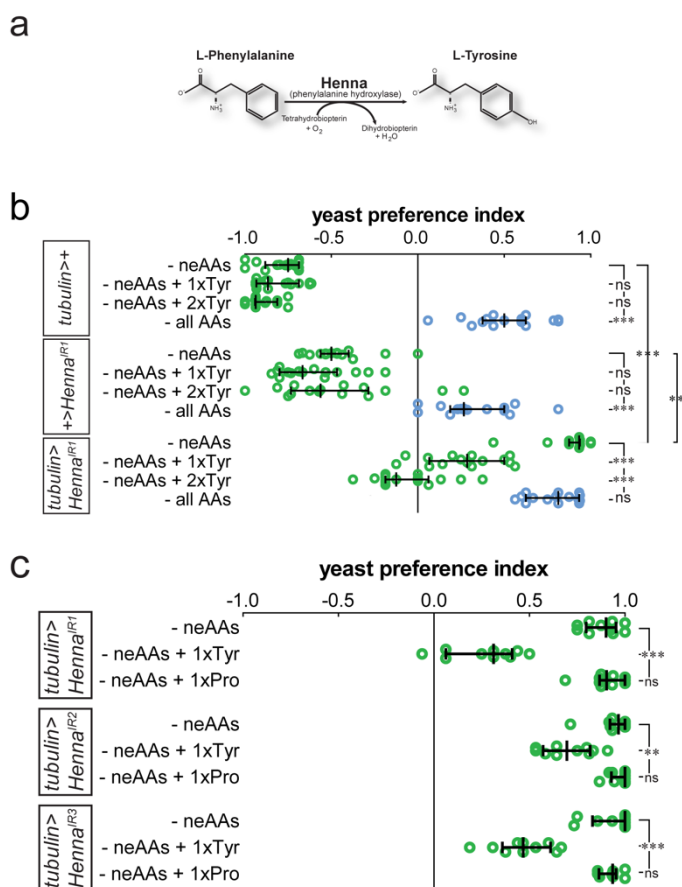


Figure 2.6 - Loss of neAAs synthesis increases yeast appetite. (a) Schematic depicting the biosynthesis of tyrosine (Tyr) in *Drosophila*. **(b)** Feeding preference of control and *Henna* whole-animal knockdown flies upon removal of all AAs, all neAAs, or all neAAs with 1x and 2x Tyr added back. $n = 15-20$. **(c)** Feeding preference of *Henna* knockdown animals using three independent hairpins. Animals were kept on holidic medium lacking neAAs, or holidic diet lacking neAAs with 1x Tyr or 1x Pro added back. $n = 10$.

In mammals, neAAs are mainly synthesized in the liver (Udenfriend & Cooper, 1952; Vajro et al., 1993), and it is thought that in insects, the fat body fulfills a similar role (Arrese & Soulages, 2010; Kilby & Neville, 1957; Neckameyer et al., 2007). We tested the importance of the fat body in guiding nutrient choice by interfering with the ability of this organ to synthesize Tyr. Knockdown of *Henna* using a fat body driver *Cg-Gal4* rendered the animal sensitive to the absence of dietary neAAs, with induction of a strong yeast appetite (Figure 2.7a). *Henna* knockdown in neurons or trachea, in contrast, did not change the behavioral sensitivity of flies to removal of all neAAs (Figure 2.7b), indicating that the effect observed with the fat body manipulation is tissue specific. However, *Cg-Gal4* has also been shown to drive expression in hemocytes (Schmid et al., 2014). It is thus possible that this cell type also contributes to Tyr synthesis and the observed behavioral phenotype.

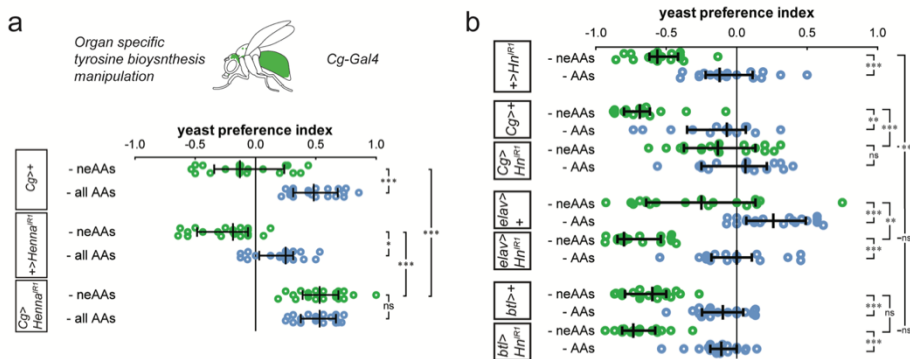


Figure 2.7 - Blocking of Tyr synthesis in the fat body increases yeast appetite. (a) Feeding preference of control and *Henna* fat body knockdown flies upon removal of either all neAAs or all AAs. $n = 15-20$. **(b)** Feeding preference of control and *Henna* (Hn) knockdown flies in different tissues upon removal of either all AAs or all neAAs. *Cg-Gal4* drives expression in fat body, *elav-Gal4* in neurons and *btl-Gal4* in trachea. $n = 14-20$.

Taken together, these data further demonstrate that AAs, be they dietary or endogenously synthesized, are able to control yeast appetite. Furthermore, our data indicate that biosynthetically active organs are important regulators of food choice, suggesting that genetic metabolic conditions such as phenylketonuria could have effects on aspects of behavior such as nutrient-specific appetites.

2.3.4 - Commensal bacteria direct feeding decisions

Mounting evidence indicate that commensal bacteria are important determinants of how nutrients are utilized (Hacquard et al., 2015; Wong et al., 2014). As such, they modulate a large set of nutrient-sensitive traits. However, whether commensals influence the selection of specific dietary nutrients is currently unknown. We therefore set out to test the effect of commensals on nutrient choice in *Drosophila*. Importantly, the flies used in our experiments had a very low baseline gut microbe load (Figure 2.8). This is likely due to the use of sterile media and the fact that upon serial passage to new food, adult flies lose a large part of their microbiota (Blum et al., 2013).

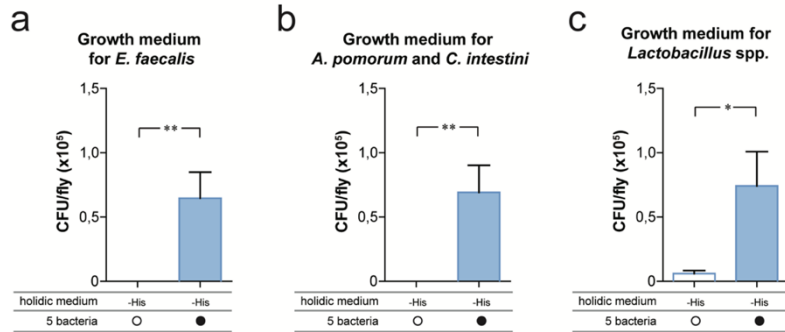


Figure 2.8 - Levels of internal microbes in non-axenic flies. The internal load of bacteria inside flies was calculated as CFU/fly after bacterial colony count on LB (a), Mannitol (b), or MRS (c) media which sustain the growth of different bacterial species as indicated in each graph. The load of bacteria was assessed for flies kept on holidic medium without His and without (empty columns) or with (filled columns) pretreatment with the commensal bacteria mix. The columns represent the mean $\pm$ SEM, $n=6$.

To test the effect of the microbiota on behavioral protein homeostasis, we removed one eAA (histidine [His]) from the holidic diet to increase the flies' preference for yeast and examined if they would show alterations in food choice when treated with a controlled microbiota (Figure 2.9a) (pure culture of five *Drosophila* gut bacteria strains: *Lactobacillus plantarum*^{WJL} (Ryu et al., 2008), *Lactobacillus brevis*^{EW} (Ryu et al., 2008), *Acetobacter pomorum* (Ryu et al., 2008), *Commensalibacter intestini*^{A911T} (Ryu et al., 2008), and *Enterococcus faecalis* (Cox & Gilmore, 2007)). Strikingly, in contrast to control flies, bacteria-treated flies did not show an increased yeast appetite upon His removal (Figure 2.9b). The effect of commensals was not limited to His but suppressed yeast appetite upon removal of any of the ten tested eAAs (Figure 2.9c). The effect of commensals on food choice was so strong that

the flies with a reconstituted microbiome were even buffered against the removal of all eAAs from the diet (Figure 2.9d).

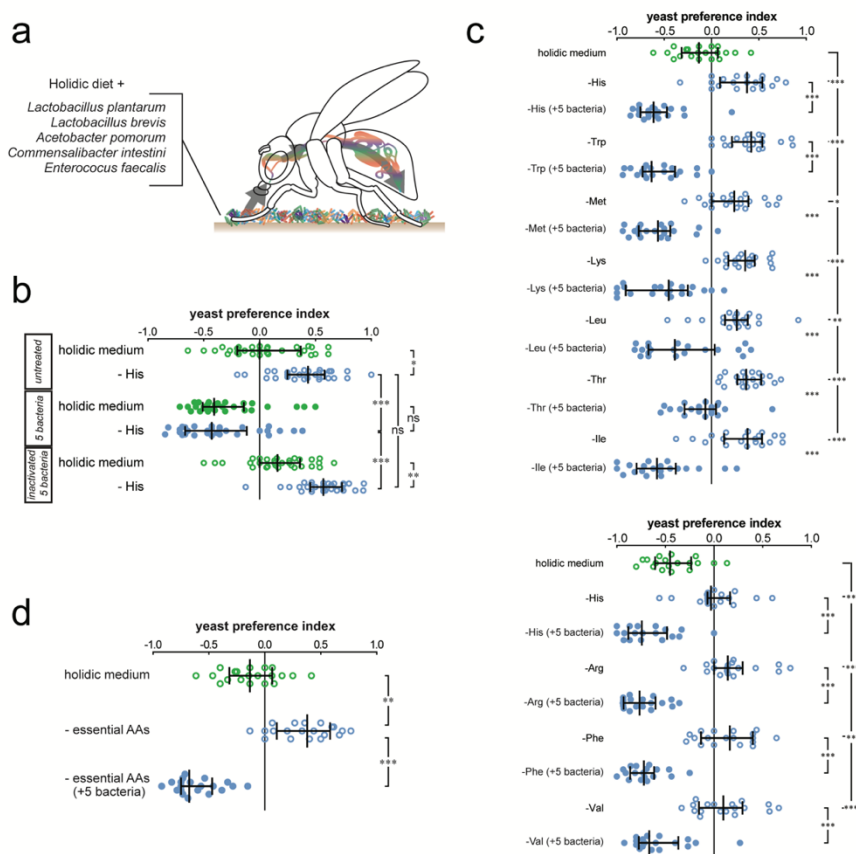


Figure 2.9 - Commensal bacteria control food choice. (a) Diagram depicting the strategy used to reconstitute the commensal population in the fly. **(b)** Yeast preference of animals kept on holidic diet with or without histidine (His). One group was not pretreated with the 5 commensals (untreated), the other was pretreated with the commensals (5 bacteria), and the third was pretreated with inactivated commensals (inactivated 5 bacteria), $n=20-30$. **(c)** Feeding preference of animals kept either on holidic medium, or holidic medium lacking one of the 10 eAAs with or without pretreatment with the 5 bacteria. Data on the different graphs were collected on two independent days, $n=18-20$. **(d)** Yeast preference of animals kept on holidic diet, holidic diet without eAAs, or holidic diet without eAAs pretreated with the 5 bacteria. $n = 20-30$.

To test if the bacteria were merely acting as food or if they needed to be metabolically active, we tested the effect of inactivated bacteria on food choice. Inactivation abolished the ability of the bacteria to alter feeding decisions, suggesting that their activity is essential to drive the change in behavior (Figure 2.9b). The behavioral effect was specific to commensal bacteria, as it was not observed when a non-commensal bacterium (*Escherichia coli*) was used in the experiments (Figure 2.10a).

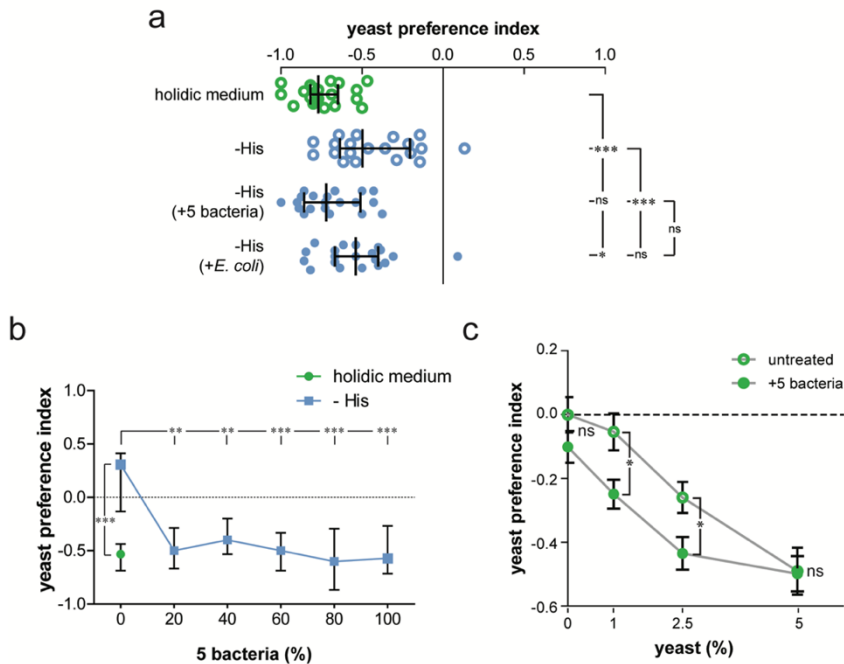


Figure 2.10 - A non-commensal bacterium does not reduce yeast preference, diluted commensal bacteria still affect yeast preference and the effect is also present on low-yeast diets. (a) Feeding preference of animals kept either on holidic medium, or holidic medium lacking His with or without pretreatment with the 5 commensal bacteria mix or *E. coli*, $n = 20$. **(b)** Yeast preference of animals kept on holidic diet with or without His and pretreated with varying concentrations of the commensal mix. Points represent median yeast preference and error bars represent the interquartile range. $n = 15$. **(c)** Feeding preference of animals kept on medium with different concentrations of yeast and with or without pretreatment with the 5 commensal bacteria mix. Circles represent mean $\pm$ SEM, $n = 20$.

That bacteria do not simply act as food is further supported by the fact that even when diluted, they are able to lower the preference for yeast (Figure 2.10b). Furthermore, to show that the effect of the bacteria is not confined to flies fed on the synthetic diet, we pretreated flies with decreasing amounts of yeast to induce a yeast appetite. As expected, flies fed with decreasing amounts of yeast showed an increase in yeast preference (Figure 2.10c). Similarly to the effect on AA-deprived flies, bacteria pretreatment reduced the yeast appetite of yeast-deprived flies when compared to non-pretreated controls (Figure 2.10c). This indicates that the effect of commensal bacteria on food choice is generalizable to ecologically relevant AA sources. Commensals are therefore strong modifiers of food choice behavior by buffering the animal from the effect of dietary lack of eAAs.

2.3.5 - Commensal bacteria can only buffer the absence of essential amino acids

Given the strong effect of commensals on food choice, we set out to test if they would also be able to suppress the food choice phenotype in flies which are impaired in Tyr synthesis. Surprisingly, addition of the five bacteria to the diet was not able to suppress the yeast appetite induced by neAA deprivation in *Henna* knockdown flies, while Tyr supplementation was able to suppress this appetite (Figure 2.11a). The bacteria mix was still able to reduce the yeast appetite induced by His deprivation in *Henna* knockdown flies, indicating that the bacteria are effective in this genetic background (Figure 2.11b). This indicates that the microbiota exerts its effect specifically in the context of eAA but not neAA depletion. This experiment also strongly suggests that the commensal pretreatment does

not alter food choice in an indirect way (e.g., by suppressing the ability of flies to choose yeast or only increasing their preference for sucrose) and that the effect of the bacteria is not due to them serving as food, as these effects should also lead to a decreased yeast preference index in these flies. Commensal bacteria are therefore strong modifiers of food choice specifically in the context of eAA deficiencies.

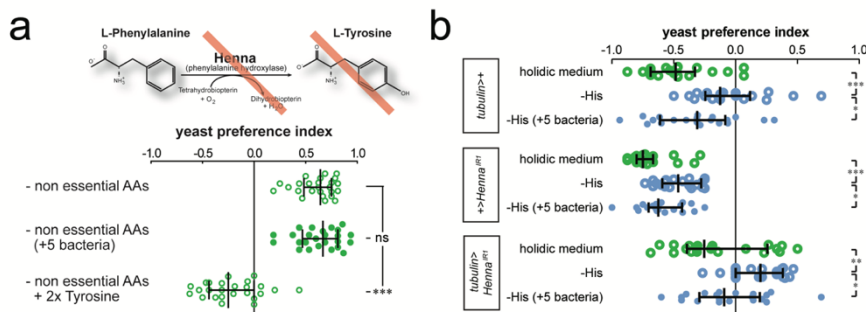


Figure 2.11 - Commensal bacteria cannot suppress yeast appetite upon neAA deprivation. (a) Yeast preference of *Henna* knockdown animals (*tubulin* > *Henna*^{IR1}) kept on holidic diet lacking neAAs, with or without pretreatment with the commensal mix, and holidic diet lacking neAAs with 2x tyrosine (Tyr) added back, $n=15$. (b) Feeding preference of control and *Henna* knockdown animals kept on holidic medium, or holidic medium lacking His with or without pretreatment with 5 commensal bacteria mix. $n = 20$.

2.3.6 - Commensal bacteria increase egg laying upon essential amino acid deprivation

Stem cell proliferation and differentiation is limited by the availability of eAAs (Drummond-Barbosa & Spradling, 2001; Hansen et al., 2004; Hosios et al., 2016). In *Drosophila* this is most evident in the context of egg production, in which depletion of eAAs strongly reduces egg laying (Grandison et al., 2009; Piper et al., 2014). We therefore tested if commensals would also be able to affect egg laying. As shown above, depriving animals of three different eAAs (His, Ile, or Val) led to the induction of yeast appetite, which was strongly suppressed in animals pretreated with commensals (Figure 2.9c). Removal of any of these three eAAs significantly decreased egg laying when compared to the complete holidic diet (Figure 2.12).

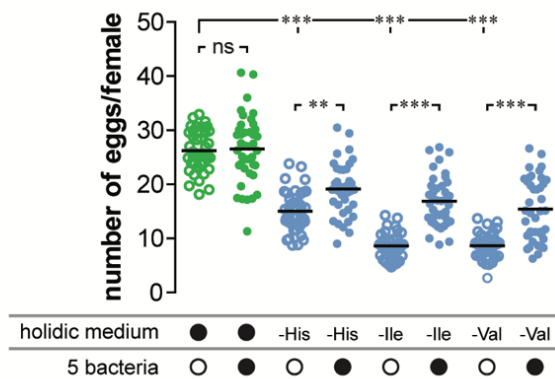


Figure 2.12 - Egg laying reduction upon eAA deprivation is increased by commensal bacteria. Number of eggs laid per female in 24 h of animals kept on holidic diet, holidic diet with all amino acids except His, isoleucine (Ile), or valine (Val), and with or without pretreatment with commensals. Black filled circles represent complete holidic medium or pretreatment with the bacteria mix. Open black circles represent flies that were not pretreated with bacteria mix. AA deprivation is indicated as –His, –Ile or –Val. Data are pooled from two different rounds of experiments performed independently on different days, $n = 39\text{--}40$. Line represents the mean.

Flies treated with the five bacteria, however, laid a significantly higher number of eggs in the context of a diet without single eAAs when compared to the flies without bacterial pretreatment (Figure 2.12). The microbiota is therefore not only able to buffer the effect of removing eAAs in the context of food choice but also in terms of physiological traits such as the reduction of egg laying triggered by removing one eAA. Given the importance of fecundity for the fitness of the animal, this effect therefore suggests that in the adult fly the host–bacteria interaction is mutualistic.

2.3.7 - Commensal bacteria specifically increase yeast appetite in the context of eAA depletion

To separately analyze the effects of the microbiome on yeast and sugar feeding, we chose to use the flyPAD assay (Itskov et al., 2014). Furthermore, to fully control the microbial conditions of the experiments, we performed the flyPAD experiments starting with flies kept in an axenic state, which ensured that our microbiome reconstitutions resulted in gnotobiotic flies. Flies kept on a full holidic diet and pretreated with the five bacteria did not show an increase in yeast feeding when compared to the germ-free controls (Figure 2.13a), but they showed an increase in sucrose feeding (Figure 2.13b). The sucrose effect is reminiscent of previous reports that the gut microbiota can increase food intake in flies (Wong et al., 2014). In agreement with the data generated using the CAFE assay, both His and Ile deprivation led to a specific increase in yeast feeding in the axenic flies (Figure 2.13a), while sucrose feeding was unaltered (Figure 2.13b). Compared with germ-free flies, the gnotobiotic flies pretreated with the five gut bacteria showed a highly significant

decrease in yeast feeding, corroborating the hypothesis that the microbiota suppresses yeast appetite (Figure 2.13a).

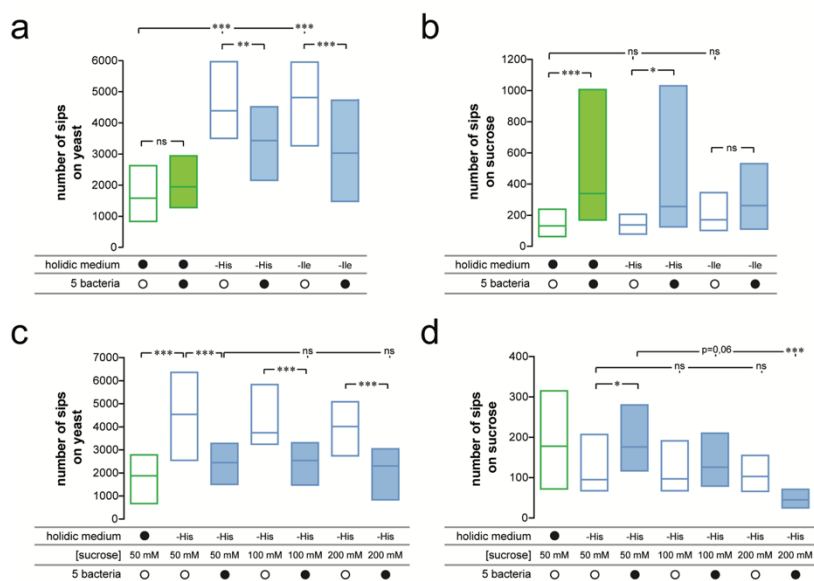


Figure 2.13 - Commensal bacteria decrease yeast appetite upon eAA deprivation.

Numbers of sips on yeast (**a**) or sucrose (**b**) of axenic flies prefed on holidic medium with different eAAs and with or without the commensal mix as measured using the flyPAD. $n = 70\text{--}82$. Numbers of sips on yeast (**c**) or sucrose (**d**) of axenic flies prefed on holidic medium containing different eAAs and different sucrose concentrations with or without the commensal mix as measured using the flyPAD. $n = 25\text{--}37$. (**a-d**) In this and following Figures, boxes represent upper and lower quartiles with the median; empty boxes represent non-bacteria-treated conditions and filled boxes represent bacteria-pretreated conditions.

In contrast to the effect on yeast appetite, the increase in sugar feeding observed in the flies treated with bacteria was more variable. While a significant effect was observed in the His-deprived flies, Ile-deprived flies did not show an increase in sucrose feeding (Figure 2.13b). These data support the idea that commensal bacteria specifically change food choice by decreasing yeast appetite in eAA-deprived flies.

It has been indicated that the microbiota competes with their host for the availability of sugars in the diet (Huang & Douglas, 2015). To test this hypothesis and rule out that the effect on yeast choice is due to the observed increase in sugar intake, we increased the amount of sugar in the holidic diet. Adding increasing amounts of sucrose to the diet decreased the sugar appetite, suggesting that the bacteria were indeed reducing the sugar available to the fly from the diet (Figure 2.13d). Importantly, neither the levels of sucrose in the diet nor the level of sugar feeding affected the yeast appetite, showing that these macronutrient appetites are independently regulated (Figure 2.13c and 2.13d). Therefore, while a decrease in the sugar content of the food could account for the previously reported increase in food intake caused by gut bacteria (Wong et al., 2014), this effect is not related to the changes in food choice we describe. Gut bacteria therefore use an independent mechanism to specifically reduce the yeast appetite of the host.

2.3.8 - Flies eat more vigorously from food containing commensal bacteria

Flies rely on the continuous replenishment of their microbiome through feeding (Blum et al., 2013). If commensal bacteria provide protection against eAA depletion, one might expect flies to prefer ingesting food containing commensals. We therefore set out to compare the appetite of flies towards food with or without commensal bacteria using the flyPAD (Figure 2.14). In agreement with our hypothesis, flies ate more vigorously from a food source containing the commensal bacteria when compared to the same food without commensals (Figure 2.14). Flies are therefore able to increase feeding behavior when bacteria are present

in the food. This suggests that flies are able to actively modulate their feeding behavior to replenish or modify their microbiota in order to profit from the physiological benefits of the commensals.

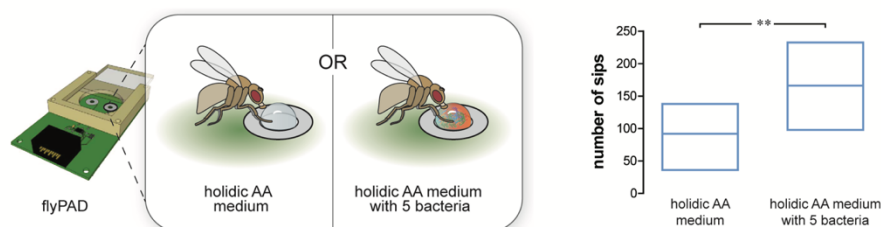


Figure 2.14 - Commensal bacteria are phagostimulators. On the left, a diagram depicting the strategy used to test the phagostimulatory power of commensal bacteria. flyPAD drawing from (Itskov et al., 2014). On the right, numbers of sips of flies feeding from holidic base medium containing AAs with or without the commensal mix as measured using the flyPAD. $n = 37-39$.

2.3.9 - *Acetobacter pomorum* acts together with *Lactobacilli* to modify food choice

Our data suggest that specific bacteria directly act on host physiology and behavior and provide evidence contrary to a generalized effect of bacterial material. We therefore decided to use the gnotobiotic model to identify which bacteria in the mix were producing the change in feeding behavior in eAA-deprived animals. To do so, we first removed each species separately from the mix and tested if the reduced sets could suppress the yeast appetite of His-deprived flies. While removal of *Acetobacter pomorum* (*Ap*) abolished the capacity of the mix to suppress yeast appetite, removal of any of the other four species had no effect

(Figure 2.15a). *Ap* alone, however, is not sufficient to change yeast appetite, indicating that it acts in concert with other bacteria in the mix.

