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Effects of the neuropeptide sNPF on honey bee behaviours

(*Apis melllifera*)

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Contents

Introduction

0.1 Animal motivation

Animals have evolved effective ways to evaluate the surroundings using sophisticated and complex chemosensory organs. Sensory inputs are conveyed and processed within the brain to drive appropriate innate or adaptive behavioural responses for survival. Although considered to be hardwired, innate behaviours are also flexible and subject to modulation by internal states, external stimuli and experience-dependant plasticity [1, 2]. A key concept in the modulation of behaviour is 'motivation', a term that can be defined as the "energizing of behaviour in pursuit of a goal" [3]. A general aspect of motivated behaviours is that they lead to a goal that is rewarding *per se*. In fact, Hull postulated precisely that behaviour occurs to reduce biological needs [4]. Later, motivation was proposed to include both a goal-directed (in the sense postulated by Hull [4]) and an arousal component [4, 5]. In this view, appetitive motivation, leading an animal to obtain food reward, is crucial for an animal's survival and should drive food-search behaviour in an appropriate way.

The regulation of motivated behaviours is achieved by the coordinated action of molecules (peptides, hormones, neurotransmitters, etc.), acting within specific circuits that integrate multiple signals to ensure the coordination between the external and internal milieu [6]. Among these molecules, neuropeptides such as the neuropeptide Y (NPY) of vertebrates and the neuropeptide F (NPF) of invertebrates play a fundamental role in determining specific motivational states. However, the question of how neuropeptides change the information flow within neuronal circuits remains largely elusive [7] and starts only to be understood *via* studies on these two neuropeptides which seem to have convergent functions in vertebrates and invertebrates.

0.2 Neuropeptides: key regulators of animal motivation

Neuropeptides are peptides functionally diverse secreted by a huge variety of neurons of the central nervous system, ranging from a few to around 100 amino acids. These neuroactive substances

regulate locomotion, odour-guided foraging, activity/sleep, feeding, aggression and reproductive behaviour, as well as learning and memory [8, 9]. They are defined as "small proteinaceous substances produced and released by neurons through the regulated secretory route and acting on neural substrate" [10]. They bind to specific membrane receptors, mainly seven transmembrane-domain G-protein coupled receptors (GPCR) [11], a binding that initiates second-message cascades resulting in distinct molecular responses [12]. Neuropeptides have a course of action lasting from several seconds to several days [13] and can diffuse over tens of micrometers to reach their specific receptor, meaning they can act at a distance from their release sites within the central or peripheral nervous system [14]. However, their spatial and temporal range of action is limited by the distance they cross and by peptidase activity. After binding, the receptor activation can be ended by desensitization and receptor internalization. Neuropeptides generally act as neuromodulators. In some cases, the same molecule can have various role such as hormone, neuromodulator at a global and local scale or as local co-transmitter [8].

0.3 The neuropeptide Y and its role in vertebrate behaviours

One of the most studied neuropeptides is the neuropeptide Y (NPY) of vertebrates, known for being a key neuromodulator at the crossroad between hunger, motivation and stress: it regulates multiple appetitive behaviours [15, 16], confers resilience to diverse nociceptive stimuli [9, 17] and modulates cognitive abilities expression [18]. It is secreted by the hypothalamus and its main effect is to increase food intake and decrease physical activity, among others.

Many studies have highlighted the stimulatory role of NPY on food intake. An acute central administration of NPY promotes food ingestion in rats [19, 20]. When NPY is chronically administrated, rats' physiology mimics metabolic abnormalities, such as an increase in the insulin responsive glucose transporter protein and triglyceride content, that are observed in the case of obesity [21]. NPY is thought to induce food consumption via an increase of motivation to eat as rats treated with NPY will endure electric shocks to obtain food [22, 23]. Central chronical injection of NPY also promotes food-seeking behaviours in rats [24].

NPY expression is subjected to stress-modulation. While a single stress event (restraint) downregulates NPY expression in the amygdala of rats, repeated stress events upregulate NPY expression [25]. NPY receptors distribute in the arcuate nucleus of hypothalamus (ARC), a structure involved in pain processing. To test whether NPY modulates nociception, rats performed nociceptive tests consisting in placing the rat's left or right hindpaw on a hot plate which was maintained at a temperature of 52°C. The time to hindpaw withdrawal was measured and referred as hindpaw withdrawal latency (HWL). Administration of NPY in the ARC increases in a dose-dependent

manner HWL to thermal and mechanical stimulation in rats [26], suggesting that NPY exerts an antinociceptive effect in the ARC.

0.4 NPF and sNPF: the invertebrate pendants of NPY

The invertebrate pendants of NPY are the neuropeptide F (NPF) and its short version sNPF [27]. They have been mostly studied in insects and molluscs which are ideal model organisms for studying the underlying molecular mechanism of hardwired innate behavioural responses as well as of behaviours acquired by learning and memory.

Neuropeptide NPF is the invertebrate orthologue of NPY, the most abundant neuropeptide in the nervous system of vertebrates [27]. NPF and NPY are named after their C-terminal end which is an aminated phenylalanine (F) and an aminated tyrosine (Y) residue, respectively. NPF is described as a superfamily of NPY as both its structure and functions are evolutionary conserved with vertebrate NPY. NPF was first identified in the platyhelminth *Moniezia expansa* [28], followed by identifications in molluscs [29, 30] and annelids [31]. Among insects, it was first characterized in the corn earworm *Helicoverpa zea* [32]. In some insect species such as in the migratory locust, *Locusta migratoria*, the desert locust, *Schistocerca gregaria*, (both belonging to the same taxonomic family) and *H. zea*, only a C-terminal fragment of NPF could be identified while the predicted "full-length" NPF peptide remained not found [33, 34]. Yet, those naturally occurring shorter peptides are truncated forms of the "long" NPFs with the same functions [33, 34, 35]. In the genome of hymenopteran species the single NPF sequence shows some variability in the C-terminus compared to other insects, which may have functional consequences [234].

NPF nomenclature is divided into two categories according to the size. While NPF is between 36-40 amino acids residues, the short version of NPF (sNPF) only consists of 8 to 12 amino acid residues [37]. The latter was first discovered in insects such as the Colorado potato beetle *Leptinotarsa decemlineata* [38] and the desert locust *S. gregaria* [39]. The way NPF and sNPF are encoded in the genome of several insect species shows some variability. For instance, NPF precursor gene encodes a single peptide in *Drosophila melanogaster* and in most insects [40] whereas in *Bombyx mor*i two separate genes encode NPF1 and NPF2 [41]. sNPF precursor encodes several peptides: four (sNPF 1-4) in the fruit fly [37] or three (sNPF 1-3) in *B. mori* [42] and only one in the honey bee [43]. In *D. melanogaster*, all sNPF peptides bind on a single G-protein-coupled receptor (sNPFR) while NPF is acting on another (NPFR). Both receptors might be related to the mammalian NPY receptor [44, 45, 46] as *Drosophila* NPFR expressed in *Xenopus* oocytes can bind with NPY-type neuropeptides and be activated [47]. Surprisingly, hymenoptera genome's does not seem to encode a clear *npfR* orthologue and only one orthologue to *snpfR* was identified

[43, 48, 49], suggesting that it may be a real gene loss. Ament et al., (2011) postulate that *npfr* gene was present in the common ancestor of bees and wasps but lost early in hymenopteran evolution [234].

Both NPF and sNPF share similarities in their carboxyterminal sequence and their range of actions, therefore sNPF-signalling is described paralogous to NPF-signalling in protostomes [35]. They originated in the common ancestor of deuterostomes (anus develops first) and protostomes (mouth develops first) but belonged to different signalling systems so they are evolutionary distant. sNPF-type neuropeptide signalling system was thought to be unique to arthropods [35]; yet it is also present in other protostomes (for instance in the nematode *Caenorhabditis elegans*) [50]. Interestingly, sNPF system might have been lost during chordate evolution, or has evolved into the prolactin releasing peptide signalling system which also modulates feeding behaviours [51, 52].

0.5 Location of NPF-sNPF in insects

The npf gene and NPF peptides are widely distributed in neurons of the central nervous system (CNS) (brain, suboesophageal ganglia) and endocrine cells of the midgut in both adults and larvae *D. melanogaster* [40], in the oriental tobacco budworm *Helicoverpa assulta* [53] or in the yellow fever mosquitoe *Aedes aegypti* [54]. In *Drosophila* larvae, NPF is expressed in four neurons in the brain whereas the adult brain contains approximately 30 NPF-positive neurons [55]. NPFR colocalizes with dopaminergic neurons in the larval *D. melanogaster* CNS which already highlights an extensive interplay between these two signalling pathways [56, 57]. *snpf* gene and sNPF peptide are broadly expressed in neurons of the CNS in adult *D. melanogaster* [58, 59], from several hundred to several thousand of neurons. These neurons are mostly Kenyon cells (intrinsic neurons of the mushroom bodies, a high order brain region of the insect's brain involved in associative learning, olfactory learning and memory [60, 61]) and in smaller proportions interneurons of the CNS, olfactory sensory neurons and neurosecretory cells innervating *corpora cardiaca* and *aorta* [59, 62]. Contrarily to adults, neither sNPF nor its receptor was expressed in sensory systems of the larva [62]. Yet, sNPF and sNPFR were mainly found in the CNS and in a subset of cells in the hypocerebral ganglion associated with the foregut and midgut of *Drosophila* larvae [62]. In honey bees, *Apis mellifera*, *npf* and *snpf* are also expressed in the brain of foragers and in distinct clusters of neurosecretory cells: *npf* was localized in 8-10 medial neurosecretory cells whereas *snpf* was found in 4-6 pairs of lateral neurosecretory cells (fig. 1) [234]. Transcripts encoding NPF and sNPF were also identified in the honey bee midgut [243].

Once these peptides have been identified in an insect's genome, functional studies have followed to unravel their influence on insect's behaviour. The following sections will describe how NPF and

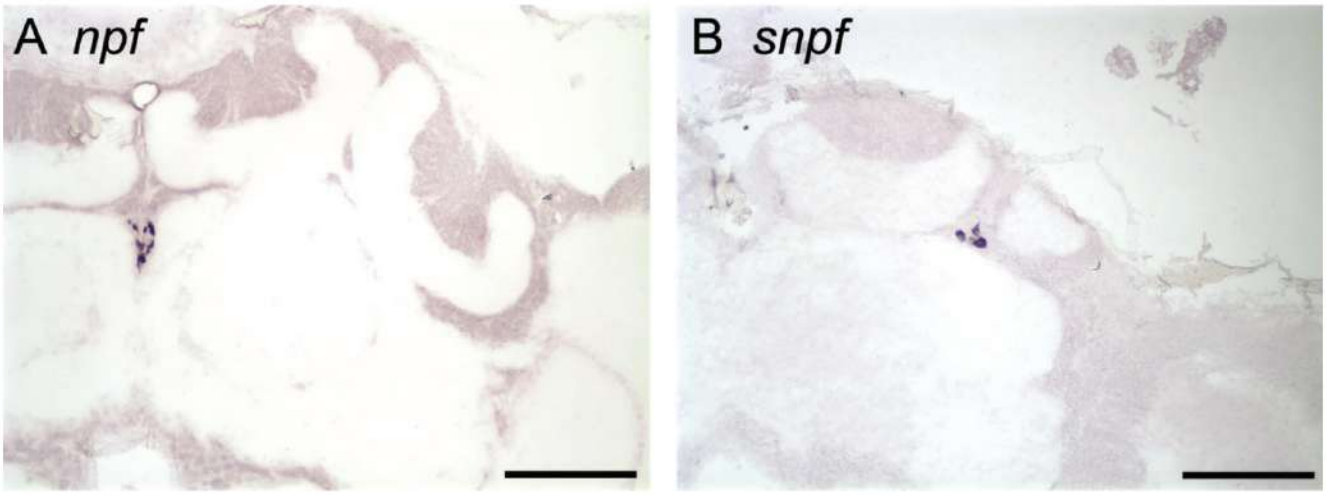

Figure 1: ***npf* and *snpf* are expressed in distinct populations of neurosecretory cells in the honey bee brain.** In situ hybridization of digoxigenin-labelled probes specific to *npf* **(A)** and *snpf* **(B)** to 10 mm coronal cryosections of forager brains. The mushroom body calyces are shown at the top of each image. *npf*-containing cells are located within a population of large cells located dorsally at the midline (within the pars intercerebralis) that can be identified from their position and morphology as neurosecretory cells. *snpf*-containing cells are located in a separate population lateral to the mushroom body calyces that resemble the lateral population of neurosecretory cells. Scale bar = 400 µm. From Ament et al. 2011 [234].

sNPF influence mostly appetitive and aversive behaviours of insects.