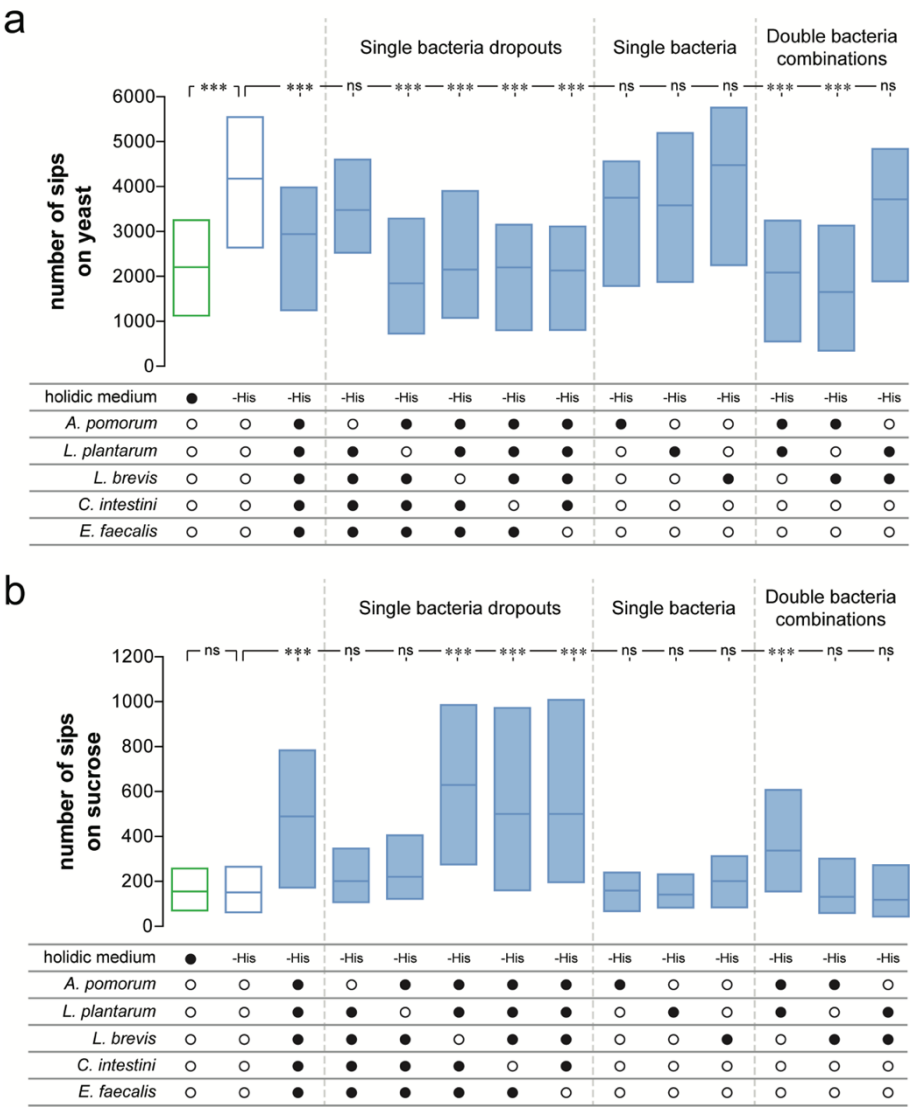


Figure 2.15 - *A. pomorum* and *Lactobacilli* are sufficient to modify yeast appetite. Numbers of sips on yeast (a) or sucrose (b) as measured using the flyPAD of axenic flies prefed on complete holidic medium or holidic medium without histidine (His) and pretreated with different bacterial mixes, $n = 46\text{--}120$.

Given that *Lactobacilli* act together with *Ap* to alter metabolite composition in flies (Newell & Douglas, 2014), we decided to test if *Ap* together with *Lactobacillus plantarum* (*Lp*) or *Lactobacillus brevis* (*Lb*) are sufficient to alter yeast appetite. Indeed, the combination of *Ap* with either *Lp* or *Lb* is sufficient to suppress the yeast appetite induced by deprivation from either His or Ile (Figure 2.15a and Figure 2.16). This result also explains why removing either *Lp* or *Lb* from the five-bacteria mix had no effect, as these species seem to act redundantly. Furthermore, neither *Lp*, *Lb*, nor the combination of both change feeding behavior, highlighting the specificity of the combined *Ap*–*Lactobacilli* effect on yeast appetite (Figure 2.15a).

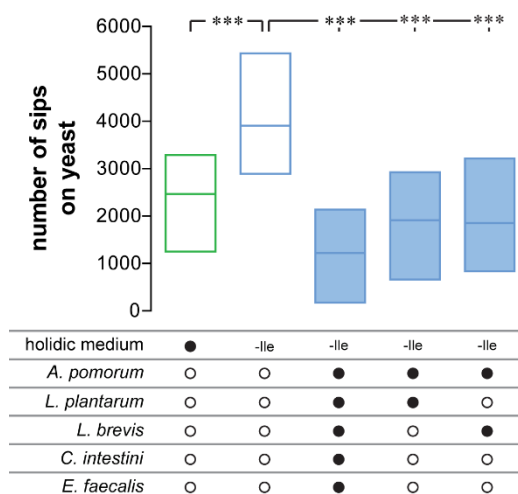


Figure 2.16 - *A. pomorum* and *Lactobacilli* are sufficient to reduce the yeast appetite induced by removal of isoleucine. Numbers of sips on yeast as measured using the flyPAD of axenic flies prefed on complete holidic medium or holidic medium without isoleucine (Ile) and pretreated with different bacterial mixes, $n = 25$ –55.

The same approach allowed us to conclude that *Ap* and *Lp* act together to increase sugar appetite (Figure 2.15b). In contrast to the effect on yeast appetite, the *Ap–Lb* combination has no effect on carbohydrate consumption (Figure 2.15b). This reinforces the previous data showing that yeast appetite is independent of sugar appetite. Taken together, these data show that *Acetobacter pomorum* can act together with either *Lactobacillus plantarum* or to a certain extent with *Lactobacillus brevis* to change food selection.

2.3.10 - Commensal bacteria do not seem to change the levels of eAAs in the host

The ability of the commensal bacteria to compensate for the effect of eAA deprivation on yeast appetite and egg laying suggests that the bacteria could supply the host with eAAs, thus buffering the animal from the absence of these important nutrients in the diet. Such an effect would be reminiscent of the role of the *Buchnera* endosymbiont in aphids, which allows this insect to thrive while feeding on sap, which contains very low amounts of AAs (Douglas, 1998). We tested this hypothesis by depriving flies from three different eAAs (His, Ile, and Val) and comparing the levels of free AAs in the heads of flies that had been either pretreated or not with the five-bacteria mix. We decided to focus on the AA levels in heads to avoid effects due to changes in the number of eggs carried by the fly and because of evidence that nutrient sensing could act at the level of the brain of the fly to change food preference (Ribeiro & Dickson, 2010). His, Ile, or Val deprivation lead to a drastic decrease in the levels of these three AAs in head extracts (Figure 2.17), which is likely to cause the previously observed increases in yeast appetite (Figure 2.5). This effect was specific

to the manipulated AAs, as the levels of nonmanipulated AAs neither increased nor decreased (Figure 2.17). AA-satiated flies treated with the bacterial mix did not show an increase in His, Ile, or Val. Surprisingly, deprived flies continued having very low titers of the measured eAA independent of the bacterial pretreatment (Figure 2.17). This stands in contrast to the clear effect of the bacterial pretreatment on yeast preference and egg laying (Figure 2.9 and Figure 2.12). Our failure to observe changes in eAA levels induced by bacterial pretreatment opens the intriguing possibility that the commensal bacteria modify food choice and egg laying through an AA-independent mechanism.

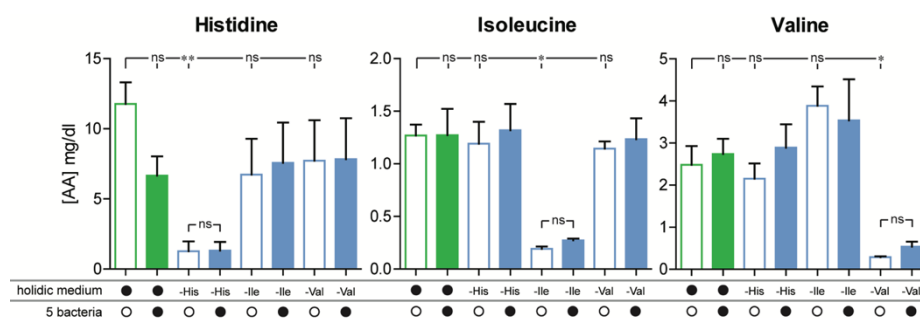


Figure 2.17 - Commensal bacteria do not seem to change the levels of eAAs in the host. Histidine (His), isoleucine (Ile), and valine (Val) concentrations in the heads of flies prefed on complete holidic medium (green) or holidic medium lacking His, Ile, or Val (blue), without (empty columns) or with (filled columns) commensals pretreatment. The columns represent the mean $\pm$ SEM of three independent experiments.

2.4 - DISCUSSION

The complexity of natural foods makes it very difficult to identify the nutrients which guide behavior and physiology. Furthermore, while the microbiota has been suggested to affect behavior, until now its influence on nutrient-specific appetites has not been explored. This study identifies two factors as strong and specific modulators of feeding decisions and reproduction: eAAs and commensal bacteria (Figure 2.18).

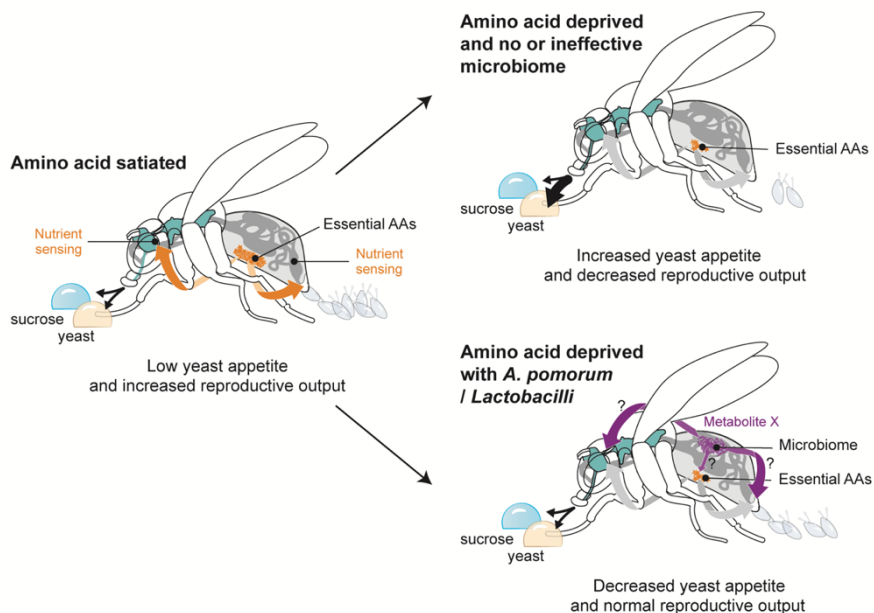


Figure 2.18 - Model of the impact of eAAs on food choice and reproduction, depending on the presence of the microbiota of the host. The nervous system is highlighted in turquoise, AAs in orange, and commensal bacteria in purple. Arrow weight from the proboscis to the food drops indicate amount of feeding, and the number of eggs reflect the reproductive output. The orange and purple arrows indicate potential effects of eAAs and metabolites, respectively, at the level of the nervous and reproductive systems. Metabolite X refers to a hypothetical metabolite mimicking the presence of AAs.

2.4.1 - Amino acids as potent modulators of protein appetite

Multiple nutrients, including AAs, metals, vitamins, and sterols have been shown to be nutritional modulators of life history traits in *Drosophila* (Piper et al., 2014). Given that in an ecological setting, yeast is likely to be an important nutritional source of these nutrients, it is therefore surprising that flies only develop a yeast appetite upon the restriction of AAs. A possibility is that animals could use the lack of internal AAs as a proxy for the concomitant lack of other nutrients. Further focused analyses of the effect of specific nutrient classes on behavior will be required to identify the full capacity of *Drosophila* to maintain nutrient homeostasis.

To react to the lack of AAs, flies must be able to sense their internal deficiencies. Indeed, AA deprivation leads to a reduction in the concentration of free AAs in the head. But how does the nervous system sense the internal deficiency of AAs? Two molecular mechanisms have been proposed as mediating neuronal AA sensing: the TOR and GCN2 pathways. Neuronal TOR signaling has been shown to be involved in food selection (Ribeiro & Dickson, 2010), and in *Drosophila* and vertebrates, GCN2 has been proposed to direct behavioral nutrient homeostasis by mediating post-ingestive neuronal sensing of AAs (Bjordan et al., 2014; Hao, 2005; Maurin et al., 2005). Intriguingly, the TOR pathway has been proposed to mainly react to specific AAs (Gu et al., 2022; Hara et al., 1998). Our data using the *Henna* knockdown, however, suggest that nutrient sensing pathways could have a much broader spectrum of action. We therefore have only very rudimentary clues as to what could be the mechanisms mediating internal sensing of AA availability and subsequent changes in behavior.

2.4.2 - Host–microbiota interactions in nutrient choice

The extent to which the microbiota affects nutrient-specific appetites has not been previously explored. We show that when flies are AA deprived, commensal bacteria reduce their compensatory yeast appetite. The increase in sugar appetite observed in flies harboring commensals further decreases the ratio of protein to carbohydrate intake, an important determinant of life history traits in animals, including vertebrates (S. J. Simpson & Raubenheimer, 2012). Given that a reduction in yeast and AA intake leads to an increase in lifespan, our observation that commensal bacteria reduce the intake ratio of proteins to carbohydrates could account for the shorter lifespan of axenic flies (Brummel et al., 2004). It is interesting to note that the flies harboring commensals are able to increase their reproductive output (our study and (Elgart et al., 2016)) despite their lower protein intake. Commensal bacteria could therefore have a highly beneficial impact on the fly, enabling it to simultaneously maximize lifespan and reproductive output.

Why do flies suppress their yeast appetite in the presence of commensal bacteria? One simple explanation could be that these bacteria act as nutrients, which has been proposed for yeast and other fungi (Yamada et al., 2015). However, our findings indicate that the microbiota acts in a very specific way to alter food choice. A key question is thus: what could be the mechanisms by which gut bacteria change yeast appetite and increase egg laying? They could provide the lacking AAs, as seen with aphids and the intracellular symbiotic bacteria *Buchnera* (Douglas, 1998). However, we were not able to detect changes in the levels of free eAAs in flies pretreated with the commensal mix. In spite of this, it is still possible, for example, that in an eAA-deprived situation, bacterially produced eAAs

are immediately utilized without increasing the pool of free AAs. Our data, however, suggest that commensal bacteria do not act by providing eAAs to the host. Thus, the main question of how commensal bacteria influence behavior and egg production still remains. They could secrete metabolites that help the host to increase its ability to use its remaining AAs or that mimic the availability of eAAs (Metabolite X in Figure 2.18). Distinguishing between these hypotheses will require comprehensive metabolome analyses of flies in different bacterial and nutrient states as well as careful genetic and behavioral studies, both at the level of the host and the bacteria.

Our understanding of how the microbiota influences behavior remains extremely rudimentary. In vertebrates, this task is made especially daunting by the complexity of their microbiota. *Drosophila*, on the other hand, has proven to be an especially powerful model for understanding microbe–host interaction because of the ability to isolate a single bacterial species promoting physiological effects such as improved growth (Erkosar et al., 2013; W.-J. Lee & Brey, 2013). The identification of only two bacterial species as mediators of food choice paired with the powerful genetic toolkit available in *Drosophila* provides a unique opportunity to identify the mechanisms by which microbes interact to shape the behavior of the host.

2.5 - MATERIALS & METHODS

2.5.1 - *Drosophila* stocks and genetics

Unless stated otherwise, all experiments were performed with mated *w¹¹¹⁸* female flies. Ubiquitous (*tubulin-Gal4* (T. Lee & Luo, 1999)), pan-neuronal (*elav-Gal4* (Luo et al., 1994)), tracheal (*btl-Gal4* (Förster & Luschnig, 2012)), or fat body (*Cg-Gal4* (Asha et al., 2003), BL #7011) expression of RNAi delivering transgenes against *Henna* (*CG7399*) was achieved by crossing Gal4-carrying female flies with three independent UAS-*Henna*-RNAi stocks (*Henna^{IR1}*: VDRC #35240; *Henna^{IR2}*: NIG-RNAi #7399R-3; *Henna^{IR3}*: BL #29540). The full genotypes of stocks used in this chapter are listed in Supplementary Table 2.1.

2.5.2 - *Drosophila* rearing, media, and dietary treatments

Flies were reared on yeast-based medium (YBM) (per liter of water: 8 g agar [NZYTech, PT], 80 g barley malt syrup [Próvida, PT], 22 g sugar beet syrup [Grafschafter, DE], 80 g corn flour [Próvida, PT], 10 g soya flour [A. Centazi, PT], 18 g instant yeast [Saf-instant, Lesaffre], 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]). To ensure a homogenous density of offspring among experiments, fly cultures were always set with 5 females and 4 males per vial and left to lay eggs for 7 d. Flies were reared in YBM until adulthood. Holidic media (HM) were prepared as described previously (Piper et al., 2014) using the HUNTaa formulation without food preservatives, with the exception of the HM used for pretreating axenic and gnotobiotic flies, for which we used an HM with an improved AA composition (Piper et al., 2017). The different HM used in this chapter are

described in Supplementary Tables 2.2 and 2.3. In all experiments where we refer to all neAAs removal, L-glutamate was still present in the diet in order to prevent any possible adverse effects in neuronal function. Sucrose medium consisted of Kleenex tissue soaked with 5 ml of a 100 mM sucrose (Sigma-Aldrich, #84097) solution. For all experiments using the HM, the following dietary treatment protocol was used in order to ensure a well-fed state and minimize the microbial load in the flies (Blum et al., 2013) (Supplementary Figure 2.2): groups of 1–5-d-old flies (16 females and 5 males) were collected into fresh YBM-filled vials and transferred to fresh YBM after 48 h. Following a period of 24 h, flies were transferred to different HM for 72 h and immediately tested in the indicated assay. Flies treated using this protocol had a low titer of commensal bacteria (Figure 2.8). For yeast dilution experiments presented in Figure 2.10, flies were kept for 72 h prior to the behavioral assay on media containing 200 mM sucrose (Sigma-Aldrich, #84097), 2% agar (Difco, # 214530), and variable instant yeast concentrations: 5%, 2.5%, 1%, and 0% (Saf-instant, Lesaffre). After preparation, all yeast-based media were autoclaved before pouring into culture vials. Fly rearing, maintenance, and behavioral testing were performed at 25 °C in climate-controlled chambers at 70% relative humidity in a 12-h light–dark cycle (Aralab, FitoClima 60000EH). Polypropylene fly vials (VWR, #734-2261) were used.

These protocols are available in the following collection in protocols.io [dx.doi.org/10.17504/protocols.io.hdtb26n](https://doi.org/10.17504/protocols.io.hdtb26n).

2.5.3 - Axenic *Drosophila* generation and rearing

The protocol to generate axenic *w¹¹¹⁸* fly cultures by sterilizing embryos was adapted from (Wayland et al., 2014): embryos were put for 2 min in 2.5% active chlorine (50% bleach) followed by 2 min in 70%

ethanol and 2 min in autoclaved distilled water. The embryos were then transferred onto sterile food (autoclaved before pouring into culture vials) containing antibiotics (final concentrations: 416.7 µg/ml tetracycline [high dose], 41.67 µg/ml chloramphenicol, 41.67 µg/ml ampicillin, and 8.333 µg/ml erythromycin). In order to compensate for the developmental delay observed in axenic larvae (Storelli et al., 2011), the yeast content of the medium was increased to 41.67 g per liter. Axenic *w¹¹¹⁸* flies were regularly transferred into vials containing freshly prepared, antibiotic-supplemented, high-yeast food (Supplementary Figure 2.2). The absence of bacteria was assessed by grinding flies in sterile 1x PBS and spreading the suspension on LB, MRS, or Mannitol plates. LB and MRS plates were incubated at 37 °C and Mannitol plates at 30 °C, respectively, before assessing the presence of bacterial colonies. The antibiotic treatment did not lead to any apparent malaise in the treated flies. Furthermore, to ensure that the antibiotics exposure would not directly affect the experimental animals, these were raised in sterile food without antibiotics (Supplementary Figure 2.2). Importantly, the results obtained using the gnotobiotic flies fully recapitulate the results obtained with the conventionally reared “low bacteria titer” flies.

All experiments in Figures 2.13, 2.15 and Figure 2.16 were performed using axenic or gnotobiotic flies.

These protocols are available in the following collection in [protocols.io dx.doi.org/10.17504/protocols.io.hdtb26n](https://protocols.io/10.17504/protocols.io.hdtb26n).

2.5.4 - Bacterial species and cultures

The following bacterial species and strains (kindly provided by François Leulier, IGFL, France, and Won-Jae Lee, SNU, South Korea) were used in this study: *Lactobacillus plantarum*^{WJL} (Ryu et al., 2008),

Lactobacillus brevis^{EW} (Ryu et al., 2008), *Acetobacter pomorum* (Ryu et al., 2008), *Commensalibacter intestini*^{A911T} (Ryu et al., 2008), and *Enterococcus faecalis* (Cox & Gilmore, 2007). *Lactobacilli* were cultured in 10 ml of liquid MRS medium (Sigma-Aldrich, #69966) in 14 ml culture tubes (Thermo Fisher Scientific, #150268) at 37 °C for 24 h without agitation. *C. intestini*^{A911T} and *A. pomorum* were cultured in a liquid mannitol medium (3 g/l Bacto peptone [Difco, #0118-17], 5 g/l yeast extract [Difco, #212750], 25 g/l D-mannitol [Sigma-Aldrich, #M1902]) at 30 °C for 48 h under 170 rpm agitation. *C. intestini*^{A911T} was cultured in 20 ml of medium in 50-ml tubes (Falcon), and *A. pomorum* was cultured in 200 ml of medium in 500-ml flasks. *E. faecalis* was cultured in 200 ml of liquid LB medium in 500-ml flasks at 37 °C for 24 h under 220 rpm agitation. Liquid cultures were set with colonies grown in fresh solid media (15 g/l agar [Difco, # 214530]).

These protocols are available in the following collection in protocols.io [dx.doi.org/10.17504/protocols.io.hdtb26n](https://doi.org/10.17504/protocols.io.hdtb26n).

2.5.5 - Inoculation of HM with bacteria

Prior to transferring the flies, each HM vial was inoculated with either single or different combinations of the following bacterial species: *L. plantarum*^{WJL} (6.4×10^4 CFU), *L. brevis*^{EW} (5.31×10^3 CFU), *C. intestini*^{A911T} (9.04×10^4 CFU), *A. pomorum* (9.5×10^4 CFU), *E. faecalis* (1.11×10^5 CFU), and *E. coli* (0.924×10^9 CFU). To prepare this mixture, the necessary volume of liquid culture for each bacterial species was centrifuged three times at 3,000 rpm for 10 min and repeatedly resuspended in 1x PBS. To exclude an effect from residual components of bacterial media, the equivalent volume of sterile bacterial media was centrifuged in parallel and used as a control. After the final centrifugation,

both the control and the bacterial pellet were resuspended in sufficient 1x PBS to achieve an inoculation volume of 50 μ l per vial. For the experiments with heat-inactivated bacteria, the bacterial suspension was incubated at 100 °C for 10 min before inoculation in HM vials. The final suspensions were added to the surface of the HM and allowed to dry for approximately 1 h before the addition of flies. Note that even when not pretreated to be axenic, because of rearing protocol flies used in all experiments had a very low starting titer of internal microbes prior to inoculation (Figure 2.8).

These protocols are available in the following collection in [protocols.io dx.doi.org/10.17504/protocols.io.hdtb26n](https://protocols.io/dx/doi.org/10.17504/protocols.io.hdtb26n).

2.5.6 - Calculation of internal bacterial load of flies

Flies were surface sterilized to remove any bacteria that could be found on the cuticle by washing them in 70% ethanol followed by two washes in sterile 1x PBS. Flies were grinded in 1x PBS (50 μ l/fly) and diluted 100X. The suspension was then plated on LB, MRS, or Mannitol medium. LB and MRS plates were incubated at 37 °C and mannitol plates at 30 °C before counting the number of bacterial colonies.

These protocols are available in the following collection in [protocols.io dx.doi.org/10.17504/protocols.io.hdtb26n](https://protocols.io/dx/doi.org/10.17504/protocols.io.hdtb26n).

2.5.7 - Two-color food choice assays

Two-color feeding preference assays were performed as previously described (Ribeiro & Dickson, 2010). Groups of 16 female and 5 male flies were briefly anesthetized using light CO₂ exposure and introduced into tight-fit-lid Petri dishes (Falcon, #351006). For the yeast choice assays, the flies were given the choice between nine spots of 10 μ l sucrose

solution mixed with red colorant (20 mM sucrose [Sigma-Aldrich, #84097]; 7.5 mg/ml agarose [Invitrogen, #16500]; 5 mg/ml Erythrosin B [Sigma-Aldrich, #198269]; 10% PBS) and nine spots of 10 μ l yeast solution mixed with blue colorant (10% yeast [Saf-instant, Lesaffre]; 7.5 mg/ml agarose; 0.25 mg/ml Indigo carmine [Sigma-Aldrich, #131164]; 10% PBS) for 2 h. For the defined nutrient-choice assays, flies were given the choice between HM lacking AAs and containing 20 mM sucrose mixed with red colorant (option 1: sucrose) and HM lacking sucrose and containing the nutrients required for the experiment mixed with the blue colorant (option 2). In these experiments, the agar concentration in the HM was changed to 1.5%. After visual inspection of the abdomen under the stereo microscope (Zeiss, Stereo Discovery.V8), each female fly was scored as having eaten either sucrose (red abdomen), yeast (blue abdomen), or both (red and blue or purple abdomen) media. The yeast preference index (YPI) for the whole female population in the assay was calculated as follows: $(n_{\text{blue yeast}} - n_{\text{red sucrose}}) / (n_{\text{red sucrose}} + n_{\text{blue yeast}} + n_{\text{both}})$. Initially, dye-swap (red yeast versus blue sucrose choice) experiments were performed in parallel, and because the change of feeding preference was observed in both conditions, we opted to exclusively perform red sucrose versus blue yeast choice experiments. In all experiments, the observer was blind for both diet and genotype. All assays were performed between ZT6 and ZT9.

2.5.8 - CAFE assays

CAFE assays were based on a protocol previously described (Ja et al., 2007; Walker et al., 2015) with some adaptations. On the assay day, flies were anesthetized with CO₂, sorted under a stereo microscope in groups of 18 females, and allowed to recover for 3 h at 25 °C. The CAFE

chamber consisted of a large plastic vial (50 x 100 mm) (Semadeni AG, #6128) with 6 5- μ l glass capillaries (Hirschmann, #9600105) inserted through a foam lid. Capillaries were filled with 20 mM sucrose or 10% yeast extract (Sigma-Aldrich #1625) solutions and placed in an alternating circular fashion. Each group of flies was aspirated into a CAFE chamber, and during the 4 h of the assay, four experimental readings per capillary were scored (t_0 , $t_{0+1\text{ h}}$, $t_{0+3\text{ h}}$, and $t_{0+4\text{ h}}$) to determine consumption. In order to correct for evaporation, each set of experimental chambers was accompanied by an empty chamber (no flies). Total sucrose or yeast extract consumption per time point was determined by subtracting the sum of the readings of the three capillaries of the respective solution in the empty chamber from the equivalent values in the experimental chamber. Consumption per fly was obtained by dividing sucrose or yeast extract total consumption by the number of living flies at the end of the assay.