0.6 NPF-sNPF signalling in appetitive context

The regulation of feeding behaviour via NPF-sNPF signalling has been extensively studied in *D. melanogaster* [49], but also in other insect species, even if to a lesser extent [49]. Here we will review basic facts about NPF and sNPF modulation of appetitive and stress related behaviour in insects, focusing on fruit flies, but also on other insect species in which relevant findings have been obtained.

0.6.1 Modulation of food search, food perception and food intake by NPF in insects

In *Drosophila* larvae, feeding is continuous until the last third instar larva stage where individuals exhibit food aversion and increase their mobility [64]. Brain expression of *npf* is high in young larvae with high feeding activity whereas its downregulation coincides with food aversion in third-

instar individuals [28, 65]. By manipulating the level of expression of *npf*, transgenic larvae without NPF-signalling precociously exhibit phenotypes displayed by older larvae (i.e. food aversion and social behaviours such as social foraging and clump aggregation) [65]. Conversely, overexpressing NPF in the larval CNS and midgut prolongs feeding and inhibits social foraging [65]. Thus, the experimental inference with NPF-signalling mimics the developmental switch. NPF is also thought to participate in the developmental regulation of physiological processes or behaviours in the pea aphid as its expression varies between the first and last larval stage [66]. In addition, the effects of NPF on feeding regulation were investigated in larvae of several lepidopteran species were an injection of dsRNA to knockdown NPF or NPFR decreases food feeding and body weight [67, 68] whereas an increase of NPF levels enhances those phenotypes [32]. Interestingly, injecting dsNPF in the 5th-instar Asian corn borer larvae (*Ostrinia furnacalis*) inhibits electrophysiological responses of the medial *sensilla styloconica* to deterrents [69], thus suggesting that NPF might mediate the sensitivity of gustatory receptor neurons.

One of the earliest experiment studying NPF-signalling in *Drosophila* larvae's appetitive behaviour shows that *npf* expression in a pair of two distinct neurons of the suboesophageal ganglion is evoked with taste perception of sugar but not sugar ingestion [70]. Additional experiments demonstrate that overexpression of *npf* or *npfr1* in *Drosophila* larvae increase intake of unpalatable food under starvation via the modulation of the reward circuitry which inhibits food avoidance normally elicited [71, 72]. In starved adult flies, NPF enhances sugar sensitivity via dopamine signalling resulting in a sensitization of the sweet-sensing gustatory receptor Gr5a [249]. Thus, it increases the acceptance of lower concentrations of sweet tastants while leaving bitter sensitivity unchanged [249] (fig. 2). These results show that hunger state results in modulation of NPF-signalling, which affect food perception and food attractiveness.

Feeding flies with a diet of the artificial sweetener sucralose leads to a chronic sweet/energy imbalance, which in turn enhances sweet taste perception and increases feeding motivation [75]. Knockdown of NPFR in sweet taste neurons suppresses sucralose-mediated increased food intake [75]. Additionally, activation of specific NPF neurons in the CNS regulate thirst behaviour by promoting water-seeking and water-intake [76].

In honey bees, injection of NPF into the brains of foragers kept in cages did not influence their consumption of sugar syrup [234], which is consistent with findings in *Drosophila* where NPF promotes indiscriminative food selection but not ingestion of rich, palatable foods [72, 71]. However, NPF seem to be a positive regulator of feeding in *L. migratoria* [77], *S. gregaria* [78] or in *B. mori* [79].

Taken together, these results suggest that in the context of food intake 1) NPF is a key neurotransmitter for larval development 2) NPF-signalling plays a role from the detection of a tastant

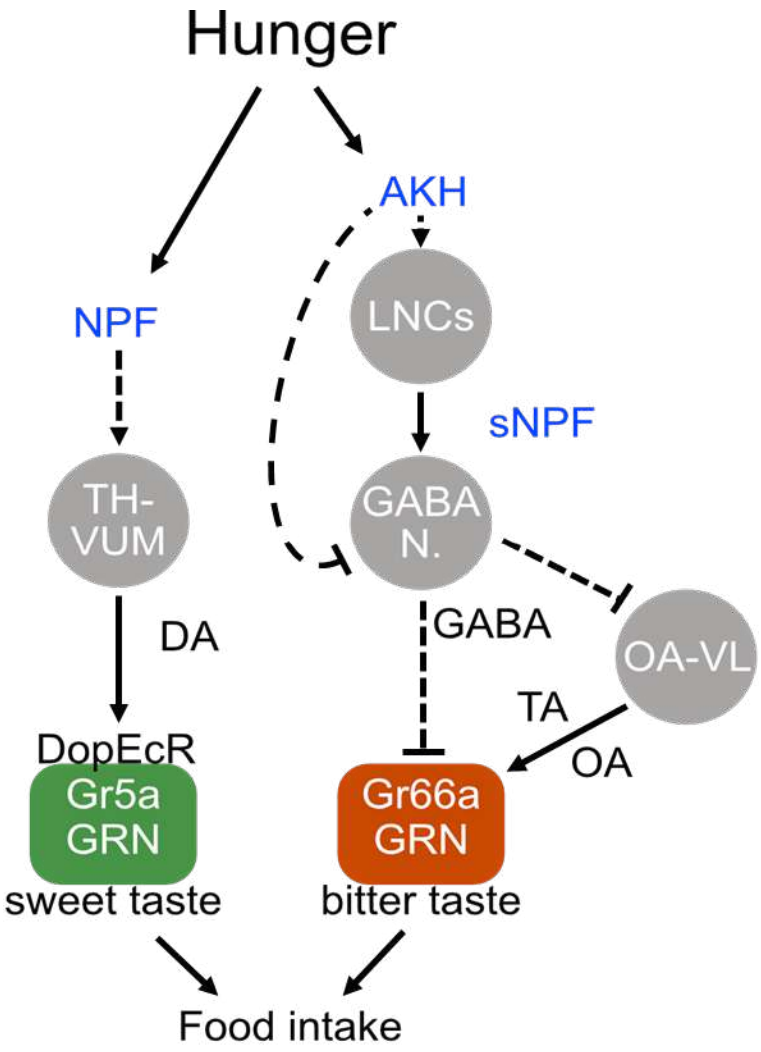

Figure 2: **Modulation of gustatory responsiveness in the adult *D. melanogaster*.** Among many Hunger modulates GRNs (orange and green) to promote food intake. Starvation increases the release of NPF, which indirectly activates the dopaminergic TH-VUM neurons that in turn potentiate sweet taste-responsive Gr5a neurons via the dopamine receptor DopEcR. Starvation also increases the release of adipokenic hormone (AKH), which indirectly activates sNPF-releasing lateral neurosecretory cells. sNPF then activates as yet unknown GABAergic neurons that inhibit the bitter taste-responsive Gr66a neurons. The same GABAergic neurons may also inhibit OA-VL neurons that can potentiate Gr66a neurons by releasing tyramine (TA) and OA. Dashed lines indicate the regulation is indirect or its underlying mechanism is not fully understood. Adapted from Lin et al. 2019 [74].

to its integration in higher-order brain centres under starvation conditions 3) NPF regulates the motivation to feed rather than directly affecting consumption.

0.6.2 Modulation of food search, food perception and food intake by sNPF

As mentioned above, NPF and sNPF signalling are evolutionary distant but both regulate, yet in different ways, feeding behaviours in insects. In *Drosophila* larvae, sNPF does not influence the behavioural switch from feeding to wandering larvae as NPF does, but rather stimulates food intake, and thus growth and development in both adult and larvae [80, 81]. The action of sNPF on growth is regulated via the insulin signalling pathway in *Drosophila*: sNPF activates extracellular-activated receptor kinases in insulin-producing cells, which in turn modulate insulin-like peptides expression [82, 83]. In addition, blocking sNPFR signals in insulin-producing cells neurons inhibits growth while overexpression of *snpfr1* in the same insulin-producing cells neurons has the opposite effect [82].

sNPF modulates food perception and acceptance in an independent modulatory pathway of NPF [249] (fig. 1). Decrease of bitter sensitivity mediated by sNPF in starved flies [249] leads to the acceptance of bitter substances that the insects would normally rejected [71]. In fact, orthogonal neuromodulatory cascades control peripheral taste sensitivity for sugar and for bitter substances. While sugar sensitivity is increased via NPF and dopaminergic signalling acting on sweet taste receptors, bitter sensitivity is reduced via the action of the adipokinetic hormone (AKH), sNPF and GABA-ergic neurons inhibiting bitter-taste receptors [249] (fig. 2). Moreover, these pathways are recruited at increasing hunger levels, such that low-risk changes (higher sugar sensitivity) precede high-risk changes (lower sensitivity to potentially toxic resources). In this way, state-intensity-dependent, reciprocal regulation of appetitive and aversive peripheral gustatory sensitivity permits flexible, adaptive feeding decisions.

Studies show that sNPF can act as a stimulating or inhibiting factor in feeding and metabolism depending on the insect species considered. In cockroaches and flies, hunger modulates sNPF-sNPFR signal as starving individuals have a higher expression level of *snpfr1* than fed individuals [84, 85]. sNPF-signalling is more ambiguous in *B. mori* where starved larvae have a decreasing expression of *snpf-1*, *snpf-2* and receptors, which allows food-seeking behaviour [86]. Yet, sNPF-2 have feeding acceleratory effects that shorten the latency to the onset of feeding [86]. In fire ants (*Solenopsis invicta*), hungry queens have a decreasing transcription level of sNPFR compared to well-fed congeners [43]. An injection of sNPF in the abdomen of desert locust reduces their food intake while knockdown of sNPFR increases feeding [87] thus suggesting that sNPF functions as

a satiety factor in this insect species. In *A. aegypti*, an injection of sNPF into the thorax reduced host-seeking behaviour at a level similar to that observed in blood fed mosquitoes [88]. In the Colorado potato beetle, sNPF regulates the diapause process, which is defined as a "physiological state of arrested metabolism, growth and development that occurs at one stage in the life cycle" [89]. In this study, sNPF is present in brain extracts of nondiaposausing individuals and absent in those of diapausing adults [89].

As previously mentioned, NPF and sNPF modulate feeding behaviours in distinct manners but both are necessary for proper control of food intake and metabolism. For instance, the increase or decrease of either npf or snpf expression dramatically reduces the resistance to amino acid starvation and lifespan of adult *Drosophila* raised on culture media lacking of amino acids [90].

0.7 Modulation of the appetitive learning and memory by NPS/sNPF

Learning consists in the acquisition of novel information based on individual experience. Memory consists in the sum of processes that allow encoding and storing the information acquired via learning, as well as retrieving it in appropriate circumstances [91]. In associative learning, animals learn to associate sensory cues with appetitive or aversive reinforcements so that these cues become predictive and provide additional incentive and direction to locate a particular food source, in the case of appetitive learning [92], or to avoid a potential harmful situation. This implies convergence between the neuronal pathways signalling cues and reinforcement information.

For animals to learn and form memories induced by learning, they have to be in the appropriate motivation to respond to appetitive reinforcements. For instance, in appetitive learning, satiated animals will have less interest in responding to food as reinforcement and may thus either exhibit impaired learning and memory, or even not learn at all the associations based on obtaining food [92, 93]. On the contrary, starved animals with an increased appetitive motivation will be better predisposed to learn and memorize cues predicting food delivery. When the food is located and consumed, the motivational drive to feed is neutralized, fitting with Hull's theory where behaviours occur to reduce biological needs [4]. The integration of neural system representing hunger and satiety with those of memory is still poorly understood. Yet, given the fact that NPF/sNPF are crucial key elements for the concept of appetitive motivation, it is of fundamental importance to study the relationship between learning and memory formation and NPF/sNPF signalling. A basic prediction that can be made given the evidence existing on NPF/sNPF signalling and appetitive motivation is that in those species in which it enhances appetitive motivation, NPF/sNPF will

have a promoting effect on appetitive learning and memory formation (e.g *Drosophila*). On the contrary, in species in which enhanced appetitive motivation is associated with downregulation of NPF/sNPF signalling (e.g. *B. mori*), learning and memory formation will be facilitated when this signalling is reduced.