2.5.9 - flyPAD assays

flyPAD assays were performed as described in (Itskov et al., 2014). For food choice experiments, single flies in different dietary conditions were tested in arenas that contained two kinds of food patches: 10% yeast and 20 mM sucrose, each mixed with 1% agarose.

To measure the phagostimulatory power of bacteria (Figure 2.14), we used a flyPAD setup that had never been exposed to yeast. All tested flies were deprived from amino acids using HM–AAs. For the flyPAD assays, one feeding well per arena was filled with HM without sucrose, either intact media (holidic AA medium) or media supplemented with the bacterial mixture (holidic AA medium with 5 bacteria) (in HM: *L. plantarum*^{WJL} 1.02×10^2 CFU, *L. brevis*^{EW} 8.49 CFU, *A. pomorum* 1.52×10^2 CFU, *C. intestini*^{A911T} 1.45×10^2 CFU, and *E. faecalis* 1.77×10^2 CFU).

These media were prepared by adding agarose (1%) as a gelling agent together with cholesterol after autoclaving. Media were prepared on the experimental day and maintained at 30 °C in a heat block. Preparation of the control and bacterial mixture pellets were performed as described above and directly resuspended in the HM without sucrose to generate holidic AA medium and holidic AA medium with five bacteria, respectively. Each medium was loaded into a single feeding well of the arena.

For all experiments, flies were individually transferred to flyPAD arenas by mouth aspiration and allowed to feed for 1 h at 25 °C, 70% relative humidity. The total number of sips per animal over this hour was calculated using previously described flyPAD algorithms (Itskov et al., 2014). Noneating flies (defined as having fewer than two activity bouts during the assay) were excluded from the analysis.

2.5.10 - Egg-laying assays

Groups of 16 female and 5 male flies were briefly anesthetized using light CO₂ exposure and transferred to apple juice agar plates (per liter, 250 ml apple juice, 19.5 g agar, 20 g sugar, and 10 ml nipagin [15% in ethanol]), where they were allowed to lay eggs for 24 h. Flies were then removed and counted and eggs were counted. Egg laying was calculated by dividing the number of eggs by the number of living females at the end of the assay.

2.5.11 - Total mRNA extraction, RT-PCR, and quantitative real-time PCR

Flies used for mRNA extraction were snap frozen in dry ice and kept at -80 °C until used. Behavioral assays were performed in parallel to

confirm that sibling flies presented the expected feeding phenotype. mRNA was extracted from flies (three flies per condition) using the following procedure: flies were grinded and homogenized for 20 s (using pestles #Z359947, Sigma) in 100 μ l of PureZOL (#732-6890, Bio-Rad). 250 μ l of PureZOL was further added and mixed by pipetting and incubated at RT for 10 min. Finally, 350 μ l of 100% ethanol was added, and the samples were mixed and transferred to a Zymo column (Direct-zol RNA MicroPrep #R2062, Zymo research). The manufacturer's instructions were followed to purify the mRNA (including DNase treatment), and samples were eluted in 15 μ l of distilled RNase/DNase-free water. The concentration of the total mRNA samples was determined by performing a spectrophotometer scan in the UV region. Total RNA (1 μ g) was reverse transcribed (RT) using the iScript Reverse Transcription Supermix for RT-PCR kit (#170-8840 Bio-Rad), following the manufacturer's instructions. The expression of *Henna* was determined using real-time PCR. Each cDNA sample was amplified using SsoFast EvaGreen Supermix on the CFX96 Real-Time System (Bio-Rad). Briefly, the reaction conditions consisted of 1 μ l of 1:10 diluted cDNA, 1 μ l (10 μ M) of each primer, 10 μ l of supermix, and 7 μ l of water. The cycle program consisted of enzyme activation at 95 °C for 30 s, 39 cycles of denaturation at 95 °C for 2 s, and annealing and extension for 5 s. The primers used in this reaction are listed in Supplementary Table 2.4. This experiment was performed using three experimental replicas and two technical replicas per genotype. Appropriate nontemplate controls were included in each 96-well PCR reaction, and dissociation analysis was performed at the end of each run to confirm the specificity of the reaction. Absolute levels of RNA were calculated from a standard curve and normalized to the internal controls (*Actin42A* and *RpL32*). The relative

quantitation of each mRNA was performed using the comparative Ct method. Data processing was performed using Bio-rad CFX Manager 3.1 (Bio-Rad).

2.5.12 - Amino acid measurements in fly heads

500 females per condition were collected on the same day as behavioral assays and were snap frozen in dry ice. Flies were kept at -80°C until head preparation for amino acids measurements. Fly heads were separated from other body parts by vortexing the Eppendorf tubes and posteriorly passing the debris through 710- μm and 425- μm sieves (Retsch GmbH). Fly heads were counted before homogenization to ensure that the same number was used for all conditions. Heads were homogenized in 200 μl of 2.5% TCA and centrifuged for 10 min at top speed at 4°C . The supernatant was recovered and stored at 4°C for analysis. Amino acid quantification was performed by HPLC at a clinical laboratory (Joaquim Chaves Laboratories, PT). Amino acids were detected using AccQ.Tag (Waters, #176001235).

2.5.13 – Statistical Analysis

Results of two-color food choice assays and flyPAD experiments were compared using the Kruskal-Wallis test, followed by Dunn's multiple comparison test where appropriate. For tests comparing only two groups, the Wilcoxon rank-sum test was used. In the CAFE assay experiments and AA measurements significance was tested using the unpaired t test with Bonferroni correction. For egg laying experiments significance was tested using the one-way analysis of variance test followed by Bonferroni's multiple correction test. All tests were two-tailed. For all figures, *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; ns $p \geq 0.05$.

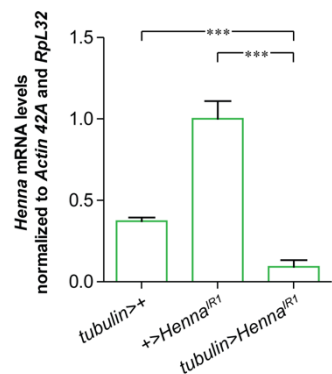
2.6 - ACKNOWLEDGMENTS

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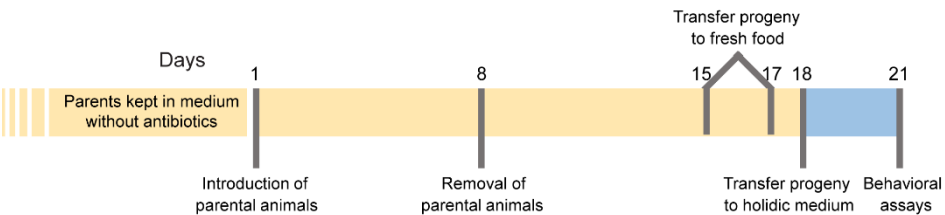
2.7 - SUPPLEMENTARY MATERIALS

2.7.1 Supplementary Figures

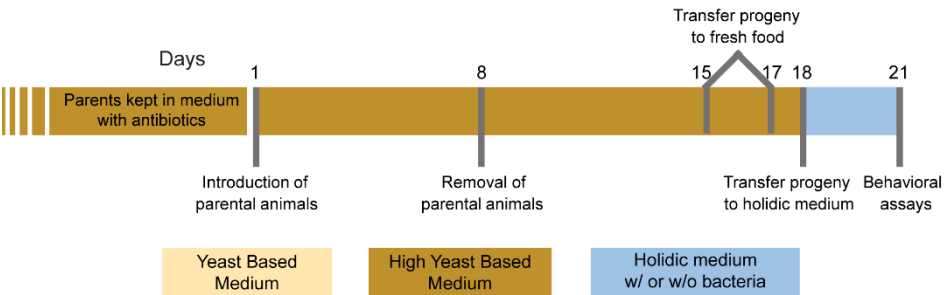


Supplementary Figure 2.1 - *Henna* mRNA levels measured from whole flies and normalized to two internal controls (*Actin 42A* and *RpL32*). The columns represent the mean $\pm$ SEM, $n = 6$.

Non-axenic flies



Axenic flies



Supplementary Figure 2.2 - Diagram depicting the chronology of the dietary and microbial manipulations of flies used in experiments.

2.7.2 Supplementary Tables

Supplementary Table 2.1 - List of stocks used in chapter 2.

Referred to as	Detailed genotype	Source
background	w^{1118}	Ribeiro Lab
<i>tubulin-Gal4</i>	$y^*, w^*; ; P\{tubP-GAL4\}$	Luo Lab
<i>Cg-Gal4</i>	$P\{Cg-GAL4.A\}2;$	BDSC #7011
<i>elav-Gal4</i>	$w^{1118}, P\{w[+mC]=UAS-Dcr-2\};$ $; P\{w[+mC]=GAL4-elav.L\}3$	Jan Lab
<i>btl-Gal4</i>	$w^*; ; P\{w+, btl-GAL4\}$	Luschnig Lab
<i>Henna^{IR1}</i>	$w^{1118}; P\{GDI2227\}^{v35240}$	VDRC #35240
<i>Henna^{IR2}</i>	$w^{1118}; ; P\{NIG.7399R\}3$	Kyoto #7399R
<i>Henna^{IR3}</i>	$y^l v^l; P\{TRiP.JF02261\}attP2$	BDSC #29540

Supplementary Table 2.2 - Recipe for generation of HM used in chapter 2. Gray fields indicate manipulated nutrients.

Referred to as	holidic medium (HUNTaa)	-all AAs	-eAAs	-neAAs	-single eAA	-neAAs + 1xTyr (or 2xTyr)	-neAAs + 1xPro
Essential amino acids	60,51 ml	0 ml	0 ml	140,45 ml(a)	140,45 ml(a,b)	140,45 ml(a)	140,45 ml(a)
L-isoleucine	1,82 g	0 g	0 g	4,21 g(a)	4,21 or 0 g(a,c)	4,21 g(a)	4,21 g(a)
L-leucine	1,21 g	0 g	0 g	2,81 g(a)	2,81 or 0 g(a,c)	2,81 g(a)	2,81 g(a)
Non-essential amino acids	60,51 ml	0 ml	97,52 ml(a)	0 ml	0 ml	0 ml	0,27 g proline(d)
L-glutamate	15,13 ml	0 ml	24,38 ml(a)	35,11 ml(a)	35,11 ml(a)	35,11 ml(a)	35,11 ml(a)
L-tyrosine	0,42 g	0 g	1,61 g(a)	0 g	0 g	0,42 g (1x) or 0,84 g (2x)	0 g
L-cysteine (HCl)	n/a(e)	n/a(e)	n/a(e)	n/a(e)	n/a(e)	n/a(e)	n/a(e)
Cholesterol	15 ml	15 ml	15 ml	15 ml	15 ml	15 ml	15 ml
CaCl ₂	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml
MgSO ₄	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml
CuSO ₄	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml
FeSO ₄	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml
MnCl ₂	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml
ZnSO ₄	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml
Nucleic acids & Lipids	8 ml	8 ml	8 ml	8 ml	8 ml	8 ml	8 ml
Vitamins	14 ml	14 ml	14 ml	14 ml	14 ml	14 ml	14 ml
Folic acid	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml
Acetic acid buffer	100 ml	100 ml	100 ml	100 ml	100 ml	100 ml	100 ml
Sucrose	17,12 g	17,12 g	17,12 g	17,12 g	17,12 g	17,12 g	17,12 g
Agar	20 g	20 g	20 g	20 g	20 g	20 g	20 g
Milli Q H ₂ O	Adjust to 1 L	Adjust to 1 L	Adjust to 1 L	Adjust to 1 L(f)	Adjust to 1 L	Adjust to 1 L	Adjust to 1 L

(a) The amount of these nutrients was increased to adjust concentration of biological active nitrogen to 197,9 mM as in the complete HM. In diets where neAAs were removed, L-glutamate was still added.

(b) A complete essential AAs solution or an essential AA drop-out solution lacking L-arg, L-met, L-val, L-phe, L-his, L-lys, L-thr or L-trp were used.

(c) L-ile or L-leu were omitted to obtain HM lacking these AAs.

(d) The amount of Proline used corresponds to the biological active nitrogen equivalent to 0,42 g of L-tyr (2,32 mmol).

(e) L-cysteine HCl was added in a separate solution only in the HM with improved AA composition.

(f) In the absence of medium preservatives, Cg-Gal4 flies were not healthy and therefore 6 ml of propionic acid and 15 ml of nipagin (100 g/l in 95% EtOH) were added.

(continues in the next page)

Referred to as	-single eAA (g)	-folic acid	-metals	-nc.ac. & lipids	-sterol	-vitamins	holidic medium (improved AA composition) (h)
Essential amino acids	60,51 ml(b)	60,51 ml	60,51 ml	60,51 ml	60,51 ml	60,51 ml	60,51 ml(i)
L-isoleucine	1,82 or 0 g(c)	1,82 g	1,82 g	1,82 g	1,82 g	1,82 g	1,12 g or 0 g(j)
L-leucine	1,21 or 0 g(c)	1,21 g	1,21 g	1,21 g	1,21 g	1,21 g	2,03 g
Non-essential amino acids	60,51 ml	60,51 ml	60,51 ml	60,51 ml	60,51 ml	60,51 ml	60,51 ml
L-glutamate	15,13 ml	15,13 ml	15,13 ml	15,13 ml	15,13 ml	15,13 ml	15,19 ml
L-tyrosine	0,42 g	0,42 g	0,42 g	0,42 g	0,42 g	0,42 g	0,93 g
L-cysteine (HCl)	n/a(e)	n/a(e)	n/a(e)	n/a(e)	n/a(e)	n/a(e)	6,83 ml(e)
Cholesterol	15 ml	15 ml	15 ml	15 ml	0 ml	15 ml	15 ml
CaCl ₂	1 ml	1 ml	0 ml	1 ml	1 ml	1 ml	1 ml
MgSO ₄	1 ml	1 ml	0 ml	1 ml	1 ml	1 ml	1 ml
CuSO ₄	1 ml	1 ml	0 ml	1 ml	1 ml	1 ml	1 ml
FeSO ₄	1 ml	1 ml	0 ml	1 ml	1 ml	1 ml	1 ml
MnCl ₂	1 ml	1 ml	0 ml	1 ml	1 ml	1 ml	1 ml
ZnSO ₄	1 ml	1 ml	0 ml	1 ml	1 ml	1 ml	1 ml
Nucleic acids & Lipids	8 ml	8 ml	8 ml	0 ml	8 ml	8 ml	8 ml
Vitamins	14 ml	14 ml	14 ml	14 ml	14 ml	0 ml	21 ml
Folic acid	1 ml	0 ml	1 ml	1 ml	1 ml	1 ml	1 ml
Acetic acid buffer	100 ml	100 ml	100 ml	100 ml	100 ml	100 ml	100 ml
Sucrose	17,12 g	17,12 g	17,12 g	17,12 g	17,12 g	17,12 g	17,12 g
Agar	20 g	20 g	20 g	20 g	20 g	20 g	20 g
Milli Q H ₂ O	Adjust to 1 L	Adjust to 1 L	Adjust to 1 L	Adjust to 1 L	Adjust to 1 L	Adjust to 1 L	Adjust to 1 L

(b) A complete essential AAs solution or an essential AA drop-out solution lacking L-arg, L-met, L-val, L-phe, L-his, L-lys, L-thr or L-trp were used.

(c) L-ile or L-leu were omitted to obtain HM lacking these AAs.

(e) L-cysteine HCl was added in a separate solution only in the HM (with improved AA composition).

(g) Used for experiments in Figures 4, 7, S4, S5 and S6.

(h) Used for experiments in Figures 5, 6, and S7.

(i) A complete essential AAs solution or an essential AA drop-out solution lacking L-his were used.

(j) L-ile was omitted to obtain HM lacking this AA.

Supplementary Table 2.3 - Detailed composition of amino acid solutions for HM used in chapter 2.

Referred to as		holidic medium (HUNTaa) (b)	holidic medium (improved AA composition) (a)
Essential amino acid stock solution (1L)	L-arginine	8 g	26,95 g
	L-histidine	10 g	10,8 g
	L-lysine (HCl)	19 g	22,5 g
	L-methionine	8 g	9,95 g
	L-phenylalanine	13 g	16,65 g
	L-threonine	20 g	18,3 g
	L-tryptophan	5 g	5,3 g
	L-valine	28 g	19,85 g
Non-essential amino acid stock solution (1L)	L-alanine	35 g	18,2 g
	L-asparagine	17 g	17 g
	L-aspartic acid	17 g	19,35 g
	L-cysteine (HCl)	0,5 g	n/a
	L-glutamine	25 g	18,55 g
	Glycine	32 g	12,7 g
	L-proline	15 g	16,15 g
	L-serine	19 g	22,8 g
L-glutamate stock solution (1L)	L-glutamate	100 g	100g
L-cysteine (HCl) stock solution (1L)	L-cysteine (HCl)	n/a	50 g

Supplementary Table 2.4 - Nucleotide sequence of primers used for qRT-PCR.

Primer	Primer sequence
<i>Henna Fwd</i>	5' ACAACCACGTCTTTCCCTTG 3'
<i>Henna Rev</i>	5' GCAGAGTGAAACCTGTGCAA 3'
<i>Actin42A Fwd</i>	5' CAGGCGGTGCTTTCTCTCTA 3'
<i>Actin42A Rev</i>	5' AGCTGTAACCGCGCTCAGTA 3'
<i>RpL32 Fwd</i>	5' GCCCAAGATCGTGAAGAAGC 3'
<i>RpL32 Rev</i>	5' GCACTCTGTTGTCGATACCCTTG 3'

CHAPTER 3

MARMITE *ENCODES* *A* *NEW*
NEUROPEPTIDE *MODULATING*
PROTEIN INTAKE IN DROSOPHILA

Note: Part of the material in this chapter is included in a preprint available in an open access repository:

Francisco AP, Tastekin I, Fernandes AB, Ezra-Nevo G, Deplancke B, Oliveira-Maia AJ, Gontijo AM & Ribeiro C. (2022). *marmite* defines a novel conserved neuropeptide family mediating nutritional homeostasis (p. 2022.12.12.520095). *bioRxiv*.
<https://doi.org/10.1101/2022.12.12.520095>

Author Contributions: **APF** performed all experiments, analyzed data and interpreted the results. RNAseq samples were collected by **APF**, APE, CB, MA and ZCS, processed by BD and analyzed by CR and GE-N. IB assisted with the acquisition, processing and analysis of the imaging data. DG analyzed the raw data and assisted with video-tracking experiments. **APF** and CR conceived and designed the project.

3.1 - SUMMARY

Nutritional homeostasis is essential for an animal's survival and wellbeing. While it is known that animals modify their food choices to respond to the lack of specific nutrients, the molecular mechanisms mediating nutrient-specific appetites remain unclear. To understand how the activity of genes in the nervous system is modulated by diet, we performed RNAseq on brains of flies kept on different amino acid diets. Using this approach, we identified the gene *CG14075*, later named *marmite* (*mnt*), which is predicted to encode a secreted peptide with an unknown function in *Drosophila melanogaster*. *mnt* expression is higher in animals maintained on amino acid rich food. To characterize whether *mnt* is involved in feeding decisions, we used the flyPAD technology, a capacitance-based setup that enables a quantitative feeding analysis, together with CRISPR-Cas9-based genome engineering techniques. Both *mnt* mutants and neuronal knockdown of *mnt* increased the intake of proteinaceous food, whereas neuronal *mnt* overexpression reduced protein appetite, suggesting that *mnt* encodes a protein-specific satiety signal. Marmite (Mnt) is expressed in a small number of neurons in the adult nervous system and activation of these neurons is sufficient to reduce yeast feeding in a *mnt*-dependent manner. Moreover, a single pair of *mnt*-expressing neurons seems to be necessary and sufficient to modulate protein appetite. Thus, we have identified a novel neuropeptide that plays a key role in nutritional homeostasis and protein appetite in *Drosophila*.

3.2 - INTRODUCTION

A balanced diet is key for an animal's health and wellbeing (Leopold & Perrimon, 2007; Münch et al., 2020; Simpson et al., 2015). Choices such as which food to eat or how much to ingest have a great impact on the overall fitness of animals. Macronutrients such as proteins (amino acids), lipids and carbohydrates are particularly important for building tissues and energy balance. While protein is especially important for reproduction, its over ingestion is known to reduce lifespan and negatively impact health (M. E. Levine et al., 2014; Piper et al., 2011; Solon-Biet et al., 2014). Therefore, the intake of this macronutrient must be tightly regulated and animals, including humans, are able to direct their food choices to compensate for the lack or over ingestion of protein. In the previous chapter we demonstrated that dietary amino acids (AAs), as building blocks of proteins, are potent modulators of protein appetite, especially essential amino acids (eAAs), which can only be acquired through the diet. A main open question is what are the molecular and neuronal mechanisms involved in translating the information of a deficit in eAAs into behavioral changes that promote protein intake.

Neuropeptides and peptide hormones are important signaling molecules involved in almost all aspects of an animal's life, from physiology to development and behavior (Andermann & Lowell, 2017; Nässel & Zandawala, 2019; L. Schoofs et al., 2017). They play a key role in the dynamic adaptation that animals have to perform in order to adjust to multiple environmental challenges. Given their essential and highly specialized role, peptide hormones are thought to be both ancient and fast evolving (Elphick et al., 2018). Together with their small size, it is however difficult to identify them using classic bioinformatics or

experimental approaches, such as proteomics (DeLaney et al., 2018). Several neuropeptides and peptide hormones have been shown to regulate different aspects of the feeding process in different species, from flies to vertebrates (Andermann & Lowell, 2017; S. Lin et al., 2019). Although there are some examples of nutrient-specific effects, most described neuropeptides mediate general hunger and satiety states, which promote or reduce feeding, respectively. Due to the challenges in identifying these molecules, it is likely that new neuropeptides and peptide hormones remain to be discovered.

3.3 - RESULTS

3.3.1 - Identification of an uncharacterized gene modulated by AA state

In the previous chapter it was shown that eAA deprivation leads to a potent preference for yeast and more specifically, AAs (Figure 2.3). The main question arising from this change in food preference was which neuronal and molecular mechanisms could be underlying this behavioral switch. We therefore decided to pinpoint which genes could be mediating this effect by examining the transcriptional profile of flies kept on different AA states. Since the brain is the critical organ responsible for generating a change in behavior, we performed RNA sequencing (RNAseq) using dissected brains in order to probe differentially expressed neuronal genes. Deprivation of any single eAA was sufficient to drive a strong yeast appetite (Figure 2.5), so we decided to use isoleucine-deprived (-Ile) flies and compare their transcriptional profile with the one of flies kept on a complete holidic medium.

The transcriptomic analysis of -Ile flies revealed several differentially expressed genes, either up- or downregulated compared to fully fed flies (Figure 3.1a). From the most differentially expressed genes, we decided to focus on *CG14075*, whose expression was downregulated upon isoleucine deprivation (Figure 3.1b).

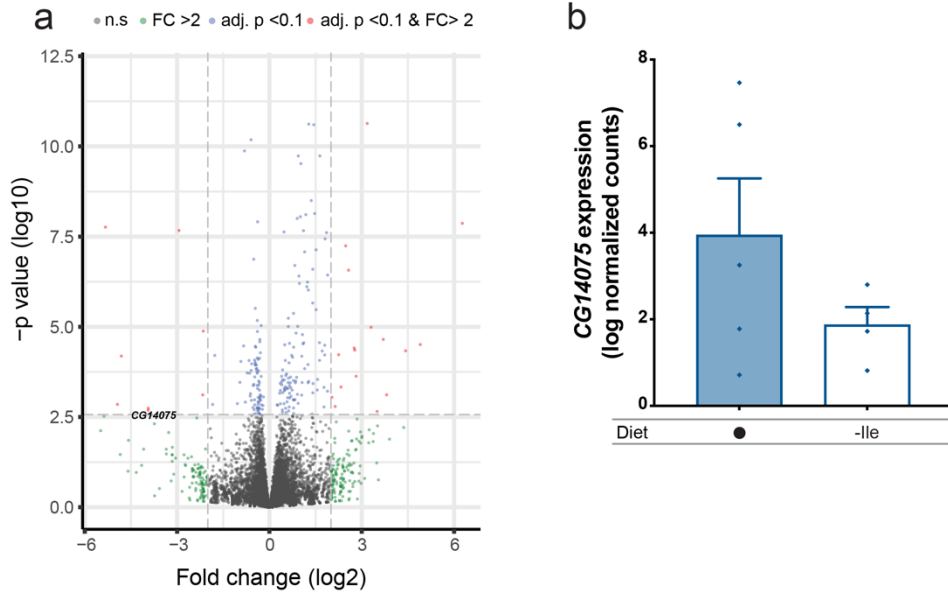


Figure 3.1 - Differentially expressed genes between flies kept on holidic medium with and without isoleucine. (a) Plot representing the p-value and fold change of all genes analyzed. Each data point is a gene. *CG14075* is labelled. Genes with negative or positive fold change are down or upregulated in -Ile, respectively. **(b)** Log normalized counts of *CG14075* in full diet (black filled circle) and upon isoleucine deprivation (-Ile) conditions. $n = 4-5$ biological replicates.

The function of *CG14075* has not been previously characterized, but according to *flybase* and single-cell RNAseq datasets (Gramates et al., 2022; Li et al., 2022) it is a neuronally expressed gene. Moreover, it is predicted to encode a small protein of 155 amino acids with no known or predicted domains. Sequence analyses (see Materials & Methods) revealed a predicted signal peptide (SP) in the amino (N)-terminus and polybasic sites towards the central part of the protein (Figure 3.2a). Both are stereotypical features of neuropeptide precursors: the SP directs the peptide to the secretory pathway, while prohormone convertases typically recognize the identified basic residue motifs for proteolytic processing (Nässel & Zandawala, 2019; Pauls et al., 2014). These features highly

suggest that *CG14075* encodes an uncharacterized peptide, which given its expression in the nervous system could act as a novel neuropeptide. We validated the RNAseq results using quantitative RT-PCR and observed a strong reduction in the expression of *CG14075* upon isoleucine deprivation (Figure 3.2b).

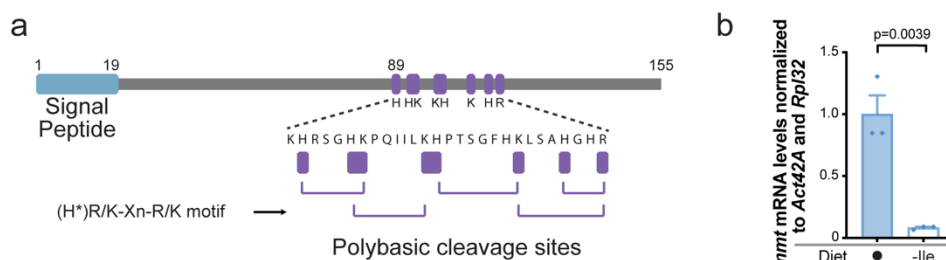


Figure 3.2 - *CG14075* predicted features and mRNA expression. (a) Scheme depicting the 155 amino acid sequence of *CG14075*/*Mmt* with predicted motifs: signal peptide in the N-terminus and polybasic residues as potential targets for protein processing. The cleavage sites predicted by the motif (H*)R/K-Xn-R/K are shown, where X is any amino acid, n is 0, 2, 4, or 6 amino acids, and an H* denotes that histidine is accepted when n is 2, 4, or 6. The sequence and the size of the different motifs are represented to scale. The numbers on top indicate the AA position. (b) *mtt* mRNA levels measured from heads of flies maintained on complete holidic medium or holidic medium lacking Isoleucine (-Ile) as normalized using the expression levels of two control genes (*Actin 42A* and *RpL32*); columns represent the mean and the error bars the standard error of the mean. Significance was tested using the unpaired *t*-test, *n* = 3.