In fruit flies, the relationship between NPF/sNPF and learning and memory has been studied using appetitive olfactory conditioning protocols as fruit flies can learn to associate an odour with sugar taste reinforcement [94]. Groups of flies were trained in a T-maze to discriminate two odorants; one was rewarded with sugar (CS+) whereas the other was not (CS-). Flies were transferred sequentially to the first arm presenting the CS- and then to the second containing the CS+. A performance index is calculated as the number of flies running toward the CS-, divided by the total number of flies in the experiment. In *Drosophila* larvae, three pair of NPF neurons that specifically modulate sugar reinforcement overlap with dopaminergic neurons [95], which form the most downstream element of the sugar reinforcing sub-circuit [96]. Interestingly, NPF neurons and NPFR are necessary for sugar reward learning using low but not high concentrations of fructose during olfactory conditioning [95]. Furthermore, artificial activation of individual NPF neurons changes the conditioned response from attraction to avoidance [95].

It is necessary for fruit flies to be hungry to effectively express appetitive memory performance processed in the mushroom bodies [97, 98]. NPF through NPFR promotes appetitive memory performance by suppressing the inhibitory activity of dopamine neurons innervating the mushroom bodies (MB) (fig. 3) [56]. Stimulation of NPF neurons in fed flies mimics the hunger state, resulting of hunger-induced expression of appetitive memory [56]. On the contrary, knockdown of npfr prevents hungry flies from expressing this memory (fig. 3) [56]. sNPF also plays a role in appetitive memory expression as its knockdown in mushroom bodies impaired olfactory appetitive memory in fruit flies [99].

In the desert locust (*Schistocerca gregaria*), sNPF has been studied in visual learning and memory performances [78, 100]. In this insect, starvation reduces the sNPF precursor transcript level in the optic lobes [101]. Yet, knocking down sNPF to mimic starvation state had no influence on the appetitive visual learning and memory of locusts [100].

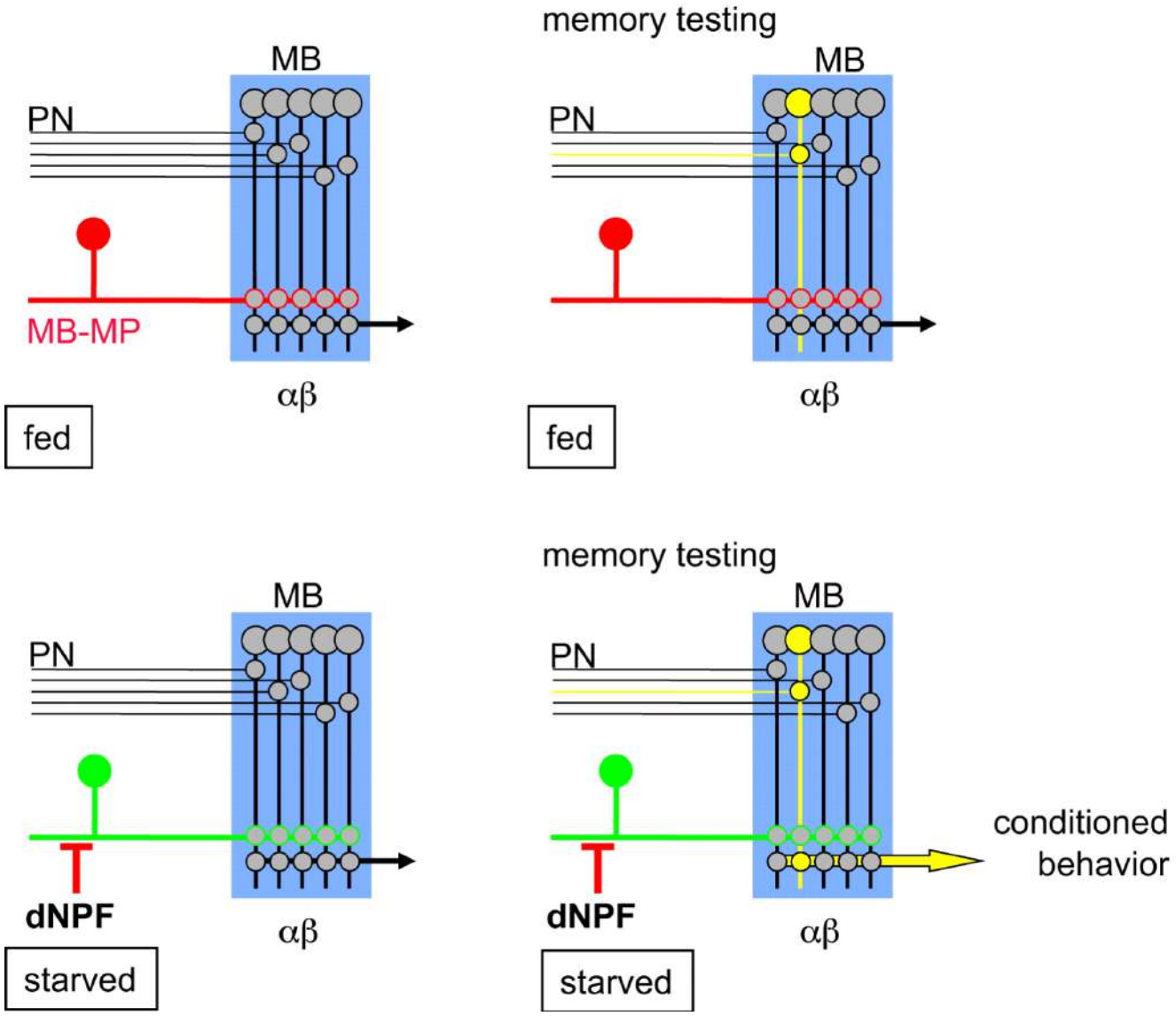

Figure 3: **Model for memory retrieval in *Drosophila.*** Left panels illustrate the state of the inhibitory control exerted upon the mushroom body (MB) in the fed state (top) and starved state (bottom). When fed flies are exposed to the conditioned odour during memory testing (right panels), the appropriate projection neurons and MB neurons are activated (yellow). However, the signal only propagates beyond the MB neurons in hungry flies when the MB-MP neuron "gate" is open. Red lines denote inhibition, and green lines denote relief from inhibition. From Krashes et al. 2009 [56].

0.8 NPF-sNPF signalling in aversive context

A function different from the regulation of appetitive motivation and food-search behaviour has been found in the fruit fly *D. melanogaster*. In *Drosophila* larvae, a downregulation of NPF coincides with food avoidance [65]. This behaviour is modulated by the activity of sensory neurons expressing the transient receptor potential ion channel A (TRPA) called PAINLESS (PAIN) on which brain NPF signalling is thought to act and inhibit the regular avoidance response to aversive stimuli [102, 103], such as thermal, mechanical, and chemical stressors [104, 105].

NPF modulation on thermonociception was tested in *Drosophila* larvae by means of an electric heating probe at 40°C [104] touching the abdominal segments. An aversive response elicited a stereotypical rolling behaviour within 1 s after the stimulation. Overexpression of NPFR1 in PAIN neurons delayed the aversive response of *Drosophila* larvae [103]. These results suggest that PAIN-mediated thermal nociception is modulated by NPF1 expressed in PAIN neurons.

As for another example in arthropods, NPF signalling is involved in aversive but not appetitive olfactory learning in *C. elegans* [106]. In appetitive learning, worms are placed on conditioning plates containing food and diluted butanone for training. To assess aversive learning, hungry worms are expose to diluted diacetyl, an appetitive odorant, without food. This conditioning switches the appetitive value of the odorant from attractive to repulsive. The results show that the NPF receptor in *C. elegans*, NPR-11, does not influence appetitive olfactory learning but mediates aversive olfactory learning through the signal of serotoninergic neurons [106].

0.9 The case of the honey bee *Apis mellifera*

0.9.1 The social life of honey bees

Honey bees are social insects living in colonies called 'superorganisms' and divided in three adult castes with distinct phenotypes: queens, workers (both females) and drones (males). Each cast plays a different role within the colony, which has consequences on lifespan and behaviours.

Queens lay eggs during their whole life, which in average lasts two years [107] or, in extreme cases up to four years [108]. In contrast, workers are sterile and vary in lifespan from 2-8 weeks during summer and several months during winter [109] when foraging ceases. Drones life span is only 4–5 weeks [110]. They die soon after mating with a new queen during mating flight. Within the worker caste, ontogeny determines the tasks executed in an age-depend manner. Young workers,

called nurses, are engaged in within-nest activities such as cleaning combs and nursing larvae. 2–3 weeks later they will become foragers collecting pollen, water, propolis or nectar for the colony [110]. This behavioural shift is accompanied with changes in diet [111], reduced blood proteins [112] and reduced lipid stores [113]. Such correlation between low internal nutrient stores and foraging has been reported in several hymenopteran species [113, 114]. However, the onset of foraging transition is flexible [115].

0.9.2 Appetitive motivation of honey bees in a foraging context

A food source will attract foraging visits of bees according to its capacity to deliver sugar- the principal energy carrier in the flower - per unit time. But the rate at which a forager can obtain sugar from this food source also depends on other related variables, such as concentration and viscosity of the sucrose solution, environmental temperature, probability of finding the food source, handling time invested to obtain sugar, etc. This leads to the concept of 'profitability' of a food source. A more profitable food source unifies the two previous aspects: it provides high power, and also allows a rapid extraction rate by the honey bee forager.

In experimental terms, the crop load attained in a foraging trip provides a suitable reading of food source profitability and of honey bee foraging motivation [116]. Individual crop load changes logarithmically with the flow rate of sucrose solution delivered at an automatic feeder, mimicking flow rates delivered naturally in flowers. It also varies with the concentration of sucrose solution delivered by the food source but if the ingestion units are converted from µl/min for every concentration assayed in mg sucrose/min, all ingestion curves obtained coincide in a single one, thus indicating that bees may evaluate the amount of sugar ingested per unit time as an essential criterion to evaluate food source profitability. Crop load also varies with the distance of the food source: yet the variation observed goes against individual optimization principles. Indeed, when the food source is close to the hive (e.g. 100 m) bees load less sucrose solution; on the contrary, when it is distant, bees load more sucrose solution. The explanation to this variation resides in the social control of foraging motivation and foraging activities: when the food source is close to the hive, bees shorten their exploitation of food sources to return faster to the hive and recruit more hive mates to a profitable food source. Indeed, the time spent away from the food source decreases with hive-food source distance and the number of recruited bees increase.

This peculiar aspects of honey bee foraging show that food search and foraging activities do not respond necessarily to individual needs, as seen in solitary insects, but are under the control of a society. Bees do not collect food for them but to satisfy colony needs and survival.

Figure 4: **Behavioural method for studying the gustatory responses of honey bees in appetitive contexts.** A bee harnessed within a cylindrical tube before, during and after antennal stimulation with a toothpick soaked in a sucrose solution. Contact of sucrose receptor neurons on the antennae with sucrose and other sweet tastants triggers the Proboscis Extension Reflex (PER), which is an appetitive response to food. From Bestea et al. 2021 [252]

0.9.3 Appetitive motivation in the laboratory

Appetitive motivation of honey bees can be assessed in lab condition by mean of a standardized protocol measuring sucrose responsiveness [117]. Proboscis Extension Response is triggered by antennal stimulation with sucrose solutions [118]. This reflex is used to examine a bee's sensitivity to varying sucrose concentrations. Bees are harnessed in tubes to they can only freely move the antennae and the mouth-parts. The experimenter stimulates the antennae of the bee (fig. 4) by mean of a toothpick soaked with increasing sucrose concentrations (0.1, 0.3, 1, 3, 10, and 30 % (w/w)). To avoid sensitization resulting from the antennal stimulation with sucrose, water stimulation was applied before 0.1 % sucrose and before each subsequent sucrose stimulation. The lower the concentrations a bee starts to respond, the more sensitive it is toward sucrose.

0.9.4 Learning and memory in the laboratory

The honey bee is a well-established model in neuroscience research to study learning and memory processes as reflex responses can be easily conditioned. Several protocols have then been established and either exploit the appetitive motivation of food search or the aversive context using a noxious stimulus. Here I will describe the mains conditioning protocols.