3.3.2 – *CG14075*/*mtt* knock-out flies increase yeast but not sucrose appetite

Since the expression of this gene is regulated by nutritional state, we then questioned whether *CG14075* could play a role in feeding decisions and nutritional homeostasis. To address this, we devised a loss-

of-function approach by generating a knock-out allele using CRISPR-Cas9-based genome engineering (Supplementary Figure 3.1) (see also Materials & Methods). More specifically, we used two single guide RNAs (sgRNAs) to generate a double cut in the genome leading to the deletion of the whole coding sequence of *CG14075*. A genetic cassette with an attP landing site was introduced by homologous recombination, allowing for later insertions of transgenes into the *CG14075* locus.

Given the nutritional regulation of its expression, we decided to test the role of *CG14075* in feeding behavior using the flyPAD technology, a capacitance-based setup for testing independent changes in protein and carbohydrate feeding (Itskov et al., 2014). When given a choice to eat from a protein (yeast) or carbohydrate (sucrose) food source, fully fed *CG14075* mutant female flies showed a significant increase in protein feeding when compared to control animals (Figure 3.3a), while sucrose feeding was not affected (Figure 3.3b). This phenotype was confirmed using a video-tracking foraging setup allowing us to quantify the feeding behavior of flies in an arena containing protein (yeast) and carbohydrate (sucrose) food spots (Corrales-Carvajal et al., 2016). Similarly to what we had observed using the flyPAD system, over the duration of the foraging assay, *CG14075* mutants showed a longer cumulative duration of micromovements (a proxy for feeding behavior) on yeast spots compared to controls (Figure 3.3c). Furthermore, we could not observe a phenotype for sucrose spots (Figure 3.3d). These results suggested that *CG14075* encodes a protein-specific satiety factor, whose loss leads to overconsumption of proteinaceous food. Given this phenotype we decided to name this gene *marmite* (*mmt*), referring to the yeast extract spread appreciated in some cultures. Our findings prompted us to further investigate the role of this gene, specifically in protein homeostasis.

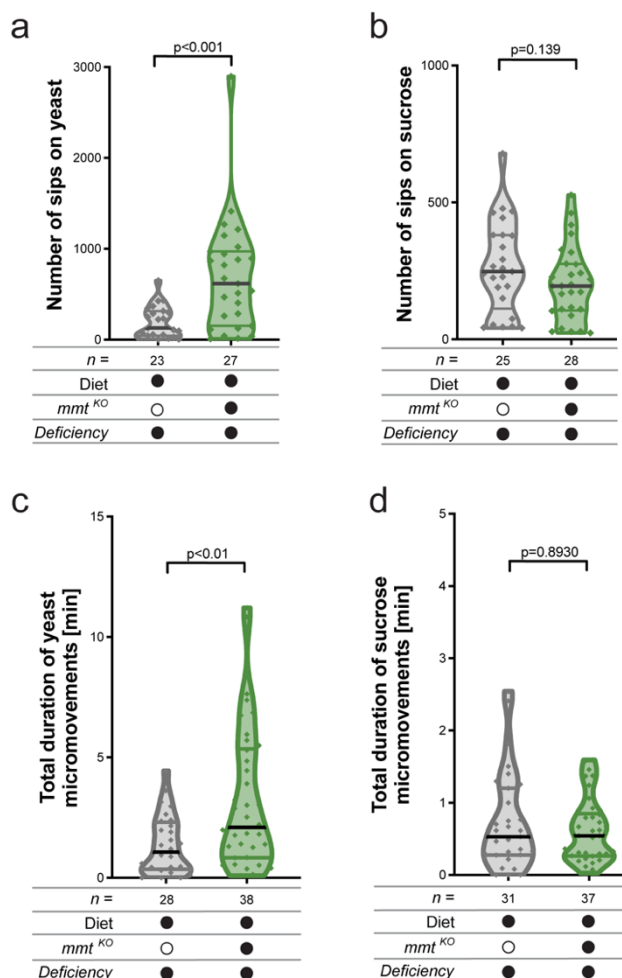


Figure 3.3 - *CG14075/mmt* mutants specifically increase protein but not sucrose appetite. Number of sips on yeast (**a**) and sucrose (**b**) of *mmt* mutant or control flies maintained on complete holidic medium. Total duration of yeast (**c**) and sucrose (**d**) micromovements of *mmt* mutant and control flies maintained on complete holidic medium, using a video-tracking setup. (**a-d**) “Deficiency” denotes a genetic deficiency spanning the *mmt* gene. In this and next figures, below the graphs, filled black circles represent the complete holidic medium or presence of a specific transgene, whereas empty black circles represent its absence; violin plots show the frequency distribution of the data, with lines representing the median, upper and lower quartiles; each point corresponds to a fly. Significance was tested using the Mann-Whitney test. The number of flies used in each condition is shown in each panel.

3.3.3 – Loss of *mnt* does not seem to affect physiological traits

Due to the effect of *mnt* mutants on food intake, we hypothesized that physiological aspects directly linked to the nutritional state of the animal could also be affected. Protein is a crucial nutrient for egg production (Carvalho-Santos et al., 2020; Drummond-Barbosa & Spradling, 2001). Therefore, we decided to test egg production and viability in control and *mnt* mutant females. Since mutant females increase their protein intake, we hypothesized that the increase would be translated into an increase in egg production. However, we could not observe any difference in either egg production or viability in *mnt* mutant females compared to control flies (Figure 3.4a and b).

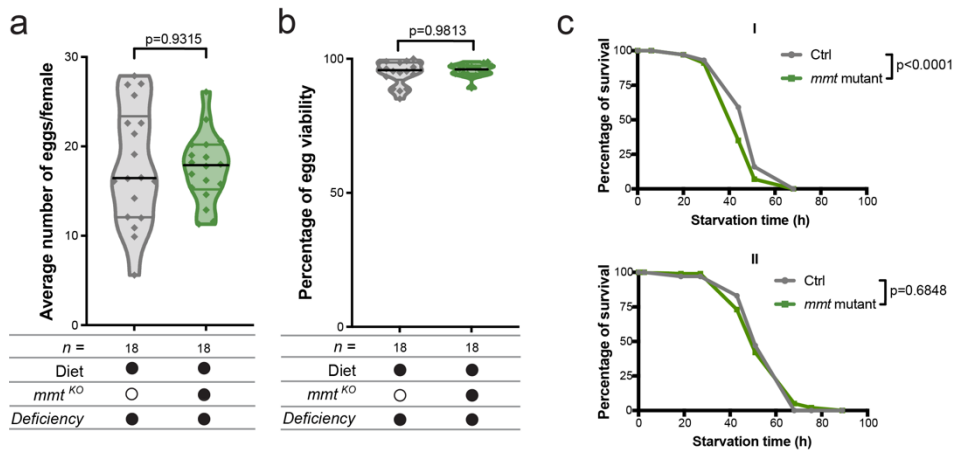


Figure 3.4 - Reproduction and starvation resistance are not affected in *mnt* mutants.

(a) Average number of eggs laid after 24h of *mnt* mutant and control flies maintained on complete holidic medium. **(b)** Percentage of viable eggs assessed after 48h of control and *mnt* mutant flies. **(a-b)** Significance was tested using the Mann-Whitney test. The number of flies used in each condition is shown in each panel. **(c)** Starvation curves of *mnt* mutant females (green) and control flies (grey) in two experiments (I and II). Significance was tested with Log-rank test (Mantel-Cox). $n = 100$.

We also investigated whether the increase in protein intake seen in *mnt* mutants could affect their internal nutrient storage and have an impact in the resistance to starvation. We kept single flies in tubes with water-soaked papers and scored their survival approximately every 12h. The effect of *mnt* loss on starvation was ambiguous, since in a first experiment *mnt* mutant flies survived less, but this effect was not observed in a second experiment (Figure 3.4c). These results suggested that *mnt* plays a specific role in feeding decisions and has no clear effect on reproductive output or starvation resistance.

3.3.4 – Neuronal expression of *mnt* is necessary and sufficient to alter protein appetite

Neuropeptides are neuronally expressed peptides that play key roles in regulating behavior and physiology. The presence of a predicted SP and potential cleavage sites suggest that *mnt* encodes a secreted peptide and according to available transcriptomic data, *mnt* is expressed in the nervous system, indicating that it could function as a neuropeptide. To test this hypothesis, we assessed if neuronal-specific knockdown of *mnt* was sufficient to phenocopy the protein appetite effect of the *mnt* knock-out. Indeed, knocking down *mnt* specifically in the nervous system led to a strong increase in the number of sips on yeast, strengthening the idea that it encodes a neuropeptide regulating protein appetite (Figure 3.5a and Supplementary Figure 3.2).

If *mnt* encodes a neuropeptide mediating protein-specific satiety, overexpressing it should reduce protein appetite. This effect should be more prominent in females deprived of an eAA as these show a high

protein appetite as seen in the previous chapter. Indeed, neuronal overexpression of *mmt* in isoleucine-deprived females resulted in a significant decrease in the number of sips on yeast when compared to deprived control females (Figure 3.5b).

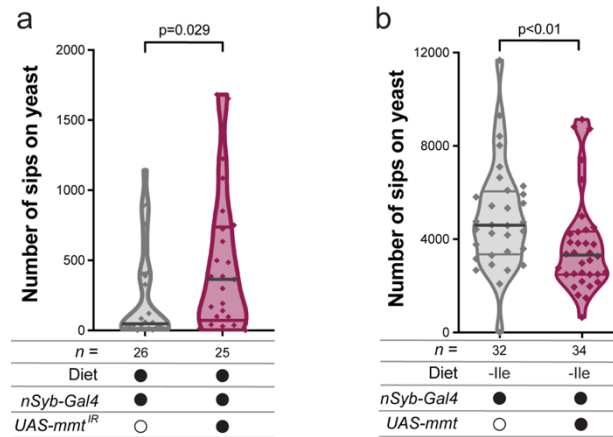


Figure 3.5 - *mmt* is necessary and sufficient to alter protein appetite. (a) Number of sips on yeast of control and *marmite* pan-neuronal knockdown flies maintained on complete holidic medium. (b) Number of sips on yeast of control and flies overexpressing *mmt* using a pan-neuronal driver maintained on holidic medium lacking Isoleucine (-Ile). (a-b) Significance was tested using the Mann-Whitney test. The number of flies used in each condition is shown in each panel.

Our data lent further support to the idea that Marmite could act as a satiety signal dedicated to specifically reducing protein intake. Moreover, the higher expression of *mmt* in fully fed flies (Figure 3.1 and 3.2b) corroborates its putative role in linking AA state to protein satiety. Overall, these results supported the idea that *mmt* encodes a neuropeptide whose expression is regulated by the animal's AA state to alter protein appetite according to its nutritional needs.

3.3.5 – Marmite is sparsely expressed in the nervous system

We have shown that neuronal *mnt* knockdown and overexpression are sufficient to alter protein feeding. Neuropeptides are usually expressed by specific, specialized, and sparsely distributed neurosecretory neurons (Nässel & Zandawala, 2019). To identify the neurons expressing the Mnt peptide, we first used a custom-generated polyclonal antibody to visualize the distribution of the peptide in the nervous system (see Materials & Methods). Mnt was sparsely expressed in the brain labeling around 28 cell bodies (Figure 3.6a). Cell bodies could be found in the Subesophageal Zone (SEZ), the Posterior Ventrolateral Protocerebrum (PVP), the Lateral Horn (LH), and the Superior Medial Protocerebrum (SMP). Mnt was also expressed in 8 neurons in the Ventral Nerve Cord (VNC) (Figure 3.6c). Importantly, in *mnt* mutant animals most of the staining was abolished, validating both the loss-of-function nature of the mutant as well as the high specificity of the antibody (Figure 3.6b and d).

Additionally, to gain genetic access to the neurons expressing *mnt*, we took advantage of the landing site inserted into the *mnt* locus in the null allele (Supplementary Figure 3.1) to generate a knock-in allele with a *Gal4* inserted in the locus of the *mnt* gene (*mnt-Gal4*) (Supplementary Figure 3.3). The expression of the *Gal4* should therefore mimic the endogenous expression of *mnt*. Accordingly, mCD8::GFP driven by *mnt-Gal4* was expressed in neurons in the brain (Fig. 3.7a) and the VNC (Figure 3.7b).

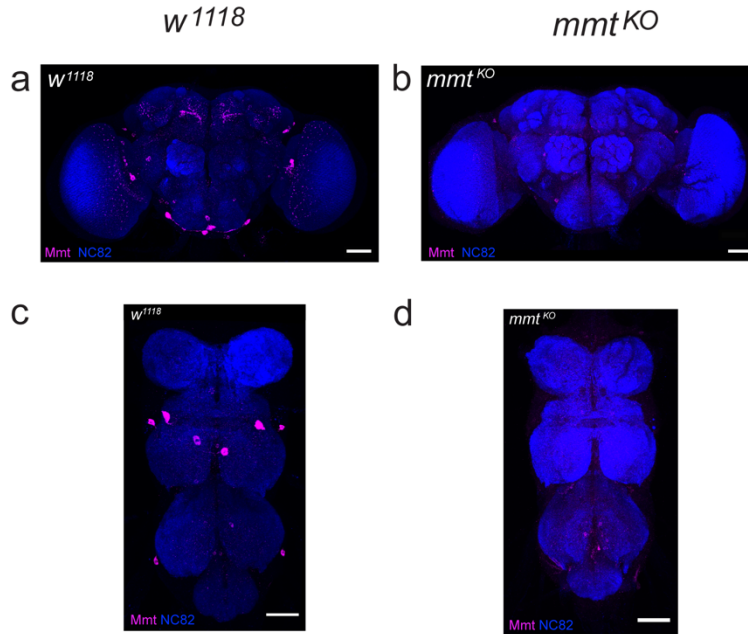


Figure 3.6 - Expression of Mmt antibody in the brain and VNC. Mmt antibody staining of brains of (a) control and (b) *mmt* mutant flies in magenta, with nc82 neuropile staining in blue. Brains are oriented with the dorsal side on top and the ventral side at the bottom. Mmt antibody staining of VNC of control (c) and *mmt* mutant (d) flies in magenta, with nc82 neuropile staining in blue. VNCs are oriented with the anterior side on top and the posterior side at the bottom. (a-d) Maximum projection images of confocal stacks; scale bars correspond to 50µm.

To validate that the *mmt-Gal4* line faithfully recapitulates endogenous *mmt* expression, we visualized the Mmt peptide expression in brains of flies in which *mmt-Gal4* drove mCD8::GFP expression. Quantification of 9 brains revealed that the large majority of neurons (23 ± 2) expressed both mCD8::GFP and Mmt, with a minority (5 ± 1) expressing only the peptide and almost none being labeled by the *Gal4* line and not the antibody (2 ± 1) (Figure 3.7c and Supplementary Figure 3.4).

One of the most prominent labeled neurons was a pair of neurons with large somata in the medial part of the brain, which we named Medial

Marmite Neurons (MMNs) (Figure 3.7a and d). Upon close inspection, we could observe strong Mmt labeling in varicosity-like structures in the projections of these neurons (Fig. 3.7d), compatible with Mmt being packed in vesicles.

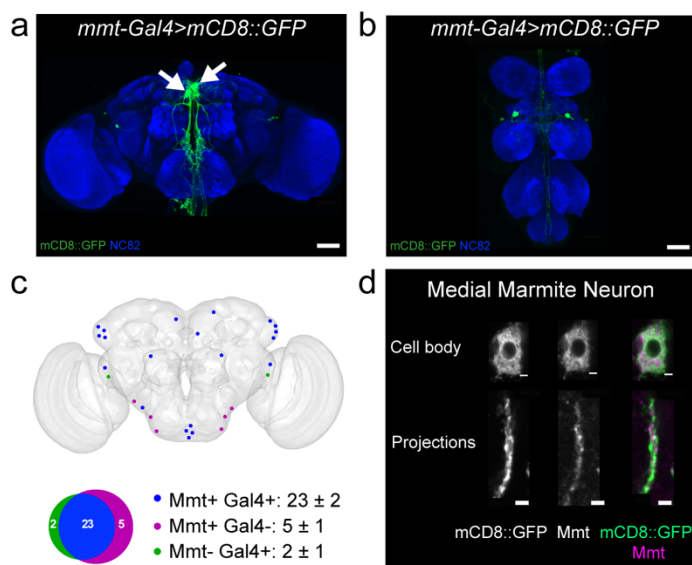


Figure 3.7 - Mmt is sparsely expressed in the nervous system. Expression pattern of *mmt-Gal4* in the brain (a) and VNC (b) as visualized using *UAS-mCD8::GFP* in green, with *nc82* neuropile staining in blue. Arrows mark the Medial Marmite Neurons (MMNs). (c) Schematic of a representative brain with the location of the soma of neurons showing labeling by both Mmt antibody and *mmt-Gal4* (Mmt + Gal4 +, blue), Mmt antibody only (Mmt + Gal4 -, magenta), or *mmt-Gal4* only (Mmt- Gal4 +, green) as well as a Venn diagram depicting the overlap of these populations. Mean ± SEM for each combination is indicated, n=9. (d) Single plane of confocal images of the cell body and projections of the MMNs showing *mCD8::GFP* and Mmt labeling; scale bar corresponds to 2µm. (a-b) Brain is oriented with the dorsal side on top and the ventral side at the bottom. VNCs are oriented with the anterior side on top and the posterior side at the bottom. Maximum projection images of confocal stacks; scale bars correspond to 50µm.

Together, our data shows that Mmt is sparsely expressed in the central nervous system of the fly. In general, Mmt neurons have large cell bodies, which are a hallmark of neurosecretory cells. Given that we also observed Mmt labeling in varicosity-like structures within neuronal projections, our neuroanatomical analysis further supports that *mmt* encodes a secreted neuropeptide.

3.3.6 – *mmt*-expressing neurons modulate protein feeding

Given the function of Mmt, we hypothesized that changing the activity of neurons expressing the peptide could also lead to specific changes in feeding. We therefore took advantage of *mmt-Gal4* to activate these peptidergic neurons using the temperature-sensitive cation channel TrpA1 (Hamada et al., 2008). Since we expected that this would lead to a reduction in protein feeding, we decided to do this manipulation in isoleucine-deprived animals. Consistent with previous results using *mmt* overexpression (Figure 3.5b), activation of *mmt*-expressing neurons led to a decrease in the number of sips on yeast when compared to the controls (Figure 3.8a). One possibility is that the neuronal activation effect on feeding is due to an increase in Marmite release in response to neuronal activation. To test this hypothesis, we compared the effect of the neuronal activation experiment in a *mmt* expressing (while still being hemizygous as *mmt-Gal4* is a *mmt* null allele) and *mmt* null background. Importantly, *mmt* null mutants showed an increase in yeast feeding when compared to hemizygous controls, confirming the reproducibility of the *mmt* phenotype in a different genetic background and nutritional condition (Figure 3.8b and c). This effect was even clearer when focusing on the top half of the

distribution (high eaters) (Figure 3.8c). This fits the rationale that flies with a low level of feeding would be less sensitive to the manipulation as they would already be subjected to a satiation signal. Importantly, whereas activation of *mmt-Gal4* in a control condition led to a significant decrease in yeast intake, the same activation in an *mmt* mutant background had no significant effect (Figure 3.8b and c). These data show that the reduction in protein feeding observed upon activating *mmt* neurons is dependent on Mmt.

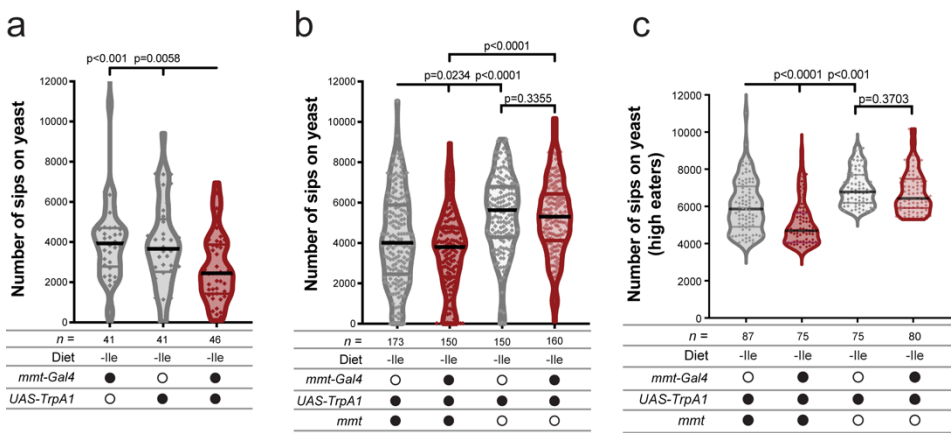


Figure 3.8 -Activation of *mmt*-expressing neurons reduces protein feeding in a *mmt*-dependent manner.

(a) Number of sips on yeast of flies in which *mmt-Gal4* neurons were thermogenetically activated using TrpA1 and corresponding controls. Full **(b)** or top half **(c)** distribution of the number of sips on yeast of flies in which *mmt-Gal4* neurons were thermogenetically activated using TrpA1 in a context of *mmt* presence or absence and corresponding controls. **(a-c)** Flies were maintained on holdic medium lacking Isoleucine (-Ile). Significance was tested using the Mann-Whitney test. The number of flies used in each condition is shown in each panel.

In this working model, we would expect that silencing *mnt*-expressing neurons could block the release of Mmt, mimicking the effect of the *mnt* loss-of-function experiments shown before. We therefore decided to use Kir2.1, an inward rectifying potassium channel that hyperpolarizes the neurons (Nitabach et al., 2002). However, silencing of *mnt-Gal4* did not change protein feeding (Figure 3.9).

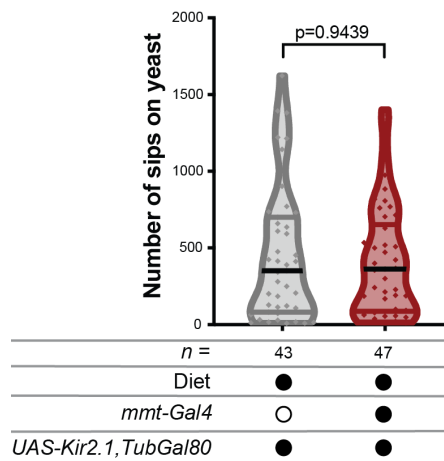


Figure 3.9 - Silencing of *mnt*-expressing neurons has no effect on protein feeding. Number of sips on yeast of flies in which *mnt-Gal4* neurons were silenced and corresponding control maintained on complete holidic medium. Significance was tested using the Mann-Whitney test. The number of flies used in each condition is shown in the panel.

3.3.7 – A single pair of *mmt*-expressing neurons mediates protein appetite.

We have shown that *mmt-Gal4* labels 25 neurons in the brain (Figure 3.7a and c). For multiple neuropeptides and neuromodulators, careful neurogenetic dissection has shown that it is possible to attribute specific behavioral functions to subsets of neurons (Q. Liu et al., 2017; Waddell, 2013). We therefore set out to test if we could identify a subset of *mmt*-expressing neurons mediating the yeast satiety effect. We identified a driver line from the Vienna Tile (VT) collection (VT013947) (Tirian & Dickson, 2017) whose expression pattern includes a pair of neurons that resembled the Medial Marmite neurons (Figure 3.7a and 3.10a). Co-staining experiments with Marmite antibody showed that the middle neurons are indeed Marmite positive, and that no other neurons labeled by this *Gal4* line expressed Marmite (Figure 3.10b and Supplementary Figure 3.5). This finding strongly suggests that the medial neurons labeled by this *Gal4* line are the Medial Marmite Neurons.

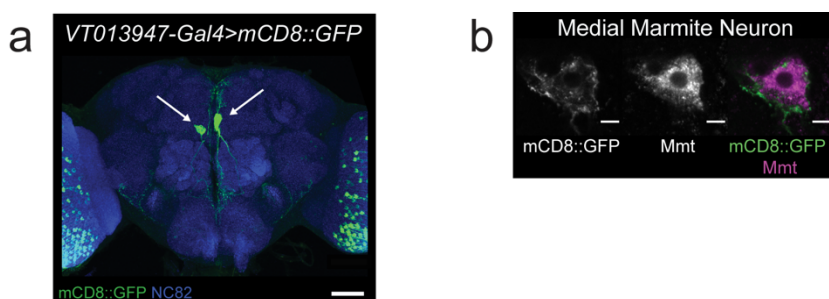


Figure 3.10 - VT013947-Gal4 labels the pair of Medial Marmite neurons. (a) Expression pattern of VT013947-Gal4 in the brain visualized using *UAS-mCD8::GFP* in green, with nc82 neuropile staining in blue; arrows highlight MMNs; maximum projection of confocal images, scale bar: 50µm. (b) single plane of one of the medial cell bodies showing mCD8::GFP and Mmt labelling; scale bars: 5µm. Brain and soma are oriented with the dorsal side on top and the ventral side at the bottom.

Acute thermogenetic activation using *VT013947-Gal4* reduced yeast feeding (Figure 3.11a and b). As observed earlier, the activation effect is stronger in animals with a higher level of yeast feeding (high eaters). To validate that the pair of middle neurons controlled yeast feeding via Mmt, we knocked down *mmt* expression using RNAi driven by *VT013947-Gal4* and tested if this recapitulates the increase in yeast feeding observed upon pan-neuronal *mmt* knockdown (Figure 3.5a). Indeed, loss of *mmt* function specifically in these neurons was sufficient to increase yeast appetite (Figure 3.11c), consistent with the idea that Mmt suppresses yeast feeding via the Medial Marmite Neurons.

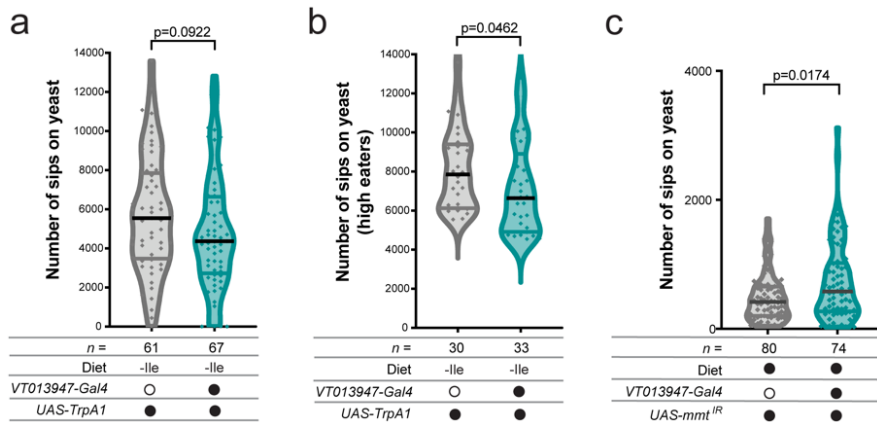


Figure 3.11 - Activation of *VT013947-Gal4* reduces feeding and loss of *mmt* specifically in these neurons is sufficient to increase protein feeding. Full (a) or top half (b) distribution of the number of sips on yeast of flies in which *VT013947-Gal4* neurons were thermogenetically activated using TrpA1 and corresponding control. Flies were maintained on holidic medium lacking Isoleucine (-Ile). (c) Number of sips on yeast of flies in which *mmt* was knocked down in *VT013947-Gal4* and control flies. Flies were maintained on complete holidic medium. Significance was tested using the Mann-Whitney test. The number of flies used in each condition is shown in each panel.

Additionally, silencing of the neuronal population labeled by *VT013947-Gal4* using Kir2.1, led to a significant increase in yeast appetite (Figure 3.12a). This is in contrast with the lack of effect observed upon silencing of the whole population labeled by *mmt-Gal4*. This apparent discrepancy could be explained by distinctive subsets of *mmt*-expressing neurons having different neuronal functions, that could mask the effect of silencing the pair of Medial Marmite neurons. This result also mimicked the effect of the *mmt* loss-of-function experiments and can be explained by a possible block of Mmt release due to the hyperpolarization of the neurons. This neuronal silencing effect observed using the flyPAD system was also supported by a significant increase observed in the total duration of yeast micromovements using the video-tracking setup (Figure 3.12b).

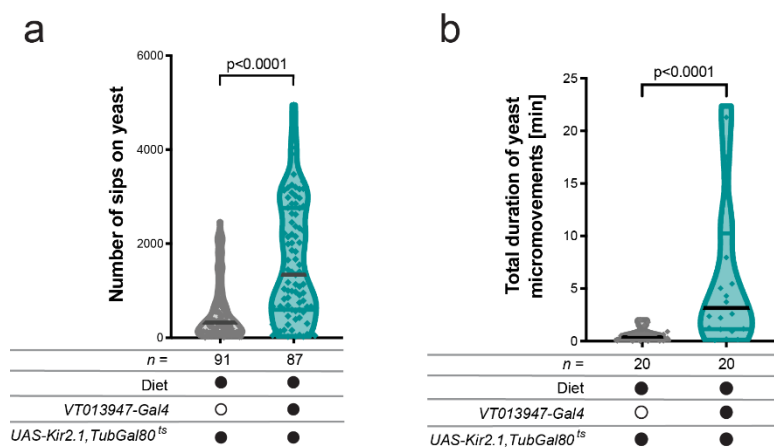


Figure 3.12 - Silencing of *VT013947-Gal4* increases protein appetite. Number of sips on yeast (**a**) and total duration of yeast micromovements (**b**) of flies in which *VT013947-Gal4* neurons were inducibly silenced using Kir2.1 and corresponding control. Flies were maintained on complete holidic medium. Significance was tested using the Mann-Whitney test. The number of flies used in each condition is shown in each panel.

We also used intersectional genetics to generate a *split-Gal4* which exclusively labels the pair of Medial Marmite neurons, named *mmt-m-Gal4* (Figure 3.13a). Co-staining experiments confirmed that these neurons are indeed Mmt-positive (Figure 3.13a). Specific silencing of this pair of neurons also led to an increase in yeast intake, corroborating our earlier results (Figure 3.13b and c).

Altogether, these results showed that activity of *mmt*-expressing neurons controls protein feeding in a Mmt-dependent manner and that this effect can be attributed to a single pair of Mmt-positive neurons.

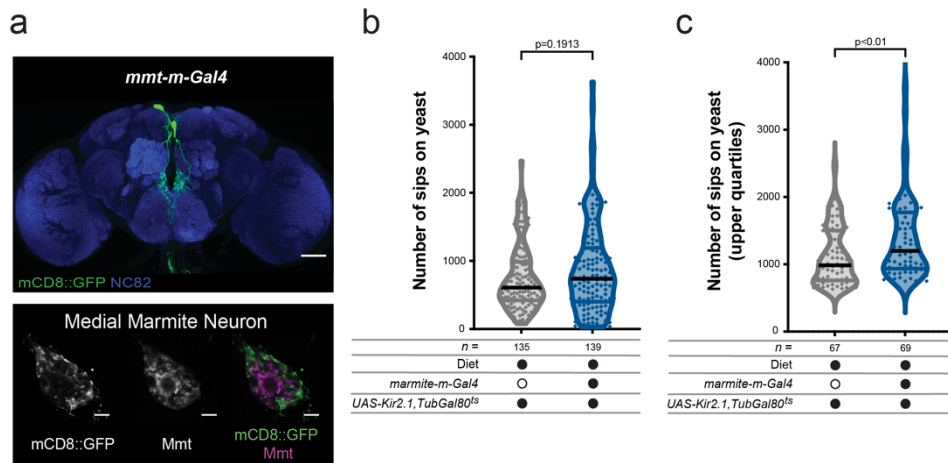


Figure 3.13 - Specific silencing of the pair of Medial Marmite neurons increase protein appetite. (a) Top: expression pattern of *mmt-m-Gal4* in the brain as visualized using UAS-mCD8::GFP in green, with nc82 neuropile staining in blue. Bottom: single plane of one of the cell bodies stained with Mmt antibody and mCD8::GFP. Total data points (b) or only the top half (c) distribution of the number of sips on yeast in which *mmt-m-Gal4* neurons were acutely silenced using Kir 2.1 and corresponding control. Flies were maintained on complete holidic medium. (b-c) Significance was tested using the Mann-Whitney test. The number of flies used in each condition is shown in each panel.

3.4 – DISCUSSION

This chapter described the function of *CG14075*, a previously uncharacterized gene in *Drosophila melanogaster*, named *marmite*, involved in nutritional homeostasis and protein appetite.

3.4.1 – *mnt* is a previously uncharacterized gene modulated by AA state

Animals do not always produce the same behavior when exposed to the same stimuli. This can be partially attributed to alterations in the physiological condition of the animal, or internal state, which are not fixed but highly dynamic. This general internal state can comprise a variety of specific states, denoting different aspects of the animal's physiology. One of these states is the nutritional state, which represents the current metabolic status of the animal and its nutritional requirements. Animals, including flies, adapt their feeding decisions in order to compensate for or anticipate the lack of a specific nutrient (Ribeiro & Dickson, 2010; Steck et al., 2018; Walker et al., 2015). In the previous chapter, we demonstrated that the lack of a single eAA was sufficient to drive a potent appetite for protein. However, how is the animal capable of translating this need for a specific nutrient into behavioral responses in order to achieve a homeostatic state again? What are the molecular changes within the brain modulating this behavioral change? To tackle this question, we decided to investigate the transcriptional profile of animals in different amino acid states. Moreover, being the brain the key organ to modulate behavior, dissected brains were used to probe the profile of neuronally expressed genes. RNAseq analysis revealed a set of genes either up- or

downregulated upon Ile deprivation and we focused on a previously uncharacterized gene, *CG14075*. Sequence analysis showed a predicted signal peptide and several basic motifs, as potential cleavage sites, both features of secreted peptides (Nässel & Zandawala, 2019). According to expression databases in *flybase* and single cell RNAseq datasets (Li et al., 2022), *CG14075* is mostly expressed in the brain, corroborating our finding as a neuronally-expressed gene. Given these results, *CG14075* became a great candidate to investigate the molecular mechanisms underlying feeding decisions.

3.4.2 – Mmt acts as a protein-specific satiety signal

The nutritional state of an animal is in constant change. The lack of nutrients and energy in the body elicits a general *hunger* state, driving animals to increase foraging and food intake. The opposite also occurs, and *satiety* leads to a cessation of feeding-related behaviors. A variety of neuronal and peripheral signals induced by hunger and satiety states has been described in *Drosophila*, and most of these signals correspond to neuropeptides or peptide hormones (Lin et al., 2019). Hugin, a proposed fly homologue of the mammalian Neuromedin U, acts as a satiety signal, where high *hugin* levels in larvae are correlated to low food-intake and reduced food-seeking behavior (Melcher & Pankratz, 2005). Other examples of satiety factors, such as FIT, MIP, DSK and Upd1 have also been described to reduce food intake (Beshel et al., 2017; Carvalho-Santos et al., 2020; Min et al., 2016; Söderberg et al., 2012; Sun et al., 2017). While most studies focus on general satiety states, few nutrient-specific satiety signals are known (Münch et al., 2020). Our data suggest that *mmt* also functions as a satiety signal, and particularly, a protein-specific satiety

signal. *mmt* mutants overconsume a protein-rich food (yeast), while having no effect on sucrose consumption, thus suggesting that the absence of *mmt* has a specific effect in protein, but not carbohydrate consumption. Using the same rationale, if Mmt acts as a satiety factor, one could expect that its overexpression would promote a reduction in feeding. Indeed, pan neuronal overexpression of *mmt* reduced yeast feeding, even in a context of eAA deprivation, when flies have a strong drive to eat yeast. A third observation supporting this idea is the higher level of *mmt* expression in fully fed flies, compared to Ile-deprived ones. These data open an important research avenue to study the role of a new signal regulating feeding and protein homeostasis. How is AA state information transformed into changes in *mmt* expression? What is the final processed form of Mmt? In which cells is Mmt acting? Future studies identifying the bioactive form of Mmt and to which receptor it is binding, will be crucial to unravel the mechanisms by which Mmt changes behavior. Additionally, animals have several satiety and hunger signals, whether and how Mmt signaling intersects with other known signals to promote protein satiety are still open questions.

3.4.3 – *mmt*-expressing neurons also play a role in protein appetite

Most *Drosophila* neuropeptides are produced in small subsets of neurons or neuroendocrine cells. Peptidergic neurons are very stereotypical, displaying large cell bodies and extended axon ramifications (Nässel & Zandawala, 2019). Visualization of the peptide distribution using a custom-made Mmt antibody and membrane-targeted GFP expression driven by *mmt-Gal4* showed that the peptide is produced in a few neurons within the CNS with large cell bodies and vesicle-like

structures in their projections, supporting the idea that *mnt* encodes a secreted neuropeptide. Like in other neuropeptides involved in food intake, not only the peptide itself plays a role, but the activity of the neuropeptide-producing neurons also mediates changes in feeding. As an example, both *mip* mutants and silencing of MIP-producing neurons increase food intake, whereas activation reduces feeding (Min et al., 2016). A simple explanation for this phenomenon could be that neuronal activation promotes the release of the neuropeptide, while silencing prevents it. Activation of *mnt*-expressing neurons also led to a reduction in feeding and this effect was abolished in a *mnt* mutant background. This shows that while these neurons might express other neuropeptides and neuromodulators, their effect on protein appetite is mediated by Mnt.

Mnt is produced in around 28 neurons in the central brain, and some neuropeptides have been mapped to only a couple of pairs in the brain (Martelli et al., 2017; Terhzaz et al., 2007). Thus, we decided to test if a subset of *mnt*-expressing neurons could be mediating the yeast satiety effect. Activation of an independent driver line (*VT013947-Gal4*) including a pair of *mnt*-expressing neurons resembling the Medial Marmite neurons, reduced feeding and specific *mnt* knockdown using this line led to an increase in yeast intake. Moreover, silencing of *VT013947-Gal4* also increased feeding, emphasizing the role of this pair of neurons in yeast appetite. The identification of MMNs as important mediators of protein-specific satiety will allow a focused dissection and identification of downstream circuits controlling protein appetite. These results also suggest that the other *mnt*-expressing neurons and, therefore, also Mnt, might exert further regulatory roles beyond controlling protein appetite. Careful neurogenetic, behavioral, and physiological analyses should allow for the identification of these roles in future studies.

In sum, we have discovered a novel neuropeptide in *Drosophila* mediating feeding behavior and nutritional homeostasis. Its expression is modulated by AA state and a functional characterization revealed a role as a protein-specific satiety signal. Moreover, our experiments suggest that one pair of neurons is required and sufficient for mediating the protein appetite effect of the neuropeptide (Figure 3.14). These data define a starting point to further investigate the molecular and neuronal circuits underlying the role of Mmt and Mmt-producing neurons in protein appetite and homeostasis.

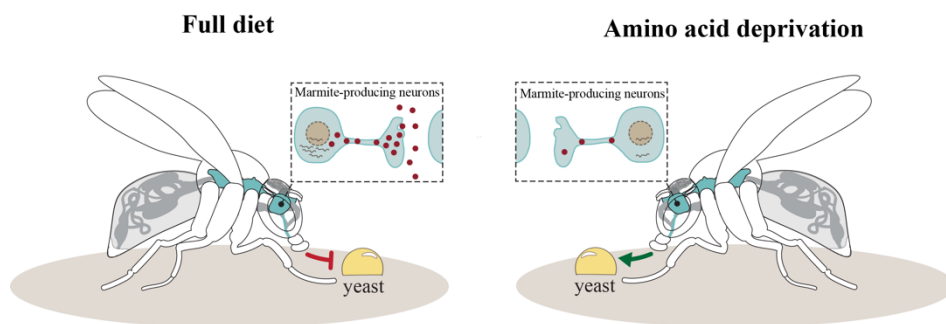


Figure 3.14 – Model depicting Mmt function in protein intake.

3.5 – MATERIALS & METHODS

3.5.1 - *Drosophila* rearing, media, and dietary treatments

Flies were reared on yeast-based medium (YBM) (per liter of water: 8 g agar [NZYTech, PT], 80 g barley malt syrup [Próvida, PT], 22 g sugar beet syrup [Grafschafter, DE], 80 g corn flour [Próvida, PT], 10 g soya flour [A. Centazi, PT], 18 g instant yeast [Saf-instant, Lesaffre], 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]). To ensure a homogenous density of offspring among experiments, fly cultures were always set with 6 females and 3 males per vial and left to lay eggs for 3 d. Flies were reared in YBM until adulthood.

Holidic media (HM) were prepared as described previously (Leitão-Gonçalves et al., 2017) using the exome match formulation with food preservatives (Piper et al., 2017). For Holidic medium -Isoleucine (-Ile), Isoleucine was not added to the diet, keeping the concentration of all other nutrients constant. For all experiments using the HM, the following dietary treatment protocol was used to ensure a fully-fed state: groups of 1±5-d old flies (16 females) were collected into fresh YBM-filled vials, 5 *Canton-S* males were added to the vials to ensure females were mated, and transferred to fresh YBM after 48 h (or 24h in flies kept at 22°C). After 24 h, flies were transferred to different HM for 3 days or 5 days (if flies are kept at 22°C for temperature-sensitive experiments) and immediately tested in the indicated assay. All experiments were performed with female flies. Flies were reared and maintained at 25°C in climate-controlled chambers at 70% relative humidity in a 12-h light-dark cycle (Aralab, FitoClima 60000EH). Behavioral testing was performed at 25°C or 30°C

(for experiments using *UAS-TrpA1*). Polypropylene fly vials (VWR, #734±2261) were used.

3.5.2 - *Drosophila* stocks and genetics

VT013947-Gal4 (VDRC #205650) and *CG14075* RNAi (VDRC #14328) were obtained from the VDRC stock center, and the deficiency spanning *CG14075* (*w¹¹¹⁸*; *Df(3L) BSC220/TM6C, Sb^l, cu^l* - BDSC #9697) was obtained from BSDC. Other stocks and the genotypes of flies used in this study are listed in Supplementary Table 3.1.

3.5.2.1 - Generation of *mnt* knock-out and *Gal4* knock-in

To generate both *mnt* knock-out line and the *Gal4* driver line, we adapted protocols from two-step CRISPR- and RMCE-based strategies for genome editing in *Drosophila* (Port & Bullock, 2016; Zhang et al., 2014). To generate the knock-out line (Supplementary Figure 3.1), we first cloned 2 single guide RNAs (sgRNAs) into a pCFD5 plasmid (Addgene #73914): sgRNA1 atcgctccggcagccagcgttcgg (189bp upstream of the start codon) and sgRNA2 tcaatcaggccgcaagtga (26bp downstream of the stop codon). We used the pTV3 (gift from Jean-Paul Vicent Lab) as a vector for the donor DNA, including an attP site (that will be inserted into the locus for later use) and *mCherry* as a selective marker under the 3xP3 promoter driving expression in all photoreceptors. 5' and 3' homology arms of around 1 Kb upstream and downstream of sgRNA1 and sgRNA2, respectively were cloned into this vector for homologous recombination. Both plasmids were injected into *y^l M{nos-Cas9.P}ZH-2A w** embryos (BDSC # 54591). F1 progeny was screened for *mCherry* expression using a fluorescent stereomicroscope, and positive flies were confirmed by PCR (for the absence of *mnt* ORF and against non-specific insertion of the knock-in

cassette) and genome sequencing of the whole locus. We also collected flies without an insertion event to be used as background controls. PCR and sequencing also confirmed these. The obtained stocks were crossed to *w**, *hs-cre*; *Sco*/CyO; *Dr*/TM3 flies to remove the *mCherry* cassette flanked by loxP sites.

To generate a line expressing *Gal4* from the *mnt* locus (Supplementary Figure 3.3), we took advantage of the attP site present in the *mnt* knock-out flies and used an RMCE-based strategy to recombine in a vector via its attB site. This vector contains a *Gal4* sequence and the upstream and downstream sequences to restore the 5' and 3' regions that were removed in the *mnt* knock-out. A *mini-white* gene was also included in the vector as a selective marker. F1 progeny was screened for red eye color, and positive flies were confirmed by PCR and sequencing. We also collected flies without an insertion event to be used as background controls. PCR and sequencing also confirmed these. The obtained stocks were crossed to *w**, *hs-cre*; *Sco*/CyO; *Dr*/TM3 to remove the *mini-white* cassette flanked by loxP sites.

3.5.2.2 - Generation of *UAS-mnt*

UAS-mnt stocks were generated by amplifying the *mnt* ORF from the corresponding cDNA and cloning it into pUASg.attB using the Gateway System (Invitrogen) and injected into embryos of *yw*, *M(eGFP, vas-int, dmRFP)ZH-2A*; *P{CaryP}attP40* flies. Flies without successful insertion of the UAS cassette were used to generate stocks for background controls.

3.5.2.3 - Generation of *mmt-m-Gal4*

The *mmt-m-Gal4* labelling MMNs was generated using the split-Gal4 system, by combination of w^{1118} ; P{VT016839-p65.AD}attP40 (BDSC# 74247) with w^{1118} ; P{VT037488-GAL4.DBD}attP2 (BDSC# 74858).

3.5.3 - flyPAD assays

flyPAD assays were performed as described in (Itskov et al., 2014). For food choice experiments, single flies in different dietary conditions were tested in arenas containing two types of food patches: 10% yeast or 20 mM sucrose, each mixed with 1% agarose.

For all experiments, flies were individually transferred to flyPAD arenas by mouth aspiration and allowed to feed for 1 h at 25°C (or 30°C for TrpA1 experiments), 70% relative humidity. The total number of sips per animal was calculated using previously described flyPAD algorithms (Itskov et al., 2014). Non-eating flies (defined as having fewer than two activity bouts during the assay) were excluded from the analysis.

3.5.4 – Egg laying and viability assays

Egg laying assays were performed as described in (Carvalho-Santos et al., 2020). In brief, groups of 16 female and 5 male flies were briefly anaesthetized using light CO₂ exposure and transferred to apple juice agar plates (per litre: 250 ml apple juice, 19.5 g agar (MB14801, NZYTech), 20 g sugar and 10 ml nipagin (10% in ethanol; 25605.293, VWR)), where they were allowed to lay eggs for 24 h. Flies were then removed and counted, and the number of eggs was assessed. Egg laying was calculated by dividing the number of eggs by the number of living females at the end of the assay. Egg viability was assessed by counting the

number of eclosed eggs after 48h. GraphPad Prism 9 was used for data and statistical analysis.

3.5.5 – Starvation resistance assays

This protocol was performed as previously described (Carvalho-Santos et al., 2020). Groups of 16 1- to 5-day-old females were collected into fresh YBM-filled vials with 5 Canton-S males and transferred to fresh YBM after 48 h to ensure that they were well fed and mated. After 1 d, single flies were transferred to tubes containing water-soaked paper for a total of 100 flies per condition. The number of living flies was scored around every 12 h until all flies were dead and the resulting survival curve was analyzed with Log-rank test (Mantel–Cox) in Prism GraphPad 9.0.

3.5.6 - Video-based tracking assays

Video tracking foraging assays were performed based on previously described protocols (Corrales-Carvajal et al., 2016; Münch et al., 2022). In brief, flies were individually transferred to circular arenas by mouth aspiration and allowed to freely forage for one hour at 25°C, 70% RH, while being video-recorded. Each arena was prepared with 12 equally distributed food spots, six containing 100 mM sucrose and six 10% yeast, each in 1% agarose. The Bonsai framework was used to acquire the data (Lopes et al., 2015), and analysis was done in Python. Based on the validation in Corrales-Carvajal et al. (2016), we used micromovements as a proxy for feeding behavior.

3.5.7 - Total mRNA extraction, RT-PCR, and quantitative real-time PCR

Flies used for mRNA extraction were snap-frozen in dry ice and kept at -80°C until head processing. Behavioral assays were performed in parallel to confirm that sibling flies presented the expected feeding phenotype. Fly heads were separated from other body parts by vortexing the Eppendorf tubes and posteriorly passing the debris through 710-µm and 425-µm sieves (Retsch GmbH). Fly heads were counted before homogenization to ensure that the same number was used for all replicates. mRNA was extracted from heads of flies (three replicates per condition) using the following procedure: samples were ground and homogenized for 20 s (using pestles #Z359947, Sigma) in 100 µl of PureZOL (#732±6890, Bio-Rad). 250 µl of PureZOL was further added and mixed by pipetting and incubated at RT for 10 min. Finally, 350 µl of 100% ethanol was added, and the samples were mixed and transferred to a Zymo column (Direct-zol RNA MicroPrep #R2062, Zymo research). The manufacturer's instructions were followed to purify the mRNA (including DNase treatment), and samples were eluted in 50 µl of distilled RNase/DNase-free water.

The concentration of the total mRNA samples was determined by performing a spectrophotometer scan in the UV region. Total RNA (1 µg) was reverse transcribed (RT) using the iScript Reverse Transcription Supermix for RT-PCR kit (#170±8840 Bio-Rad), following the manufacturer's instructions. Each cDNA sample was amplified using SsoFast EvaGreen Supermix on the CFX96 Real-Time System (Bio-Rad). Briefly, the reaction conditions consisted of 1 µl of cDNA, 1 µl (10 µM) of each primer, 10 µl of supermix, and 7 µl of water. The cycle program consisted of enzyme activation at 95°C for 30 s, 39 cycles of denaturation

at 95°C for 2 s, and annealing and extension for 5 s. The primers used in this reaction are listed in Table 2. This experiment was performed using three experimental replicates and two technical replicates per genotype. Appropriate non-template controls were included in each 96-well PCR reaction, and dissociation analysis was performed at the end of each run to confirm the specificity of the reaction. Absolute RNA levels were calculated from a standard curve and normalized to the internal controls (Actin42A and RpL32). Data processing was performed using Bio-rad CFX Manager 3.1 (Bio-Rad).

3.5.8 - RNA sequencing

w¹¹¹⁸ axenic flies were kept for 3 days either in complete holidic medium or in holidic medium lacking isoleucine. All instruments used for the dissection procedure were thoroughly cleaned using RNase away spray (Mol Biol Products). 10 brains per nutritional condition were dissected on 1X DEPC-treated PBS on an ice-cold pad. Immediately after dissection, brains were snap-frozen on dry ice. Samples were kept at -80°C until further processing for RNA extraction as described above using the Direct-zol RNA MicroPrep #R2062, Zymo research, following the manufacturer's instructions and eluted in 12 µl of distilled RNase/DNase-free water. The concentration of the total mRNA samples was determined by performing a spectrophotometer scan in the UV region. cDNA library preparation and BRB-sequencing was done in the Deplancke Lab (Alpern et al., 2019).

3.5.9 - Immunohistochemistry and image acquisition and analysis

For visualization of expression patterns, males from each *Gal4* line were crossed to females homozygous for the *UAS-CD8::GFP* reporter.

Mated female flies with both the *Gal4* and the UAS reporter were kept on a complete holidic medium 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, and Fiji (Schindelin et al., 2012) was used for imaging analysis. The expression of *mmt-Gal4*, *VT013947-Gal4* and *mmt-m-Gal4* is stochastic and Medial Marmite Neurons are not labelled in all brains.

3.5.10 - Generation of Marmite Antibody

To visualize the localization of Mmt in the nervous system, a custom anti-rabbit polyclonal antibody was generated by the company Eurogentec as a service. In brief, a peptide of 15 amino acids (EVEQDVAGDQSAGRS) between E55 and S69 of the Mmt amino acid sequence was synthesized to be used as an antigen. The service company followed an 87-day immunization protocol and the final serum was purified using an affinity column. Upon optimization, we chose a dilution of 1:1000 to be used as the concentration for histochemistry.

3.5.11 - Sequence Analysis

Signal peptide was predicted using SignalP 6.0 (<https://dtu.biolib.com/SignalP-6>) (Teufel et al., 2022). The general motif (H*)R/K-X_n-R/K, where X is any amino acid, n is 0, 2, 4, or 6 amino acids, and an H* denotes a histidine is accepted when n is 2, 4, or 6, was used to annotate putative protein cleavage sites (Devi, 1991; Hook et al., 2008; Seidah & Chrétien, 1999; Veenstra, 2000).

3.5.12 - Statistical Analysis

All violin plots show the frequency distribution of the data, with lines representing the median, upper and lower quartiles. In all behavioral experiments, each data point corresponds to a fly, and the sample sizes correspond to the number of animals tested. All statistical tests used were two-sided. Normality was tested using the Shapiro-Wilk normality test, and parametric or nonparametric tests were used accordingly. The statistical tests used in each experiment are stated in the figure legends. GraphPad Prism 9 was used for data and statistical analysis.

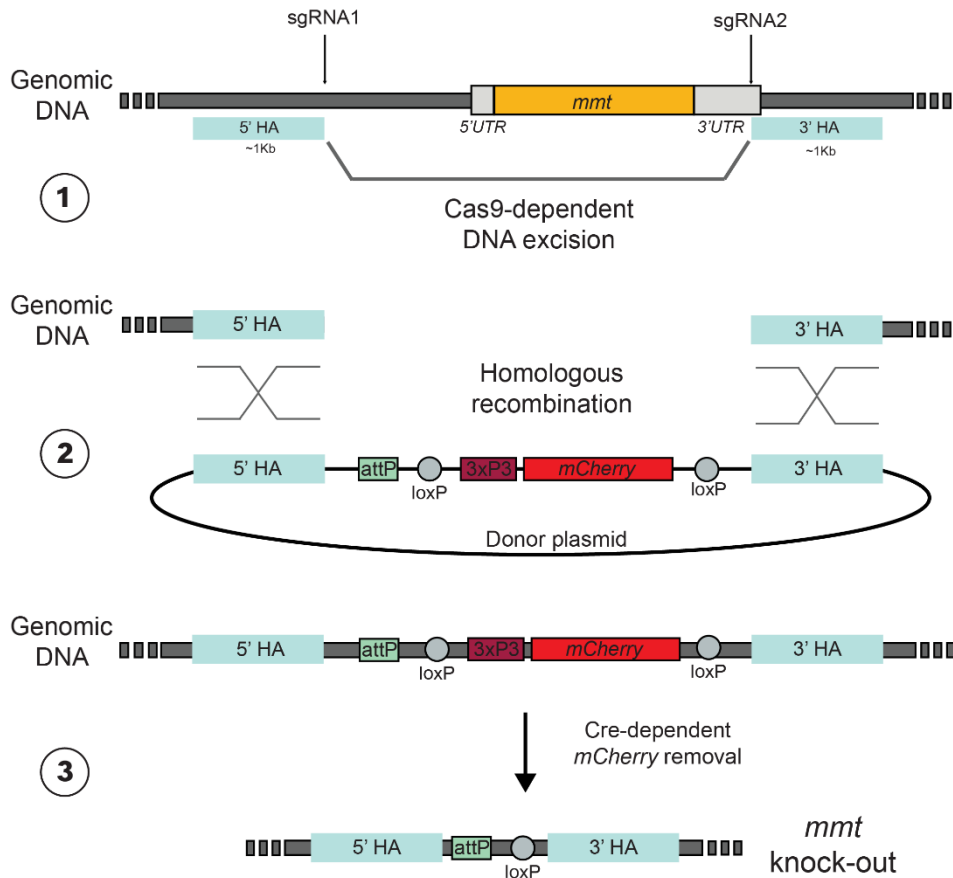
3.6 - ACKNOWLEDGMENTS

We thank Célia Baltazar, Ana Paula Elias and Inês Vicente for technical assistance at all stages of the project. We thank Zita Carvalho-Santos and members of the Behavior and Metabolism laboratory for helpful discussions and comments. We thank Moita Lab (R. Silva Neto), Vasconcelos Lab (M. Aranha), E. Chiappe, and the Jean-Paul Vicent Lab for providing fly stocks and reagents; the Champalimaud Fly Platform, Glass Wash and Media Preparation and the Molecular and Transgenic Tools Platform for technical support throughout the project. Stocks were obtained from the Bloomington *Drosophila* Stock Center (NIH P40OD018537) and the Vienna *Drosophila* Resource Center (Vienna, Austria).