0.9.4.1 Conditioning of the approach flight towards a visual target in free-flying bees

Many visual stimuli can be used to condition free-flying honey bees, such as shapes and patterns, depth and motion contrast, among others [120, 121, 122]. Each bee is marked by means of a colour

spot on the abdomen and/or the thorax to record individual performance. The experimenter displaces the marked bee towards the training/test place where it is rewarded with sucrose solution to promote its regular return. Such pre-training is performed without presenting the training stimuli in order to avoid uncontrolled learning. Once the bee has visited the place actively without being displaced by the experimenter, the training stimuli are presented and the choice of the appropriate visual target reinforced with sucrose solution.

0.9.4.2 Olfactory conditioning of the proboscis extension reflex in harnessed bees

Honeybees can be conditioned to olfactory stimuli [118, 123]. In this protocol, each bee is harnessed so it can only freely move its antennae and mouth-parts. The antennae are the bees' main chemosensory organs. The experimenter will touch the antennae of a bee with a toothpick soaked in sucrose solution which will promote the proboscis extension response (PER) if the bee is hungry. Such display enables the bee to reach out to and suck the sucrose. Classical conditioning consists on blowing a neutral odorant to the antennae (which will initially not trigger PER) immediately before sucrose solution (forward pairing) [118]. It thus forms an association which enables to odorant to release the PER in the following test. Therefore, the odorant can be considered as the conditioned stimulus (CS) and the sucrose solutions as the unconditioned stimulus (US), i.e. the reward.

0.9.4.3 Olfactory conditioning of the sting extension reflex in harnessed bees

Contrarily to the previous protocols, this form of conditioning enables to study olfactory aversive learning in honey bees [124, 125]. The bee is harnessed in a holder specifically designed for aversive conditioning. The bee is fixed between two brass plates through which an electric shock can be delivered. An electric shock can be delivered (7 V) which induces an unconditioned, defensive reaction, the sting extension reflex (SER) [126]. Pairing odorants with electric shocks enables the SER conditioning so that bees learn to extend their sting when the experiment presents an odorant previously punished [124, 125]. It is also possible to use thermal shock as a negative reinforcement [127, 128].

0.9.4.4 Gustatory conditioning of the sting extension reflex in harnessed bees

Compared to olfactory or visual modalities, gustatory learning and discrimination in bees has been hindered because sucrose or other tastants are usually used as US during classical conditioning rather than stimuli to be learned. A new aversive differential conditioning recently established

enables to study gustatory learning in honey bees [129] in which they had to learn to distinguish a punished tastant paired with a shock (CS+) from an unpunished tastant (CS-) via antennal stimulation. Harnessed the same way as aversive olfactory conditioning, bees had to learn to extend the sting only to the punished tastant.

0.10 NPF and sNPF in honey bees

Although the role of NPF/sNPF has been studied in solitary insects such as fruit flies, cockroaches, silk moths and locusts - among others – (see above) mostly focusing on their role for appetitive behaviour, functional studies investigating the impact of NPF and sNPF on the behaviour of social insects, and in particular in the honey bee, a well-established model in neuroscience research [130], are missing dramatically. Innate behaviours driving stress and hunger follow different rules in bees compared to solitary insects such as the fruit fly, as they are both under the control of social cues such as alarm pheromones [131], which trigger collective defence in the case of potential stressors, and because bees collect food for colony stores rather than for individual needs. In addition, associative learning and memory formation in both appetitive and aversive contexts are required for foraging activities, an essential task to ensure the colony's survival. Both NPF and sNPF have been identified [237] in honey bees but only sNPF has an identified receptor [43, 48], which justifies the main focus of this thesis on the short neuropeptide. Although it is possible that both NPF and sNPF bind to the same sNPF receptor (sNPFR), the more parsimonious approach is to assume that only sNPF binds to sNPFR and thus to concentrate the first studies on this short neuropeptide. A prior study addressing the role of NPF/sNPF signalling for appetitive behaviour in honey bees showed that the *npf* gene was upregulated in the brain of foragers compared to that of nurses, irrespective of their diet [36]. However, injection of NPF into the foragers' brain did not influence their sugar syrup intake; *snpf* and its receptor gene *snpfR* were partially upregulated in the brain of foragers; yet, only the latter increased its expression when colonies were food deprived (fig. 5) [36]. No upregulation of *snpfR* was observed in the brain of foragers from well-fed colonies (fig. 5). Furthermore, when levels of sNPF were compared between nectar and pollen foragers arriving at an artificial feeder, significantly higher levels were found in nectar foragers [235], thus suggesting a dependency of sNPF on food type.

The last revision of the honey bee genome annotation assigned new access number to several predicitons and genes. The sNPF sequence is GB42678-RA *A. mellifera* and the new number in Genbank is XP8006561748; as the homology assignation is automatic, it was assigned the name "prolactin-releasing peptide receptor[*Apis mellifera*]" which is its closes ortologue in vertebrates.

Studies manipulating sNPF levels and determining the impact of this manipulation on ap-

406 petitive responsiveness and other forms of sensory processing are missing. In addition, whether
407 or not sNPF signalling affects different forms of learning and memory in bees remains unknown.
408 Furthermore, the potential link between sNPF signalling and aversive responsiveness in bees has
409 not been explored so far.