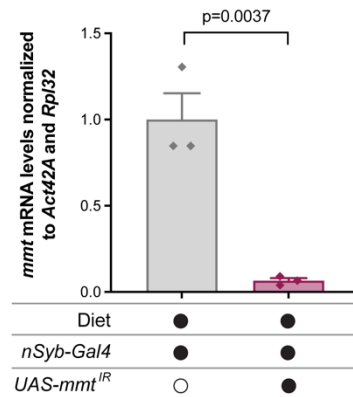
APF was supported by a doctoral fellowship PD/BD/114277/2016 from the Portuguese Foundation for Science and Technology (FCT). The project leading to these results has received funding from FCT (FCT-PTDC/BIA-MOL/30081/2017) to CR. Research at the Centre for the Unknown is supported by the Champalimaud Foundation and by Portuguese national funds through FCT - Fundação para a Ciência e a Tecnologia - in the context of the project UIDB/04443/2020, the research infrastructure CONGENTO, co-financed by Lisboa Regional Operational Programme (Lisboa2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF) and FCT under the project LISBOA-01-0145-FEDER-022170, and the PPBI - LISBOA-01-0145-FEDER-022122.

3.7 – SUPPLEMENTARY MATERIALS

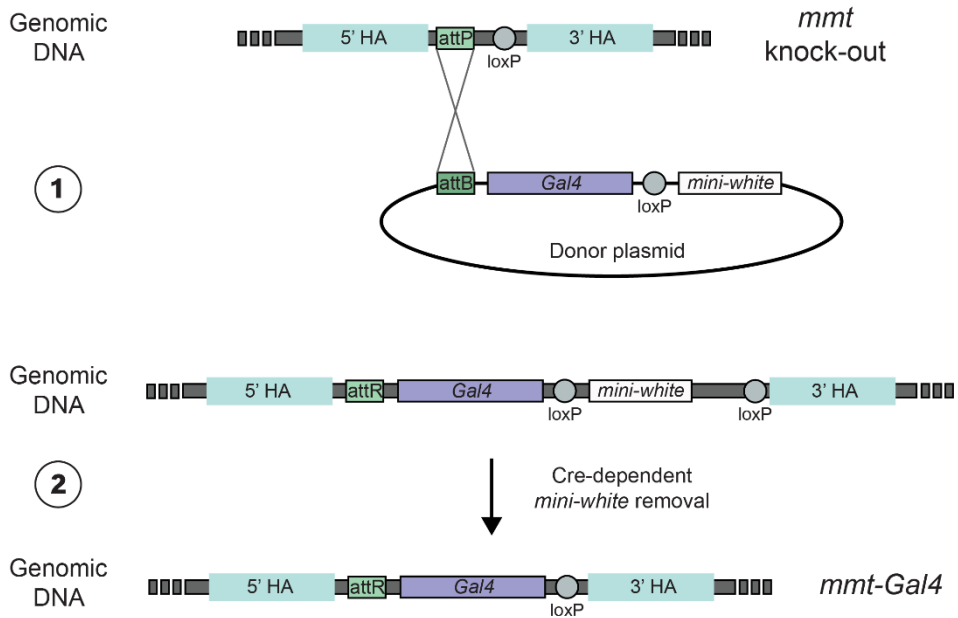
3.7.1 Supplementary Figures



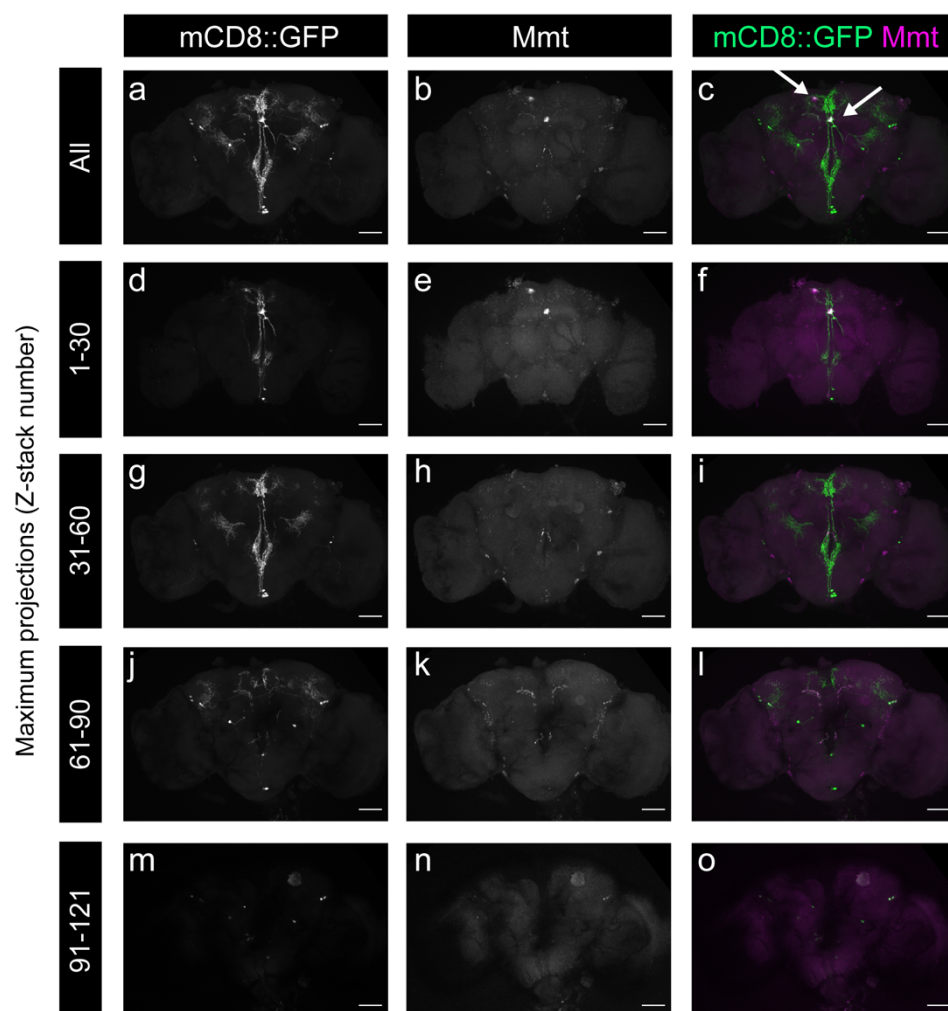
Supplementary Figure 3.1 - Generation of *mtt* knock-out using CRISPR-Cas9 genome editing techniques. Scheme depicting the generation of the *mtt* knock-out line using CRISPR-Cas9 genome editing techniques. **Step 1:** Cas-9-dependent DNA excision between the 5' and the 3' homology arms (HA) mediated by two single guide RNAs (sgRNA). **Step 2:** A donor plasmid, including an *attP* site for later insertions of transgenes and a *mCherry* as a selective marker under the 3xP3 promoter driving expression in all photoreceptors, was inserted by homologous recombination into the excised part of the genome between the 5' and 3' homology arms (HA). **Step 3:** The *mCherry* cassette was removed by inducing a Cre-mediated recombination event between the loxP sites flanking the cassette.



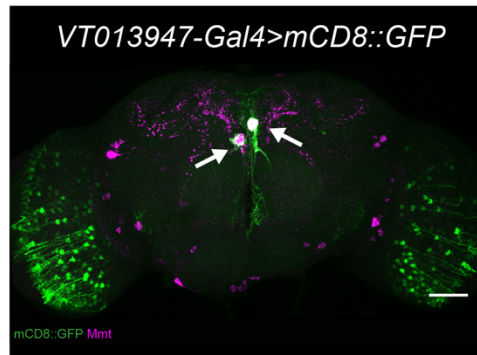
Supplementary Figure 3.2 - *mmt* RNAi leads to specific gene knockdown. *mmt* mRNA levels measured from heads of *mmt* pan-neuronal knockdown or control flies maintained on complete holidic medium as normalized using the expression levels of two control genes (*Actin 42A* and *RpL32*). Filled black circles represent the complete holidic medium or presence of a specific genetic element, whereas an empty black circle represents its absence. The columns represent the mean and the error bars the standard error of the mean. Significance was tested using the unpaired *t*-test, $n=3$. Related to Figure 3.5a.



Supplementary Figure 3.3 - Generation of *mmt-Gal4*. Scheme depicting the generation of *mmt-Gal4* knock-in. **Step 1:** A donor plasmid carrying a *Gal4* and a *mini-white* as a selection marker was inserted into the *mmt* knock-out locus via Φ C31-recombinase-mediated cassette exchange. **Step 2:** The *mini-white* cassette was removed by inducing a Cre-mediated recombination event between the *loxP* sites flanking the cassette.



Supplementary Figure 3.4 - *mnt-Gal4* recapitulates Mmt antibody expression. (a-o) Maximum projections of all or sub-sections of confocal stacks of brains of *mnt-Gal4* flies driving *UAS-mCD8::GFP*, showing mCD8::GFP, Mmt antibody or both; scale bars correspond to 50 μ m.



Supplementary Figure 3.5 - Expression pattern of *VT013947-Gal4* with Mmt staining. Maximum projection images of confocal stacks of the brain of *VT013947-Gal4* driving UAS-mCD8::GFP in green, co-stained with Mmt antibody in magenta. Arrows mark the Medial Marmite Neurons (MMNs). The brain is oriented with the dorsal side on top and the ventral side at the bottom.; scale bar corresponds to 50µm.

3.7.2 Supplementary Tables

Supplementary Table 3.1 – List of stocks used in chapter 3.

Line	Full genotype	Source
<i>w¹¹¹⁸</i>	<i>w¹¹¹⁸; +; +</i>	Ribeiro Lab
<i>nos-Cas9</i>	<i>y¹ M{nos-Cas9.P}ZH-2A w*</i>	BDSC #54591
<i>cre</i>	<i>w*, hs-cre; Sco/CyO; Dr/TM3</i>	Chiappe Lab
<i>mnt^{KO}</i>	<i>w¹¹¹⁸; +; mnt^{KO 30.2.1}</i>	this thesis
<i>background control for mnt^{KO}</i>	<i>w¹¹¹⁸; +; +</i>	this thesis
<i>Deficiency</i>	<i>w¹¹¹⁸; +; Df(3L)BSC220/TM6c</i>	BDSC #9697
<i>nSyb-Gal4</i>	<i>w¹¹¹⁸, UAS-DCR2/Yhs-hid; +; nSyb-Gal4 2.1/ TM6b</i>	Ribeiro Lab
<i>UAS-mmt^{IR}</i>	<i>w¹¹¹⁸, P{GD6177}v14328;</i>	VDRCL#14328
<i>nSyb-Gal4</i>	<i>w¹¹¹⁸/Yhs-hid; +; nSyb-Gal4 2.1/ TM6b</i>	Ribeiro Lab
<i>attP40</i>	<i>y¹ w* P{nos-phiC31\int.NLS}X; P{CaryP}attP40</i>	Champalimaud Fly platform
<i>UAS-mmt</i>	<i>w¹¹¹⁸; UAS-mmt^{8.1}/CyO</i>	this thesis
<i>background control for UAS-mmt</i>	<i>w¹¹¹⁸; +; +</i>	this thesis
<i>mnt-Gal4</i>	<i>w¹¹¹⁸; +; mnt^{Gal4 13.1.1}/TM6b</i>	this thesis
<i>background control for mnt-Gal4</i>	<i>w¹¹¹⁸; +; mnt^{KO 13.4.1}/TM6b</i>	this thesis
<i>UAS - mCD8::GFP</i>	<i>w¹¹¹⁸/Yhs-hid; mCD8::GFP</i>	Ribeiro Lab
<i>UAS - mCD8::GFP; UAS-redstinger</i>	<i>w*; mCD8::GFP; UAS-redstinger</i>	Moita Lab
<i>UAS-TrpA1</i>	<i>w¹¹¹⁸/Yhs-hid; UAS-TrpA1</i>	Ribeiro Lab
<i>UAS-TrpA1; Deficiency</i>	<i>w¹¹¹⁸; UAS-TrpA1/CyO; Df(3L)BSC220/TM6b</i>	this thesis
<i>UAS-kir2.1, tubGal80^{ts}</i>	<i>w*/Yhs-hid; +; UAS-kir2.1::eGFP, tubGal80^{ts}/TM6b</i>	Vasconcelos Lab
<i>VT013947-Gal4</i>	<i>P{VT013947-GAL4}attP2</i>	VDRCL#205650
<i>empty-Gal4</i>	<i>w¹¹¹⁸; P{GAL4.1Uw}attP2</i>	BDSC#68384
<i>mnt-m-Gal4</i>	<i>w¹¹¹⁸; VT016839-p65.AD/CyO; VT037488-GAL4.DBD/TM6b</i>	this thesis
<i>empty-split Gal4</i>	<i>w¹¹¹⁸; P{p65.AD.Uw}attP40; P{GAL4.DBD.Uw}attP2</i>	BDSC#79603

Supplementary Table 3.2 - Nucleotide sequence of primers used for qRT-PCR.

Primer	Primer Sequence
<i>mnt Fwd</i>	5' ATCTGCTGCTCCACTGTGC 3'
<i>mnt Rev</i>	5' TTCGATTGGCCTCATTTTC 3'
<i>Actin42A Fwd</i>	5' CAGGCGGTGCTTTCTCTCTA 3'
<i>Actin42A Rev</i>	5' AGCTGTAACCGCGCTCAGTA 3'
<i>RpL32 Fwd</i>	5' GCCCAAGATCGTGAAGAAGC 3'
<i>RpL32 Rev</i>	5' GCACTCTGTTGTCGATACCCTTG 3'

CHAPTER 4

*MARMITE AND THE VERTEBRATE
NEUROPEPTIDE B/W DEFINE A
NEW CONSERVED NEUROPEPTIDE
FAMILY INVOLVED IN NUTRITIONAL
REGULATION*

Note: The material in this chapter is included in a preprint available in an open access repository:

Francisco AP, Tastekin I, Fernandes AB, Ezra-Nevo G, Deplancke B, Oliveira-Maia AJ, Gontijo AM & Ribeiro C. (2022). *marmite* defines a novel conserved neuropeptide family mediating nutritional homeostasis (p. 2022.12.12.520095). *bioRxiv*.
<https://doi.org/10.1101/2022.12.12.520095>

Author Contributions: **APF** performed all the *Drosophila* experiments and analyzed and interpreted data. **AMG** performed sequence and phylogenetic analysis. Mice experiments were designed by **APF**, **CR**, **ABF** and **AJO-M** and performed by **ABF**. **APF** and **CR** conceived and designed the project.

4.1 - SUMMARY

Neuropeptides play a key role in regulating animal's physiology and behavior. One of their important functions is to mediate information about the internal state of the animal, which is essential for controlling several behaviors, such as feeding. Due to their key role, several neuropeptides have been maintained throughout evolution and orthologues can be found in different species. In the previous chapter, we described the function of Marmite, a new predicted neuropeptide in *Drosophila* involved in protein appetite and nutritional homeostasis. In this chapter, we investigate the evolutionary history of Marmite and identified the vertebrate neuropeptides B and W as part of the same neuropeptide family. Functional experiments showed that Mmt and vertebrate NPB and NPW modulate food intake in both flies and mice. Therefore, we discovered an ancient family of neuropeptides involved in controlling feeding across phyla.

4.2 - INTRODUCTION

A high degree of genetic and cellular mechanisms, as well as a wide range of molecules have been maintained throughout evolution and are shared by animals, from flies to humans. Among these different molecules, neuropeptides are a good example of conserved signals across the animal kingdom (Nässel & Zandawala, 2019; L. Schoofs et al., 2017). They are critical for the dynamic adaptation that animals have to perform in order to adjust to challenging environmental conditions. Thus, it is not surprising that they have been shown to be involved in different aspects of the animal's life, from metabolism to behavior (Nässel & Zandawala, 2019).

Feeding is fundamental for survival and general well-being. Several neuropeptides and peptide hormones have been shown to regulate different aspects of the feeding process. Molecules signaling either satiety or hunger, reducing or promoting feeding, respectively, have been identified in several species, from flies to vertebrates (Andermann & Lowell, 2017; S. Lin et al., 2019). Neuropeptide Y in mammals and its orthologue neuropeptide F in *Drosophila* are examples of two conserved peptides involved in promoting feeding (Brown et al., 1999; Horio & Liberles, 2021). However, signals regulating nutrient-specific appetites and how they act to control food choice remain less well characterized.

In vertebrates, two neuropeptides that have been proposed to control feeding are the poorly studied neuropeptide B (NPB) and neuropeptide W (NPW), which share structural and functional similarities (Brezillon et al., 2003; Fujii et al., 2002; Shimomura et al., 2002; Takenoya et al., 2010; Tanaka et al., 2003). NPB and NPW are expressed in brain regions previously related to feeding, such as hypothalamic areas.

Furthermore, intracerebroventricular administration of NPB or NPW in rodents has been shown to lead to changes in food intake (Aikawa et al., 2008; Baker et al., 2003; Levine et al., 2005; Mondal et al., 2003; Naso et al., 2014; Samson et al., 2004; Shimomura et al., 2002; Tanaka et al., 2003). Additionally, *NPB* knock-out mice have been reported to develop adult-onset obesity (Kelly et al., 2005). However, the precise function of NPB and NPW remains enigmatic as some studies observed an increase in food intake upon NPB or NPW injection (Baker et al., 2003; A. S. Levine et al., 2005; Samson et al., 2004; Shimomura et al., 2002), while others observed a reduction (Aikawa et al., 2008; Mondal et al., 2003; Naso et al., 2014; Tanaka et al., 2003). Therefore, while NPB/W signaling seems to play a role in controlling food intake and homeostasis, the mechanisms underlying their mode of action remain unclear. Likewise, the evolutionary origin and history of NPB/W remain unknown. Identifying evolutionary orthologues could open the door to a comparative mechanistic dissection of the function of this poorly understood peptide class and hence a deeper understanding of appetite regulation.

4.3 - RESULTS

4.3.1 - Marmite is part of a new neuropeptide family, including the neuropeptides B and W

In chapter 3, we identified a previously uncharacterized gene in the *Drosophila* genome, predicted to encode a secreted peptide. Transcriptional, anatomical and functional data all supported the idea that Marmite (Mmt) is a new *Drosophila* neuropeptide involved in feeding and nutritional homeostasis. Several neuropeptides have been shown to be conserved between insects and mammals, including some involved in food intake (Nässel & Zandawala, 2019). We therefore set to investigate whether Mmt could share sequence similarities with known vertebrate neuropeptides.

A simple amino acid sequence analysis using BLAST (Basic Local Alignment Search Tool) algorithms did not retrieve any similar peptide in higher taxa, such as vertebrates. This is not unexpected since neuropeptides are usually very small peptides, with only a few conserved residues, making it very challenging to discover evolutionary relationships by sequence-based searches (Elphick et al., 2018). To understand the molecular evolutionary history of Mmt and gain further insight into possible critical residues, we first aligned it with Mmt-like proteins from other *Drosophila* and closely related insect species. Not surprisingly, we observed relatively poor amino-acid conservation across the protein sequence, with the notable exception of a conserved motif corresponding to four cysteine residues in the carboxy (C)-terminus of the prepropeptide (Figure 4a and Supplementary Figure 4.1). Using this motif, which we named the Marmite domain, and filtering hits for those predicted to also

encode a signal peptide and have a similar length (see Materials & Methods), we found a group of proteins in several vertebrate species corresponding to the peptide precursor (prepropeptide) of neuropeptide B (NPB), a known vertebrate neuropeptide (Brezillon et al., 2003; Fujii et al., 2002; Tanaka et al., 2003) (Supplementary Figure 4.1). In vertebrates, this family of neuropeptides includes the *neuropeptide W* (*NPW*) gene (Shimomura et al., 2002; Tanaka et al., 2003), which encodes a prepropeptide with a very similar amino (N)-terminal peptide sequence to NPB. Our sequence analysis suggests that *NPW* has likely duplicated from an *NPB*-like ancestral gene. Intriguingly, *NPW* seems to have exchanged the conserved C-terminal region, containing the conserved cysteine residues, for a different C-terminal part (Supplementary Figure 4.2).

Further sequence- and motif-based searches identified similar sequences in other invertebrates, including ecdysozoans (Nematoda and Panarthropoda), lophotrochozoans (Mollusca and Annelida), and deuterostomes (e.g., in Echinodermata) (Figure 4.1b and Supplementary Figure 4.1). Hence, *mnt*-like neuropeptide genes are found in all major bilaterian clades (Ecdysozoa, Lophotrochozoa, and Deuterostomia). Even though we did not find examples of *Mnt*-like sequences in some major clades (such as Platyhelminthes), the most parsimonious interpretation of these results is that an *mnt*-like gene must have been present in the last common ancestor of all bilaterians.

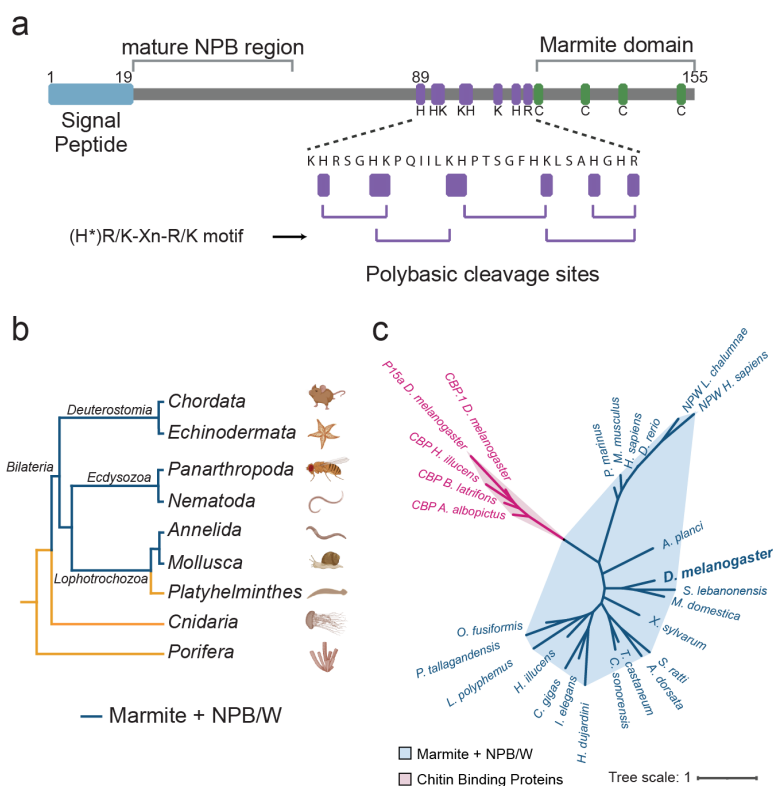


Figure 4.1 - Mmt is part of a new neuropeptide family, including neuropeptide B and W. (a) Scheme depicting the 155 amino acid sequence of the Mmt prepropeptide with predicted motifs: signal peptide in the N-terminus, region corresponding to the vertebrate mature NPB, polybasic residues as potential targets for protein processing and the four conserved cysteines in the C-terminus (Marmite domain). The cleavage sites predicted by the motif (H*)R/K-Xn-R/K are shown, where X is any amino acid, n is 0, 2, 4, or 6 amino acids, and an H* denotes that histidine is accepted when n is 2, 4, or 6. The size of the different motifs and the sequence are represented to scale. The numbers on top indicate the AA position. The size of the different motifs and the sequence are represented to scale. The numbers on top indicate the AA position. **(b)** Cladogram with representative phyla in the animal kingdom highlighting the taxa where Mmt or NPB/W sequences can be found in blue and absent in orange. Created with Biorender.com. **(c)** Phylogenetic tree showing the evolutionary relationship between AA sequences of Mmt-like, NPB/W proteins and members of the chitin binding protein family. Mmt-like and NPB/W sequences form a specific cluster in blue, separated from the CBP cluster in magenta.

Additionally, to rule out that the identified relationship between Mmt and NPB neuropeptides is spurious and/or both peptide families are part of an even larger family, we performed further relaxed sequence searches for other proteins with any resemblance to the identified Mmt/NPB C-terminal motif, retaining those hits that satisfied the criteria of being a putatively secreted protein in the hundred-AA-length range. This search identified insect members of a Chitin Binding Protein (CBP) family, which comprises chitinases and peritrophic matrix proteins (Shen & Jacobs-Lorena, 1999). However, two major pieces of evidence indicate that Mmt/NPB are part of a distinct family of proteins: first, CBP proteins are characterized by a 6-cysteine motif instead of the 4-cysteine motif found in Mmt/NPB, and a few highly conserved aromatic residues (notably at position -3 before cysteine 3, and position 6 after cysteine 4), none of which are present in Mmt/NPB precursors. Second, whereas a minority of predicted CBP proteins have 4 cysteines (e.g., CG14300 in *Drosophila melanogaster*), their sequence otherwise aligns well with the other CBPs (hence their detectability as CBPs in PFAM domain searches), which is not the case of Mmt/NPB precursors. Consistent with this, phylogenetic analysis showed that Mmt-like sequences and the vertebrate NPB and NPW form a separate cluster from CBP proteins, including those containing only 4-cysteines, supporting our finding that Mmt, NPB and NPW are part of a new, ancient, and conserved neuropeptide family (Figure 4.1c). Thus, we propose to call this new family the Marmite Neuropeptide Family.

4.3.2 – Expression of *neuropeptide B* and *W* genes in *Drosophila* alters food intake

The previous phylogenetic analysis suggested that Mmt and NPB/W are part of the same conserved family of neuropeptides. Intriguingly, NPB and NPW have been implicated in feeding regulation in vertebrates (Aikawa et al., 2008; Baker et al., 2003; A. S. Levine et al., 2005; Mondal et al., 2003; Naso et al., 2014; Samson et al., 2004; Shimomura et al., 2002; Tanaka et al., 2003). This raises the possibility that the Mmt/NPB neuropeptide family has evolved to regulate feeding across species and that their conservation covers the functional level, extending beyond pure sequence conservation. Therefore, we decided first to test whether the human NPB and NPW gene products could alter feeding in *Drosophila*.

To test for functional conservation of the encoded neuropeptides, we developed a similar gain-of-function strategy previously used to overexpress *mnt* pan-neuronally (see section 3.3.4). We generated UAS constructs of both human *NPB* and *NPW* genes and focused on phenocopying the decrease in yeast feeding observed upon neuronal *mnt* overexpression (Figure 3.5b). Strikingly, pan-neuronal overexpression of either *NPB* or *NPW* led to a clear decrease in yeast intake, which mimicked the phenotype observed upon *mnt* overexpression (Figure 4.2). These results strongly suggest that the *mnt*, *NPB*, and *NPW* genes are functionally conserved, as they can exert the same effect on feeding in flies. Furthermore, given that the NPW prepropeptides lack the Marmite domain (the cysteines in their C-terminal domain are not conserved) (Supplementary Figure 4.2) suggests that the N-terminal part of the prepropeptide, just downstream of the SP, exerts the observed behavioral

effect. Importantly, it is the N-terminal part that has been shown to give rise to the neuropeptides exerting the feeding effect in rodents, lending further support to our hypothesis (Aikawa et al., 2008; Baker et al., 2003; A. S. Levine et al., 2005; Mondal et al., 2003; Naso et al., 2014; Samson et al., 2004; Shimomura et al., 2002; Tanaka et al., 2003).

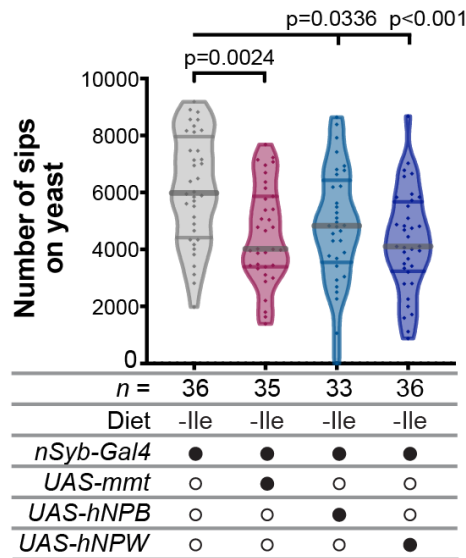


Figure 4.2 - Pan-neuronal expression of *mtt*, human *NPB* or *NPW*. Number of sips on yeast of control flies and flies expressing *mtt*, the human *NPB* or *NPW* genes using a pan-neuronal driver. Flies were maintained on holidic medium lacking Isoleucine (-Ile). In this and following figures, below the graph, filled black circles represent the presence of a specific genetic element, whereas an empty black circle represents its absence. The violin plot shows the frequency distribution of the data, with lines representing the median, upper and lower quartiles; each point correspond to an animal. Significance was tested using the Mann-Whitney test with Bonferroni correction. The number of flies used in each condition is shown in the figure.

4.3.3 – *mnt* expression in mice changes food intake

To further strengthen the argument for functional conservation between Mmt and NPB/W, we decided to test if overexpression of the *Drosophila mnt* gene can induce changes in food intake in rodents. Intracerebroventricular administration of either NPB or NPW peptides has been shown to alter food intake in rodents (Aikawa et al., 2008; Baker et al., 2003; A. S. Levine et al., 2005; Mondal et al., 2003; Naso et al., 2014; Samson et al., 2004; Shimomura et al., 2002; Tanaka et al., 2003). Thus, we decided to use a targeted gain-of-function strategy to test if Mmt can alter feeding in mice. To do so, we engineered AAV viruses to neuronally express either the mouse *NPB* or the *Drosophila mnt* gene. For each group, we injected one of these viruses or a fluorophore control bilaterally in the Ventromedial Hypothalamic nucleus (VMH), as this brain area has been shown to express NPB in mice (Fujii et al., 2002) (Supplementary Figure 4.3). Injected mice were kept in single cages and their food consumption was monitored for 8 weeks using a diet containing 14% protein. Consistent with what we observed in flies, overexpression of both *mnt* and *NPB* led to changes in feeding compared to controls (Figure 4.3). Animals overexpressing either of these neuropeptides' genes showed a significant increase in cumulative food intake over 8 weeks when compared to controls. These data, therefore, suggest that the *Drosophila mnt* gene encodes a neuropeptide that can similarly alter food intake as NPB, supporting the idea that the function of the ancient Marmite Neuropeptide Family is conserved across evolution.

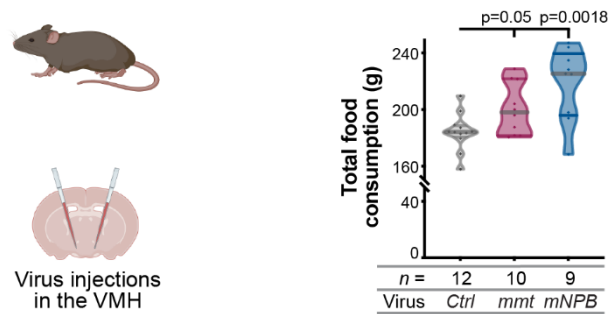


Figure 4.3 - Food intake of mice injected with *mtt* or *NPB*-expressing virus in the VMH. Left: scheme depicting the virus injection in the VMH of a mouse brain. Created with Biorender.com. Right: total food consumption of mice injected with control virus or virus expressing either *Drosophila mmt* or mouse *NPB*. Significance was tested using the unpaired *t*-test with Bonferroni correction. The number of animals used in each condition is shown in the figure.

4.4 - DISCUSSION

4.4.1 - Mmt and vertebrate neuropeptide B/W define a new conserved neuropeptide family

Neuropeptides are signaling molecules that emerged early in animal evolution (Elphick et al., 2018; Nässel & Zandawala, 2019; L. Schoofs et al., 2017). Thus, it is not surprising that some neuropeptides have maintained similar sequences and functions, and that orthologues are found among different taxa. However, due to their small size and the conservation of only a few critical residues in most cases, establishing evolutionary relationships based on sequence queries has been challenging (Elphick et al., 2018). Mmt and Mmt-like gene products in related insect species share a fully conserved motif of four cysteines in the C-terminal part of the protein (Marmite domain). This domain, together with the size range of the proteins and the presence of a signal peptide, led us to identify a group of vertebrate gene products with similar features, which corresponded to the NPB prepropeptide. Once processed, this previously characterized neuropeptide is very similar to NPW (Chottova Dvorakova, 2018). However, the prepropeptide of NPW, does not have the Marmite Domain. This could suggest that the precursor of NPW originated from a duplication of an NPB-like ancestral and its C-terminus diverged after that. Hence, even though the NPW prepropeptides have lost the Marmite domain, they share a common ancestor with Mmt and NPB prepropeptides. Moreover, we found Mmt/NPB-like sequences in other non-vertebrate taxa, strongly suggesting that the Mmt neuropeptide family is ancient and was already present in the common ancestor of all bilaterians.

Almost all members of the Mmt neuropeptide family have the Marmite domain. In general, this domain is by far the most strongly conserved portion of all members of the Marmite neuropeptide family (except NPW). Considering all of this evidence, we conclude that the Marmite domain is unique and most likely originates from evolutionarily-related genes with a separate evolutionary history from CBP-encoding genes, at least since the evolution of Bilateria. This raises the question of what could be the function of this domain. Prepropeptides often encode multiple peptides with different biological activities (Jékely et al., 2018). In other cases, domains within prepropeptides play important roles in processing or chaperoning the peptide which harbors the receptor binding and hence signaling activity (Vejrazkova et al., 2020). Currently, all functional studies in rodents have focused on the peptide encoded by the N-terminal region of NPB and NPW, leaving us with no information about the function of the conserved C-terminal region. Identifying the function of the Marmite domain will be an exciting avenue for future research.

4.4.2 - Mmt and neuropeptide B/W are functionally conserved

Our data on the functional characterization of the *mmt* gene and *mmt*-expressing neurons demonstrated the role of this peptide and the corresponding neurons in regulating food intake, specifically the appetite for protein-rich food (Chapter 3). NPB and NPW neuropeptides have previously been linked to feeding and body weight regulation (Aikawa et al., 2008; Baker et al., 2003; Kelly et al., 2005; A. S. Levine et al., 2005; Mondal et al., 2003; Naso et al., 2014; Samson et al., 2004; Shimomura et al., 2002; Tanaka et al., 2003). We, therefore, tested whether the

conservation between these vertebrate neuropeptides and Mmt could extend to the functional level. Indeed, in *Drosophila*, the neuronal overexpression of the human forms of both *NPB* and *NPW* led to a change in yeast intake, similar to when *mmt* was overexpressed. Likewise, in mice, the overexpression of the mouse *NPB* and *Drosophila mmt* genes in the brain led to increased feeding. Even though we observed a decrease in food intake in flies and an increase in mice, these data suggest that similar to what has been observed for other peptides, Marmite Neuropeptide Family members can exert similar functions across vast evolution spans. However, further studies will be necessary to elucidate the exact mode of function of Mmt, NPB and NPW. Even in rodent studies, it is not clear the effect of NPB or NPW, since both are shown to either promote or reduce feeding. Another open question is which part of the Mmt prepropeptide is responsible for the behavioral phenotype? Despite the potential cleavage sites, we currently do not know whether and how the precursor is cleaved and processed and what is the mature bioactive form.

Overall, we unraveled novel evolutionary relationships and a new neuropeptide family, which also shares a conserved functional role. Our data open a new research avenue to further dissect not only how Mmt functions in flies, but also to clarify how NPB and NPW act in the vertebrate brain. Multi-phyla and combined studies, profiting from the advantages of each model, will help elucidate the mechanisms by which this new family of neuropeptides modulates food intake and nutritional homeostasis.

4.5 - MATERIALS & METHODS

4.5.1 - *Drosophila* rearing, media, and dietary treatments

Flies were reared on yeast-based medium (YBM) (per liter of water: 8 g agar [NZYTech, PT], 80 g barley malt syrup [Próvida, PT], 22 g sugar beet syrup [Grafschafter, DE], 80 g corn flour [Próvida, PT], 10 g soya flour [A. Centazi, PT], 18 g instant yeast [Saf-instant, Lesaffre], 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]). To ensure a homogenous density of offspring among experiments, fly cultures were always set with 6 females and 3 males per vial and left to lay eggs for 3 d. Flies were reared in YBM until adulthood.

Holidic media (HM) were prepared as described previously (Leitão-Gonçalves et al., 2017) using the exome match formulation with food preservatives (Piper et al., 2017). For Holidic medium -Isoleucine (-Ile), Isoleucine was not added to the diet, keeping the concentration of all other nutrients constant. For all experiments using the HM, the following dietary treatment protocol was used to ensure a fully-fed state: groups of 1±5-d old flies (16 females) were collected into fresh YBM-filled vials, 5 *Canton-S* males were added to the vials to ensure females were mated, and transferred to fresh YBM after 48 h. After 24 h, flies were transferred to different HM for 3 days and immediately tested in the indicated assay. All experiments were performed with female flies. Flies were reared and maintained at 25°C in climate-controlled chambers at 70% relative humidity in a 12-h light-dark cycle (Aralab, FitoClima 60000EH). Behavioral testing was performed at 25°C. Polypropylene fly vials (VWR, #734±2261) were used.

4.5.2 - *Drosophila* stocks and genetics

Genetic backgrounds of the control animals were matched as closely as possible to the experimental animals. The stocks and full genotypes used in this chapter are listed in Supplementary Table 4.1.

4.5.2.1 - Generation of UAS-*mnt*, and UAS-*hnpb* and UAS-*hnpw*

UAS-*mnt* stocks were generated by amplifying the *mnt* ORF from the corresponding cDNA and cloning it into pUASg.attB using the Gateway System (Invitrogen) and injected into embryos of *yw*, *M(eGFP, vas-int, dmRFP)ZH-2A*; *P{CaryP}attP40* flies. Flies without successful insertion of the UAS cassette were used to generate stocks for background controls. DNA encoding the Human isoforms of *neuropeptide B* (based on the sequences NP-202, NM_148896.5) or *neuropeptide W* (based on the sequences NPW-201, NM_001099456.3) were chemically synthesized (GenScript) and UAS-NPB and UAS-NPW were generated using the same protocol described for UAS-*mnt*.

4.5.3 - flyPAD assays

flyPAD assays were performed as described in (Itskov et al., 2014). For food choice experiments, single flies in different dietary conditions were tested in arenas containing two types of food patches: 10% yeast or 20 mM sucrose, each mixed with 1% agarose.

For all experiments, flies were individually transferred to flyPAD arenas by mouth aspiration and allowed to feed for 1 h at 25°C (or 30°C for TrpA1 experiments), 70% relative humidity. The total number of sips per animal was calculated using previously described flyPAD algorithms (Itskov et al., 2014). Non-eating flies (defined as having fewer than two activity bouts during the assay) were excluded from the analysis.

4.5.4 - Sequence and phylogenetic analysis

Relaxed (Expect threshold = 100) BLASTp searches against all non-redundant GenBank CDS translations+PDB+SwissProt+PIR+PRF excluding environmental samples from WGS projects were used to find proteins with similarities to CG14075 (NCBI Reference Sequence: NP_649080.1). This search yielded hits only from relatively closely-related brachyceran flies, which were aligned using MULTIPLE Sequence Comparison by Log-Expectation, MUSCLE (Edgar, 2004) (<https://www.ebi.ac.uk/Tools/msa/>). This alignment was used to identify conserved features in brachyceran CG14075 (Mmt) sequences and to derive the relaxed motif M-X(20,80)-W-{C}(20,80)-C-{CH}(9,13)-C-{CH}(9,13)-C-{CH}(9,13)-C. Signal peptides were predicted using SignalP 6.0 (<https://dtu.biolib.com/SignalP-6>) (Teufel et al., 2022). The general motif (H*)R/K-X_n-R/K, where X is any amino acid, n is 0, 2, 4, or 6 amino acids, and an H* denotes a histidine is accepted when n is 2, 4, or 6, was used to annotate putative protein cleavage sites (Devi, 1991; Hook et al., 2008; Seidah & Chrétien, 1999; Veenstra, 2000).

To identify Mmt orthologues outside of brachycera, a pattern search against Eukaryote KEGG genes was performed using the Motif search tool from the Kyoto University Bioinformatics Center (<https://www.genome.jp/tools/motif/MOTIF2.html>). Hits were retained if they had the motif mentioned above, had a signal peptide, were less than 200 AA in length, and were not an obvious member of an annotated protein family with superior reciprocal blast hits in *Drosophila melanogaster*. The sole protein satisfying these rules for *Homo sapiens* was Neuropeptide B (NP_683694.1), which was not considered to have orthologues outside of Deuterostomia. After this identification, relaxed BLASTp (or TBLASTN) searches were run with CG14075 or NPB propeptides as queries to identify

further hits, following the same selective criteria described above. Divergent hits from distantly-related taxa were selected as queries, and relaxed-BLASTp (or TBLASTN) searches were exhaustively repeated until no new clade could be identified. Considering all identified Mmt-like sequences, the consensus motif for the Marmite domain was refined to <M-X(15,30)-{C}(4,110)-C-{CW}(2)-[DE]-{W}(7,12)-C-{C}(8,12)-C-{C}(11,14)-C-{C}(1,50)>.

For phylogenetic analysis, a tree was created with the (https://www.ebi.ac.uk/Tools/phylogeny/simple_phylogeny/), Simple phylogeny tool, using CG14075-like, NPB-like and NPW-like prepropeptide sequences aligned using the T-COFFEE multiple sequence alignment program with the BLOSUM62 matrix (<https://www.ebi.ac.uk/Tools/msa/>) (Notredame et al., 2000). CBP family proteins were included as outgroups. Sequences were then clustered using the neighbor-joining algorithm to construct trees from the distance matrix with gap included and with distance correction to correct for multiple substitutions at the same site (Kimura, 1980; Saitou & Nei, 1987). Unrooted trees were displayed, annotated, and managed using The Interactive Tree Of Life tool (<https://itol.embl.de>) (Letunic & Bork, 2021).

Sequence alignments were visualized and edited using Jalview (Waterhouse et al., 2009). All sequences used for the phylogenetic analysis are listed in Supplementary Table 4.2.

4.5.5 - Mice

All experiments were approved by the Champalimaud Foundation and Portuguese Direção Geral de Veterinária, and performed in accordance with the European Union Directive for Protection of Vertebrates Used for Experimental and other Scientific Ends

(86/609/CEE). Male and female C57BL/6J mice were used from the inbred colony of the Champalimaud Foundation Vivarium. All animals started the procedure at 8-9 weeks of age.

4.5.6 - Surgical procedures and viral injections in mice brain

C57BL/6J mice were anesthetized using a mix of oxygen (1-1.5 L/min) and 1-3% isoflurane (Vetflurane, Virbac). Briefly, the hair on the top of the head was clipped, the mouse was placed on a surgical table, covered with a heating pad set at 37°C and stabilized in the stereotaxic apparatus (David Kopf Instruments). A skin incision was performed to expose the skull, connective and muscle tissue was carefully removed, and the skull surface was leveled at less than 0.05 mm by comparing the height of bregma and lambda, and medial-lateral directions. Two small holes were drilled for bilateral viral injections. Bilateral viral injections were performed using glass pipettes with 3µl *marmite (mmt)*, *neuropeptide B (NPB)* or control viral stock solution. *mmt*: AAV5-hSyn-*dmmt*-2A-*mCherry*; *NPB*: AAV5-hSyn-*mNPB*-2A-*mCherry*; *Ctrl*: AAV.CAG.Flex.GCaMP6f.WPRE.SV40 (AAV5) (Addgene 100835) or AAVsyn1-tdTomato-WPRE(AAV5) (Addgene 51506). *mmt* and *NPB*-expressing viruses were produced by the Molecular and Transgenic Tools Platform at Champalimaud Foundation.

0.5 µl of virus were bilaterally injected in the Ventromedial Hypothalamus (VMH) (Antero-posterior (AP) -1.3 mm; Medio-lateral (ML): +/-0.3 mm and Dorso-ventral (DV):-5.6 mm (from brain surface; Paxinos 2008), at a rate of 0.96 nL/s using nanoject II (Drummond Scientific), with the pipette retracted only twenty minutes after the injection was completed. After the surgery, animals recovered in their

home cage with a heating pad set at 37°C and were closely monitored until they fully recovered.

4.5.7 - Food intake measurements of mice

C57BL/6J animals were first separated one per cage and allowed an acclimatization period of 1 week to ensure weight stabilization. Food intake measurements were performed every 2 days over 10 weeks: 2 weeks before surgery (baseline measurements) and 8 weeks after *mmt*, *NPB* or Ctrl viral injections. 2014 Food pellets of Teklad global 14% protein rodent maintenance diets (Envigo) were used. Food intake was deduced by measuring the difference between the initial and final weight of the provided food using a scale (Ohaus bench scale).

4.5.8 - Histology and immunohistochemistry of mice samples

After feeding measurement experiments were completed, mice were anesthetized with an intraperitoneal injection of ketamine-xylazine (100 mg/kg and 5 mg/kg, respectively), and perfusion was performed with 1x phosphate buffered saline (PBS) and 4 % paraformaldehyde (PFA). Brains were gently extracted and placed in 4% PFA overnight and then transferred to PBS at 4°C for further histological processing. Brains were sectioned coronally in 50 µM slices using a vibratome (Leica VT1000S), and sections were collected into a 24-well plate with PBS before mounting. After mounting, images of the sections were taken using an automated wide-field fluorescence microscope (Zeiss AxioScan.Z1). The acquisition features were standardized for all the slices and acquired with Plan - Apochromat 10 X/0.45 M27 (Zeiss). A green and a red wavelength laser (channel Alexa Fluor (AF) 488, channel red (Cy3) 570, excitation filter 555 nm) were used with exposure times of 50 ms and 75ms, respectively,

and a LED power of 10 %. Finally, the output images were transformed into 2D maximum intensity images using the ZEN 3.4 edition (Zeiss) software.

4.5.9 - Statistical analysis

All violin plots show the frequency distribution of the data, with lines representing the median, upper and lower quartiles. In all behavioral experiments, each data point corresponds to one animal, and the sample sizes correspond to the number of animals tested. All statistical tests used were two-sided. Normality was tested using the Shapiro-Wilk normality test, and parametric or nonparametric tests were used accordingly. The statistical tests used in each experiment are stated in the figure legends. GraphPad Prism 9 was used for data and statistical analysis.

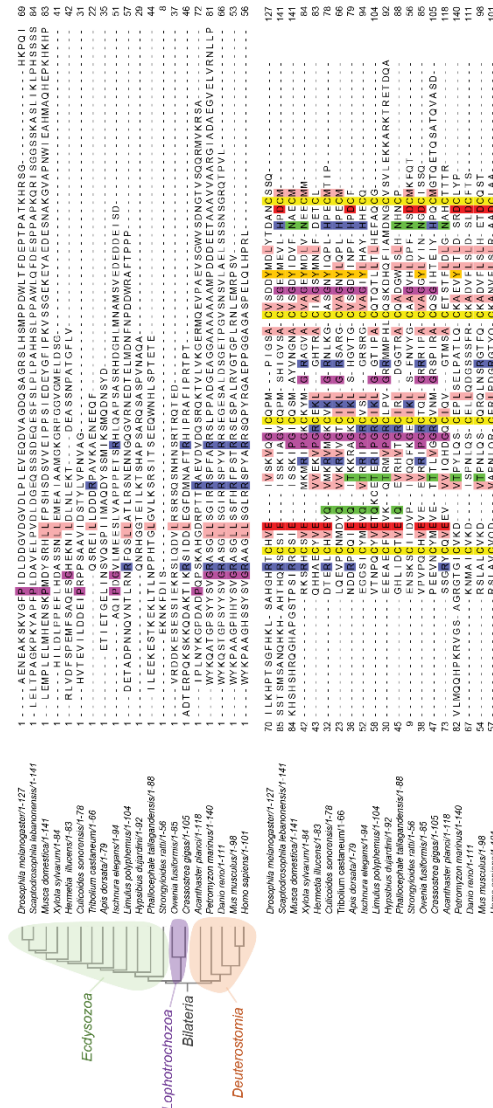
4.6 - ACKNOWLEDGMENTS

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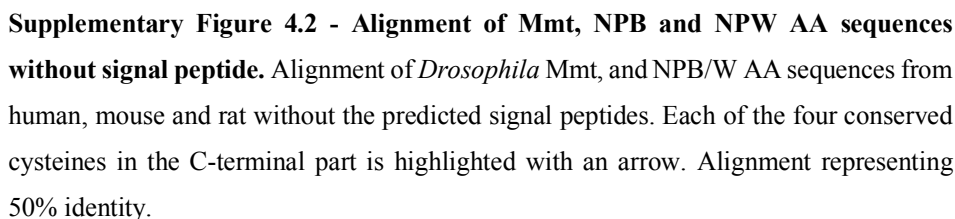
APF was supported by a doctoral fellowship PD/BD/114277/2016 from the Portuguese Foundation for Science and Technology (FCT). The project leading to these results has received funding from FCT (FCT-PTDC/BIA-MOL/30081/2017) to CR. Research at the Centre for the Unknown is supported by the Champalimaud Foundation and by Portuguese national funds through FCT - Fundação para a Ciência e a Tecnologia - in the context of the project UIDB/04443/2020, the research infrastructure CONGENTO, co-financed by Lisboa Regional Operational Programme (Lisboa2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF) and FCT under the project LISBOA-01-0145-FEDER-022170, and the PPBI - LISBOA-01-0145-FEDER-022122.

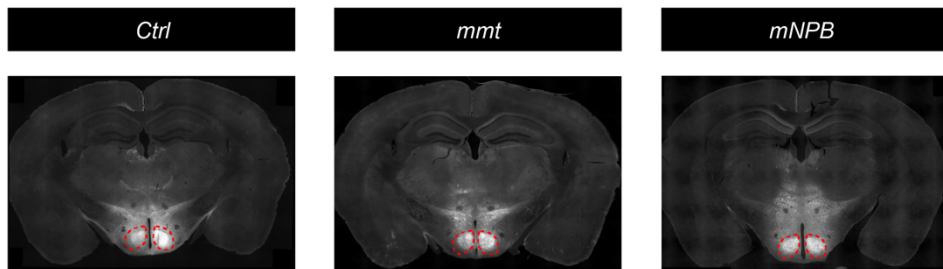
4.7 – SUPPLEMENTARY MATERIALS

4.7.1 Supplementary Figures



Supplementary Figure 4.1 - Alignment of Mmt-related and NPB AA sequences without signal peptide. Alignment of Mmt-like and NPB AA sequences without the predicted signal peptides from invertebrate and vertebrate species. The main taxa to which the different species belong are shown. Each of the four conserved cysteines in the C-terminal part is highlighted with an arrow. Alignment representing 30% identity.





Supplementary Figure 4.3 – *mmt* and *NPB*-virus expression in mice. Representative image of brains of mice injected with control (left), *mmt*-expressing virus (middle) or *NPB*-expressing virus (right) stained for fluorescent proteins. A dashed red line indicates the site of injection (VMH).

4.7.2 Supplementary Tables

Supplementary Table 4.1 – List of stocks used in chapter 4.

Line	Full genotype	Source
<i>nSyb-Gal4</i>	<i>w¹¹¹⁸/Yhs-hid; +; nSyb-Gal4 2.1/TM6b</i>	Ribeiro Lab
<i>attP40</i>	<i>y¹ w* P{nos-phiC31\int.NLS}X; P{CaryP}attP40</i>	Champalimaud Fly platform
<i>UAS-mmt</i>	<i>w¹¹¹⁸; UAS-mmt^{8.1}/CyO</i>	this thesis
<i>background control for UAS-mmt, UAS-hNPB and UAS-hNPW</i>	<i>w¹¹¹⁸; +; +</i>	this thesis
<i>UAS-hNPB</i>	<i>w¹¹¹⁸; UAS-hNPB¹/CyO</i>	this thesis
<i>UAS-hNPW</i>	<i>w¹¹¹⁸; UAS-hNPW¹/CyO</i>	this thesis

Supplementary Table 4.2 – List of sequences used for phylogenetic analysis.

Reference	Species	Common name	Protein
AAF52668.1	<i>Drosophila melanogaster</i>	Vinegar fly	CBP
NP_652325.2	<i>Drosophila melanogaster</i>	Vinegar fly	CBP
KXJ76498.1	<i>Aedes albopictus</i>	Asian tiger mosquito	CBP
XP_037906351.1	<i>Hermetia illucens</i>	Black soldier fly	CBP
XP_018785622.1	<i>Bactrocera latifrons</i>	Fruit fly	CBP
NP_001092926.2	<i>Homo sapiens</i>	Human	NPW
XP_014350484.1	<i>Latimeria chalumnae</i>	Coelacanth	NPW
NP_695206.1	<i>Rattus norvegicus</i>	Rat	NPW
NP_001093134.1	<i>Mus musculus</i>	Mouse	NPW
NP_683694.1	<i>Homo sapiens</i>	Human	NPB
NP_001334545.1	<i>Mus musculus</i>	Mouse	NPB
NP_001120841	<i>Danio rerio</i>	Zebrafish	NPB
NP_695205.1	<i>Rattus norvegicus</i>	Rat	NPB
XP_032826114.1	<i>Petromyzon marinus</i>	Sea lamprey	NPB-like
NP_649080.1	<i>Drosophila melanogaster</i>	Vinegar fly	CG14075/Mmt
XP_022103320.1	<i>Acanthaster planci</i>	Crown-of-thorns starfish	Mmt-Like
CAC9654597.1	<i>Owenia fusiformis</i>	Annelida	Mmt-Like
XP_024499497.1	<i>Strongyloides ratti</i>	Nematode	Mmt-Like
TSA: _isolate_PT2011_Phta_Filter15_CLC_contig_27382	<i>Phallocephale tallagandensis</i>	Onychophora	Mmt-Like
TSA: _transcribed_RNA_sequence_GBZR01002010.1	<i>Hypsibius dujardini</i>	Tardigrade	Mmt-Like
XP_022243359.1	<i>Limulus polyphemus</i>	Horseshoe crab	Mmt-Like
XP_046389499.1	<i>Ischnura elegans</i>	Blue-tailed damselfly	Mmt-Like
XP_031368331.1	<i>Apis dorsata</i>	Giant honey bee	Mmt-Like
KYB28121.1	<i>Tribolium castaneum</i>	Red flour beetle	Mmt-Like
LN483579.1	<i>Culicoides sonorensis</i>	Biting midge	Mmt-Like
CAD7080404.1	<i>Hermetia illucens</i>	Black soldier fly	Mmt-Like
LR999959.1	<i>Xylota sylvarum</i>	Hoverfly	Mmt-Like
XP_005177225.1	<i>Musca domestica</i>	House fly	Mmt-Like
XP_030387299.1	<i>Scaptodrosophila lebanonensis</i>	Fly	Mmt-Like
XP_019929219.2	<i>Crassostrea gigas</i>	Giant Pacific Oyster	Mmt-Like

CHAPTER 5

GENERAL DISCUSSION

The work presented in this thesis revealed modulators of nutrient appetite as well as novel molecular and neuronal mechanisms underlying feeding decisions and protein appetite. For each section of this general discussion I will integrate the findings reported in this thesis with the current state of the field, providing a further discussion of the remaining open questions for future work.

5.1 – MODULATION OF FEEDING BEHAVIOR BY AMINO ACID STATE

The behavior of an animal can be defined by the set of actions performed in order to obtain a certain outcome. The brain is the main organ coordinating the incoming information from the environment and organizing an adequate behavioral response to achieve the desired goal. However, animals not always perform the same behavior in response to the same stimuli. This behavioral variability can be mainly explained by different conditions within the body, or internal state.

It had been previously shown that animals can adapt their feeding decisions depending on their nutritional state. In *Drosophila*, previous work has demonstrated that yeast deprivation led mated females to significantly increase the preference for yeast in a food preference assay (Ribeiro & Dickson, 2010). In the wild, yeast is thought to be a main source of protein but also other nutrients such as metals, sterols and vitamins (Phaff et al., 1956). However, it was not known which nutrient depletion was driving this increase in yeast preference. In this thesis, we showed that only AA depletion, but not the deprivation of other nutrients (vitamins, lipids, sterols, etc) led to an increase in yeast preference. From

an energetic point of view, I would argue it would be very costly to the animal having to respond to every single nutrient depletion and adapt its behavior to search for that nutrient in particular. Moreover, in the wild, nutrients are not found independently, but as part of complex foods. Therefore, animals could have evolved mechanisms by which the depletion of more essential nutrients, such as proteins or carbohydrates, could be used as a proxy for the depletion of micronutrients. Indeed, yeast is not only a source of protein but also micronutrients, thus, the increase in yeast consumption triggered by AA depletion would be enough to compensate for the lack of these other nutrients as well. Nevertheless, to fully understand the ability of flies to regulate nutritional homeostasis, further systematic studies are required to investigate the effects of specific nutrients on behavior.

AAs can be divided in nonessential (neAA) and essential (eAA). Deprivation of any of the ten single eAAs in *Drosophila* led to a potent increase in yeast preference, indistinguishable from lack of all AAs. However, dietary deprivation of neAAs had no effect on yeast preference, most probably since animals can synthesize these AAs themselves. An interesting aspect is that while the yeast preference was different upon eAA or neAA deprivation, the phagostimulatory power of these different types of AAs was not correlated with their nutritional importance. One question that arose from our data was whether any limiting AA could drive a change in feeding or if this was restricted to the specificity of the ten eAAs in *Drosophila*. *Henna* knockdown revealed that even a neAA, tyrosine, when its synthesis is blocked, led to an increase in yeast appetite. This indicates that the brain could be sensing the availability of AAs in general and mount a response leading to the behavioral changes. But how are the AA levels sensed by the brain? It could sense AA concentration

directly or indirectly, via signals sent by peripheral organs, such as the gut or fat body. Regardless of the mechanism, our data suggest that AA sensing is not restricted to each eAA, but at least also to the lack of the neAA tyrosine in a background where this AA cannot be synthesized.

GCN2 and the TOR pathways are the canonical molecular mechanisms for neuronal AA sensing (Dong et al., 2000; Wolfson & Sabatini, 2017). They have been shown to mediate food preference in *Drosophila* (Bjordan et al., 2014; Ribeiro & Dickson, 2010). However, the TOR pathway, for example, has been proposed to mainly react to specific eAAs, such as leucine, whereas our data suggest a wider range of AA sensing (Gu et al., 2022; Hara et al., 1998). Recently, tyrosine has been shown to play a role in the response to low protein diets (Kosakamoto et al., 2022). Tyrosine acts in the fat body as a nutritional cue to promote the response to low-protein diets via GCN2-independent induction of ATF4 target genes in the fat body (Kosakamoto et al., 2022). Our experiments with neAA depletion did not lead to an increase in yeast appetite, but blocking tyrosine synthesis did. Therefore, it is possible that in this context, the lack of tyrosine could lead to the activation of these mechanisms to increase protein appetite.

Besides direct sensing of AAs, their depletion could be read indirectly by the nervous system. On one hand, peripheral organs could be responsible for the direct sensing of AAs levels autonomously or in circulation, leading to the expression and/or secretion of signals, including metabolites, to transmit this information to the nervous system. On the other hand, AAs are necessary to produce proteins, thus, decreases in translation in the nervous system or other organs could trigger a signal for AA depletion. AA sensing is a key aspect in the field of nutritional

homeostasis and we are only beginning to unravel the molecular mechanisms by which AA sensing occurs in the nervous system.

Nutrients can directly modulate neuronal activity and feeding related behaviors. For example, Dh44-producing neurons are specifically activated by nutritive sugars, leading to increase in proboscis extension responses and excretion (Dus et al., 2015). For protein, the activity of a population of *IR76b*-expressing neurons has been recently shown to respond to yeast stimulus. Moreover, the magnitude of this response is increased upon yeast or AA deprivations (Steck et al., 2018). This population of neurons is necessary for yeast intake induced by protein deprivation and different subsets modulate specific aspects of the feeding microstructure, either to initiate or sustain feeding. This work provided an entry point to understand how the brain integrates nutritional state and chemosensory information to modulate nutrient-specific appetites towards protein (Steck et al., 2018). In the future, identifying the second order and further downstream neurons of this sensory population and map the complete sensory-motor circuit will allow us to better understand how AA state drives feeding decisions.

5.2 – DIET-MICROBIOTA-HOST MODULATION OF BEHAVIOR AND PHYSIOLOGY

Microbiota have been previously shown to improve growth and development under poor nutrient conditions in both mice and flies (Schwarzer et al., 2016; Storelli et al., 2011). However, the effect of commensal bacteria in directing nutrient-specific appetites has not been previously explored. In chapter 2, we identified two commensal bacteria

which act together to modulate food choice and reproduction in *Drosophila* in a context of eAA deprivation. In these eAA poor conditions, flies increase their yeast intake to compensate for the lack of eAAs. Nevertheless, in the presence of specific commensal bacteria, the increase in yeast intake is suppressed. At first, this reduction could suggest a detrimental effect for the flies, but our results demonstrated that commensal bacteria can also improve the reproductive output, despite the low AA intake, implying a positive effect for the flies. Moreover, lower AA intake is linked with increased lifespan (Piper et al., 2011), thus reinforcing that the association with commensal bacteria could have a beneficial impact on the fly's fitness.

Commensal bacteria modulated yeast intake but also sucrose intake to a certain extent. For the sucrose intake, the increase observed was simply explained by the usage of this nutrient by the bacteria, as shown by Huang & Douglas (2015), resulting in a sugar deficit in the flies. For yeast, a straightforward explanation would be that bacteria could directly provide the lacking nutrients via digestion by the host of their cellular components, buffering the flies from the AA deprivation, as it has been previously shown (Yamada et al., 2015). However, in this study, several pieces of evidence do not support this explanation. First, live bacteria are required to exert their effect and are not just being used as biomass. Second, bacteria were also unable to suppress yeast appetite induced by a neAA deprivation in the context of the *Henna* knockdown. This result is particularly interesting, suggesting that the effect of the commensal bacteria is specific to eAA deprivation and not to any limiting AA. Since in normal conditions, animals can synthesize neAAs, the buffering effect of commensal bacteria would not be necessary. Our results might reveal a process of co-evolution whereby host and bacteria specifically adapted to eAA depletion. Finally,

not any commensal bacteria, but only the joint effect of *A. pomorum* (*Ap*) with either *L. plantarum* (*Lp*) or *L. brevis* (*Lb*) was sufficient to suppress yeast appetite. Therefore, all these findings indicate that microbiota affects food choice in a very specific way, not just as a food source. The open question that arises from our study is therefore what could be the mechanisms underlying the change in yeast appetite and improved reproductive output?

As discussed earlier, our data do not support the scenario where bacteria are producing eAAs, triggering a reduction in yeast appetite. Nevertheless, it is still possible that bacteria provide eAAs to the flies in a very local manner, in the gut for instance, leading to a ready utilization of these AAs, without increasing the overall levels in circulation. For example, these AAs could be immediately directed to maintain egg production. A recent study proposed that the expression of the gut peptide CNMamide is modulated by AAs, and that bacteria-derived AAs are sufficient to mimic AA availability. In the future, experiments addressing the effect of this peptide in our paradigm will help elucidate its role in the suppression of yeast appetite. However, a follow-up study from my research group, using isotope-resolved metabolomics data, showed that the amount of eAAs produced by bacteria is very small, especially compared to the amount necessary in the diet to suppress yeast intake (Henriques et al., 2020). Nonetheless, the level of AAs produced by bacteria was sufficient to alter egg laying, indicating a functional separation of the impact of the microbiome on behavior and reproduction (Henriques et al., 2020).

If bacteria-derived eAAs are not sufficient to explain the suppression of yeast intake, what mechanisms could be underlying this effect? One possibility is that bacteria are secreting specific metabolites

acting on AA sensing pathways to mimic the availability of AAs, and therefore reducing yeast appetite. Indeed, bacteria have been proposed to modulate the nutrient sensing TOR pathway in flies (Storelli et al., 2011). Another explanation could be the secretion of metabolites that would promote the use of the last AA storages. Increasing the rates of autophagy, for example, could be a way to increase free AAs in circulation. Bacteria could also produce metabolites acting on neuronal populations and modulating their activity to directly regulate feeding behavior. For example, they could act on neuronal subsets involved in chemosensory processing. In this context, the use of video-tracking systems to investigate the effect of bacteria in both exploitation and exploration of food sources could help unravelling a possible role in the modulation of sensory modalities. To disentangle among these hypotheses, the use of isotope-resolved metabolomics will be crucial to identify the metabolites secreted by the bacteria. Genetic, behavioral and functional characterization through gain- and loss-of-function approaches, both at the bacteria and host level, will be essential to start unraveling how gut bacteria are modulating feeding decisions.

Flies have a very simple microbiota composition, especially compared to vertebrate species, making them a powerful model for studying host-microbiota interactions. This allowed for the identification of a single bacterial species mediating physiological effects, such as the case of *L. plantarum* in growth (Storelli et al., 2011). Our data identified *Acetobacter pomorum* (*Ap*) and *Lactobacilli* as the bacteria influencing food choice. This finding provides a framework to understand how host-microbiota interactions modulate behavior and brain function. Moreover, since neither of these bacteria alone is sufficient to modulate yeast appetite, it is also important to understand how the bacteria-bacteria interaction

occurs to alter physiology and behavior in the host. In one scenario, both species could produce metabolites that are responsible for inducing changes in behavior. Another possibility is that one species supports the growth and survival of the other, allowing it to modify the host's behavior. The fact that *Ap* needs either *L. plantarum* (*Lp*) or *L. brevis* (*Lb*) already suggests that regardless of the mechanism, both *Lactobacilli* species should be providing the same component. Henriques *et al* (2020) explored these two hypotheses and found that *Ap* and *Lp* form a syntrophic relationship to overcome poor host dietary conditions. More specifically, *Lp* produces lactate, which supports the growth of *Ap* and its production of eAAs. These eAAs, in turn, allow the survival of *Lp* in eAA-deprived conditions, since it cannot produce some eAAs. Additionally, they showed that lactate is necessary and sufficient for *Ap* to modify yeast appetite in the fly. Therefore, this study identified *Ap* as the sole bacteria modifying feeding behavior in flies (Henriques et al., 2020). Future multidisciplinary studies involving metabolomic, genetic and behavioral analysis with manipulations in both host and *Ap* will now be crucial to investigate the metabolites and mechanisms underlying the effect of *Ap* on feeding decisions.

This thesis provided the first evidence of how commensal bacteria can affect nutrient-specific appetites, namely protein appetite. The paradigm developed here opens a new avenue to investigate the interaction between host, microbiota and nutrition. In a broader sense, these interactions also form a powerful framework to understand how the gut-brain axis functions to modulate behavior and physiology in animals.

5.3 – MARMITE IS A NOVEL SATIETY FACTOR REGULATING PROTEIN INTAKE IN *DROSOPHILA*

Low or abundance of nutrients in the body makes animals fluctuate between *hunger* or *satiety* states, respectively. Therefore, in order to maintain nutritional homeostasis, animals need to tightly regulate their intake of nutrients. But how these states are integrated in the nervous system and translated into behavioral changes to meet the nutrient requirements is still a main question in the field. In order to pinpoint the molecular mechanisms occurring in the brain that could underlie the behavioral responses associated to protein appetite, we generated a transcriptional profile of brains of flies kept in complete vs eAA-lacking diet. The transcriptomic dataset revealed an uncharacterized gene, *CG14075* - later named *marmite* (*mmt*) - as one of the top downregulated candidates in eAA-deprived flies.

How AA state is translated into changes in *mmt* expression remains an open question. How do *mmt*-expressing neurons sense AA state? As discussed previously, the mechanisms underlying neuronal AA sensing are still poorly understood. Moreover, besides direct AA sensing, these neurons could be receiving indirect signals from other neurons or from the periphery informing about AA levels. *mmt*-expressing neurons show arborizations in the pars intercerebralis, an area of the *Drosophila* brain known to harbor several neurosecretory cells, including insulin-producing cells, which directly sense circulating nutrients (Rajan & Perrimon, 2012). It is therefore possible that *mmt*-expressing neurons integrate information provided by other neuropeptides or peptide hormones to modulate Mmt function. A possible strategy to tackle this question is to identify the receptors expressed by Mmt neurons. The recent release of publicly

available Single-cell RNAseq datasets (Li et al., 2022) could provide insightful information about the repertoire of receptors expressed in these neurons. Future work will be required to dissect which and how molecular and neuronal inputs modulate *mnt* expression and the activity of Mnt-producing neurons.

Besides the *mnt* identification in a RNAseq experiment using brain samples, expression datasets from flybase and the fly cell atlas consortium also indicate that *mnt* is neuronally expressed (Gramates et al., 2022; Li et al., 2022). Moreover, sequence analysis revealed stereotypical features of a secreted peptide: a predicted signal peptide in the N-terminus and polybasic sites as potential targets for proteolytic cleavage. Together, these data suggest that *mnt* encodes a neuropeptide. As described in the introduction, several neuropeptides have been proposed to mediate the effect of internal nutrient states in behavior (S. Lin et al., 2019). However, the exact mechanisms by which they are orchestrated in the brain to produce a coordinated behavioral response remain unclear.

Since *mnt* expression changes according to AA state, I next sought to investigate whether this gene would be involved in regulating feeding behavior. Both loss- and gain-of-function manipulations supported a role of Mnt as a neuronal protein-specific satiety signal. FIT, a female-specific peptide, has been shown to act as a satiety hormone, with higher expression upon consumption of protein-rich food (Sun et al., 2017). However, it has also been shown to be involved in carbohydrate appetite (Carvalho-Santos et al., 2020). This peptide is produced in the head fat body and even though its receptor is still unknown, it seems to act via the insulin pathway to regulate food intake. It would be interesting to investigate whether Mnt and FIT are part of the same or parallel circuits to regulate protein intake.

Neuropeptides are often produced in small subsets of neurons with large cell bodies (Nässel & Zandawala, 2019). Both Mmt labeling via a custom-made antibody and genetic insertion of a *Gal4* driver into *mmt* locus revealed a sparse population of neurons labeled in the central nervous system. Interestingly, besides labelling in the cell body, vesicle-like structures were also observed in the neuronal projections, reinforcing our prediction of *mmt* encoding a secreted neuropeptide. The use of high-resolution microscopy will be crucial to investigate if Mmt is secreted at synaptic sites. Moreover, an important aspect to clarify in future work is whether Mmt is secreted into circulation. The use of cell culture assays or hemolymph analysis will be critical to investigate this.

In several examples, manipulating the activity of neurons secreting neuropeptides can mimic the loss- and gain-of function manipulations of the molecule itself (Hergarden et al., 2012; Min et al., 2016; Schoofs et al., 2014). Activation of neurons labeled by *mmt-Gal4* showed a clear decrease in yeast intake, which was *mmt*-dependent. However, silencing of *mmt-Gal4* had not observable effect. A possible explanation is that the silencing was not sufficient to prevent Mmt release from all neurons and therefore the induction of feeding was not observed. Another possibility is that not all cells labeled by this line lead to the same behavioral effects. For example, silencing of a subset of this cellular population might lead to a decrease in yeast intake, independent of Mmt. This opposing effect could mask the behavioral response of blocking Mmt release, resulting in no visible differences.

Drosophila is a powerful system to narrow down effects of a neuronal population to a single neuron. The expression pattern of *mmt-Gal4* and antibody co-staining experiments revealed a pair of neurons with

large cell bodies in the anterior medial part of the brain. Neuronal manipulations suggested that this pair of *mmt*-expressing neurons is necessary and sufficient to alter yeast intake. Thus, now that we have established an entry point to the Mmt circuitry, subsequent analysis to identify where these neurons receive their inputs and extend their axonal projections will provide insights to their neuronal partners. Moreover, the use of downstream labeling techniques will be crucial to provide an anatomical and functional identification of post-synaptic neurons. To my knowledge, this pair of *mmt*-expressing neurons has not been previously identified as producing feeding-related neuropeptides in *Drosophila*. Nevertheless, in the future, it will be important to explore whether or not *mmt*-expressing neurons produce other peptides or are connected with neuronal populations already described to play a role in feeding behavior.

At the molecular level, downstream neuropeptide signaling is mediated by its receptor. Most neuropeptides bind to G-protein-coupled receptors (GPCRs). The *Drosophila* genome is predicted to encode over 100 GPCRs and for around 20% of them, no ligand has been identified, making these orphan GPCRs (Hanlon & Andrew, 2015). A key open question from this thesis is what is the Mmt receptor. Historically, GPCR ligands have been found using heterologous expression systems, where specific GPCRs can be exposed to individual ligands to test for functionality. Alternatively, new *in silico* methods exploring protein-protein interactions could be used to predict ligand-receptor pairs. Another possibility is to take advantage of the powerful genetic toolkit of the *Drosophila* model to perform a functional screen to identify receptors specifically involved in the phenomena being studied. In the Mmt case, one could imagine a scenario combining *mmt* overexpression with specific

GPCR knockdown. The GPCRs whose knockdown abolished the overexpression phenotype would be good candidates for follow-up analysis. The identification of Mmt's receptor will be crucial to investigate the cellular populations downstream of Mmt signaling and to unravel the mechanisms underlying the effect of Mmt in protein appetite.

5.4 – DISCOVERY OF A NOVEL FUNCTIONAL AND EVOLUTIONARILY CONSERVED FAMILY OF NEUROPEPTIDES

Neuropeptides are ancient signaling molecules that emerged very early in animal evolution (Elphick et al., 2018). Thus, animals, from flies to humans have neuropeptides that share evolutionary and functional conservation. Neuropeptides involved in food intake and nutritional regulation are not exception, and there are several cases of conserved neuropeptides between vertebrates and insects: NPY/NPF, CRF/Dh44, CCK/DSK and Neuromedin U/Hugin are some examples (Nässel & Zandawala, 2019). However, neuropeptide discovery is not easy. Their small size and the conservation of only a few critical residues in most cases, makes the discovery of evolutionary relationships based on sequence queries very difficult (Elphick et al., 2018).

Our bioinformatic sequence analysis showed that the newly identified Mmt peptide shares a conserved C-terminal domain including four cysteine residues with other *Drosophila* and invertebrate species. Further sequence analyses revealed that this domain is also present in AA sequences corresponding to the prepropeptide of the vertebrate neuropeptide B (NPB). In turn, the mature peptide derived from the NPB prepropeptide, which corresponds to its N-terminal part, shares sequence

and functional similarities with neuropeptide W (NPW), another vertebrate neuropeptide.

In chapter 3, we demonstrated that Mmt and Mmt-producing neurons play a role in the regulation of food intake. NPB and NPW have also been linked to feeding regulation in rodents (Chottova Dvorakova, 2018). Therefore, we investigated whether Mmt and NPB/W could also be functionally conserved. Our gain-of-function experiments showed that, like *mmt*, overexpression of the human *NPB* or *NPW* genes led to a decrease in protein intake in flies. This result was very interesting, corroborating the idea that Mmt and NPB/W conservation also extends to the functional level.

An important question that arises from this functional experiment is which part of the Mmt prepropeptide is mediating the effect on food intake. At the moment, it is not known whether and how this precursor is cleaved and processed and what is its bioactive form. As mentioned above, the most conserved part between Mmt and NPB prepropeptides is the C-terminal domain (Marmite domain). However, NPW does not possess the Marmite domain comprised of the four cysteines. This suggests that maybe the N-terminal part of the Mmt prepropeptide between the signal peptide and the potential cleavage sites is the one mediating the biological effect. Indeed, for NPB and NPW, it is the N-terminal part that has been shown to regulate food intake (Aikawa et al., 2008; Baker et al., 2003; A. S. Levine et al., 2005; Mondal et al., 2003; Naso et al., 2014; Samson et al., 2004; Shimomura et al., 2002; Tanaka et al., 2003). Further studies, overexpressing different regions of the Mmt and NPB/W prepropeptides could provide insights into what is the bioactive peptide mediating the feeding regulation in flies.

After the results with *Drosophila*, a mouse model was used to further investigate the functional conservation between Mmt and NPB on food intake. Using a gain-of-function approach, both *mmt* and *NPB*-expressing virus injected in the mouse brain led to an increase in total food consumption. Even though NPB and NPW have been shown to affect food intake in rodents, the mechanisms underlying their mode of action remain unclear. Some studies reported anorexic effects (Aikawa et al., 2008; Mondal et al., 2003; Naso et al., 2014; Tanaka et al., 2003) whereas others showed an orexigenic effect of these peptides (Baker et al., 2003; A. S. Levine et al., 2005; Samson et al., 2004; Shimomura et al., 2002). *mmt* had the same effect as *NPB* expression in mice, suggesting that their biological activity is related. However, the fact that in the fly model there was a reduction in feeding and in the mouse model there was an increase in food intake cannot be ignored. These results might appear contradictory, but several factors could be underlying these different effects. First, as stated above, the actual mode of action of NPB in rodents is not clear, thus, complexities of its function could lead to different outcomes, especially in different species. Second, NPB could exert different effects depending on its target site in the brain, since both the peptide and the receptor have a complex expression pattern. This could also explain the distinct results in rodent studies. Another important aspect to consider is the nutritional state of the animals as well as the nutrient composition of the food presented. In *Drosophila*, *mmt* acts as a protein-specific signal. A further systematic analysis of feeding regulation with different nutrient options would be important to clarify whether, in mice, Mmt and NPB also have a protein-specific effect. Related to this, it is worth mentioning that a previous study showed that gastric *NPW* levels in mice seem to be mainly responsive to the protein content of the food, suggesting a role in protein regulation (Li

et al., 2014). Based on the rodent studies and the results presented in this thesis, it seems clear that NPB/W signaling plays a role in feeding regulation but the mechanisms underlying their effects are still unclear. A deeper understanding of how these neuropeptides act will require a careful molecular and circuit dissection in the context of precise conditions, with special attention to the nutrient content of the food tested.

5.5 – CONCLUDING REMARKS

Animals are constantly performing actions to achieve desired outcomes. Depending on the behavior, more or less complex neuronal computations are employed as well as the integration of contextual information from both the internal and external worlds. Thus, the nervous system has the important role of integrating these two types of information to generate an adaptive behavioral output in order to maintain the organism in a relatively constant and optimal state.

Animals do not always execute the same behavior to the same stimulus. How the nervous system mediates this behavioral flexibility is still an open question. One important factor is the internal context of the animals, which is not always the same. In this thesis, I showed how different internal states - nutritional and microbial - play a key role in modifying animals' behavioral responses to the same context. Therefore, these data highlight the importance of considering the internal milieu when designing experiments to study animal behavior. The precise manipulation of these or other states is critical to establish more accurate conclusions. In this sense, the use of quantitatively approaches, both at the level of

Careful state manipulations, and precise behavioral measurements are essential aspects to be considered in future studies.

Drosophila melanogaster is a powerful model organism for scientific research. It has an immense genetic toolkit, it is easy to maintain in laboratorial conditions and it is a great system for high-throughput screens. Moreover, even though *Drosophila* has a less complex nervous system than vertebrates, it exhibits a vast repertoire of adaptive and rich behaviors. The discovery of Mmt provides a great example of how fruit flies are important models for the identification and functional characterization of new molecules and neuronal mechanisms. I hope that this discovery acts as an inspiration to keep using this animal model to further our understanding of how genes, molecules and behaviors are interconnected. Indeed, it is very likely that other molecular mechanisms remain to be revealed. The recent sophisticated methods and technologies to perform molecular, neuronal, and genetic manipulations are key instruments to advance our knowledge of how behavior, and specifically feeding behavior is generated.

In this thesis, I identified Mmt as a novel neuropeptide mediating nutrient-specific appetite in *Drosophila*. This work provided the first functional characterization and laid the foundations for future studies to expand our knowledge of how the function of this neuropeptide and its neuronal circuitry is integrated at the organismal level to modulate food intake. Another major finding of this thesis is the identification of evolutionary relationships and functional conservation between Mmt and the vertebrate neuropeptides B and W. This discovery showcases how multi-phyla comparative approaches are important to improve our

understanding of conserved biological processes, taking advantage of the power of each model organism.

Neuropeptides remain among the most difficult molecules to study due to their small size, multidimensional regulatory mechanisms and complex mode of action. Nonetheless, their important regulatory roles in physiology and behavior make them key players in understanding biological functions in both physiological and disease contexts. The identification of novel neuropeptides, such as Marmite, opens new, fascinating and important research avenues to expand our understanding of the molecular and neuronal mechanisms by which animals modulate food intake to maintain nutritional homeostasis.

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