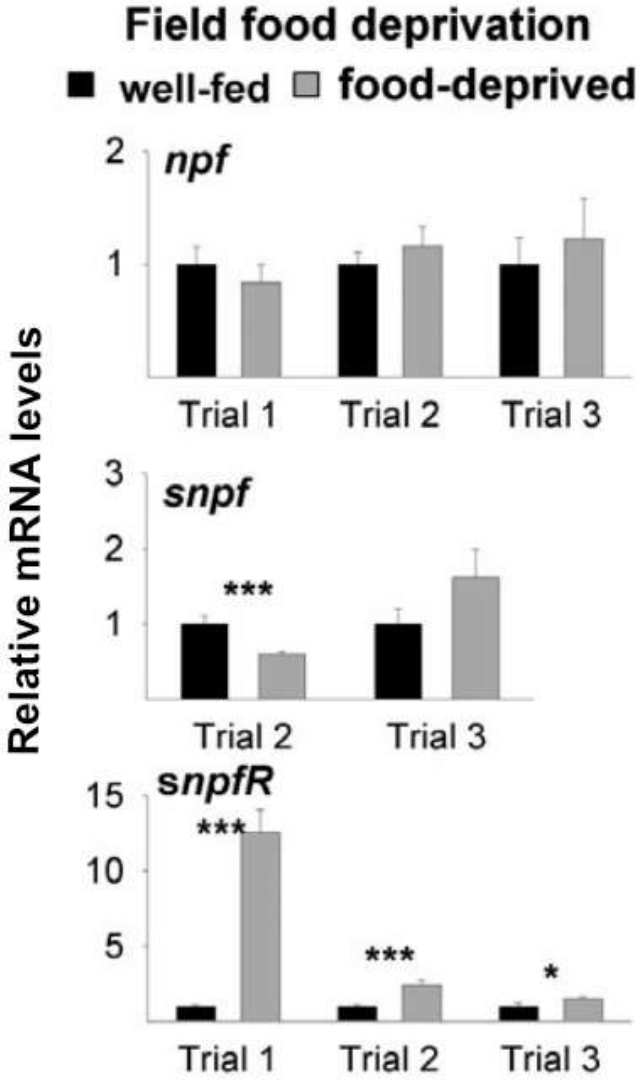

Figure 5: **Expression of *npf*, *snpf*, and *snpfR* in brains of worker honey bees following colony food deprivation.** One-day-olds were placed into miniature colonies in the field and were either 'well-fed' with *ad libitum* pollen and honey or 'food-deprived' by feeding only honey for 2 days followed by 2 days without food. N = 9–10 bees/trial. Mixed Model ANOVA for food-deprivation: *npf*: $P_{trt(1,42)} > 0.05$, $P_{trtxtrial(2,42)} > 0.05$; *snpf*: Ptrt(1,27) > 0.05, Ptrt x trial(2,27) < 0.01; *snpfR*: $P_{trt(1,44)} < 0.0001$, $P_{trtxtrial(2,44)} > 0.05$. Student's t-test for individual trials: *P $<$ 0.05, **P < 0.01, ***P < 0.001. From Ament et al. 2011 [234].

0.11 Objectives

My thesis aims at answering the following questions, which constitute the main goals of my work:

Does sNPF signalling affect sensory responsiveness in an appetitive and in an aversive context? (Chapter 1)

In this chapter, I used various behavioural, pharmacological and brain-imaging approaches to explore the effect of sNPF on innate appetitive and aversive responsiveness of honey bees in which the feeding state was controlled and manipulated. I increased artificially sNPF levels in partially-fed bees with a reduced appetitive motivation and determined the effect of this increase on appetitive sucrose and olfactory responsiveness and on aversive electric-shock and thermal responsiveness. Multiphoton imaging was used to record neural activity of glomeruli in the antennal lobe (AL), the primary olfactory centre in the honey bee brain. We thus determined if sNPF changes the encoding of odour information in the AL and if such encoding changes with the feeding state of bees. Focusing on aversive responsiveness (electric and thermal shocks) was important to determine if, as in *D. melanogaster* and mammals, sNPF plays a role in the response to stressful stimuli.

Does sNPF signalling affect visual learning and memory formation in appetitive context? (Chapter 2)

In this chapter, we addressed the topic of the impact of sNPF on appetitive learning and memory in free-flying honey bees. Given the impact of sNPF on appetitive sucrose responsiveness, we reasoned that it should also affect appetitive learning and memory in which sucrose is used as a reinforcement. We thus manipulated the feeding state of honey bee foragers trained to make decisions in a miniature maze and increased artificially sNPF levels as in the previous chapter. We studied if sNPF modulates the acquisition and formation of colour memories when free-flying bees were trained to discriminate a blue from a yellow target within the miniature maze without having the possibility to return to the hive (which would change their feeding state). The main goal was to assess whether sNPF is a critical component of motivational processes underlying learning and memory formation in bees and thus, of their foraging activities, which rely on these capacities.

Does sNPF signalling affect appetitive olfactory learning and aversive gustatory learning? (Chapter 3)

In this chapter, we further extended the study of the impact of sNPF on learning and memory processes by contrasting its effects on a further appetitive-learning protocol, the olfactory conditioning of the proboscis extension response (PER), and on an aversive-learning protocol, the gustatory conditioning of the sting extension response (SER). Given the results obtained in the chapter 1 and 2, we predicted a facilitation effect of sNPF on appetitive learning and memory but not on aversive learning and memory. To address this hypothesis, we varied the feeding state of honey bees and increased artificially sNPF levels as in the previous chapters and subjected them to these two different conditioning protocols. We thus incorporated two new modalities to our study of sNPF action, the olfactory one (*via* PER conditioning) and the gustatory one (*via* SER conditioning).

Chapter 4

Finally, (Chapter 4), as an annex related to the question of gustatory processing which underlies the gustatory protocol used in Chapter 3, I provide an extensive review on peripheral gustatory processing in honey bees. Given the impact of sNPF on feeding and gustatory processes, I considered relevant to review the field of honey bee gustation, which lacked until now from an integrative view. My chapter provides such a view and will hopefully help other researchers to address new questions on honey bee gustation.

In this way, my thesis addressed the role of sNPF in various sensory domains (olfactory, visual, gustatory), hedonic contexts (appetitive, aversive) and organization levels (individual behaviour and neural processing). Unravelling the role of sNPF in appetitive context is essential to better understand how nutritional state influences food-decision making. However, the gustatory sense, which is critical for ingestion processes, is still poorly studied and understood in honey bees. Hence, we described the current state of knowledge of peripheral taste detection in honey bees.

Three of the four goals described in this list of objectives have been either published (Chapter 4: Bestea et al., 2021, European Journal of Neuroscience, Peripheral taste detection in honey bees: what do taste receptors respond to? DOI: 10.1111/ejn.15265) or have been accepted in the journals iScience (Chapter 1, pre-proof journal) and Biology Letters (Chapter 2). The remaining goal (Chapter 3) will require further replications to be done in the future to reach solid conclusions.

Chapter 1

Neuropeptide sNPF modulates appetitive but not aversive responsiveness of honey bees

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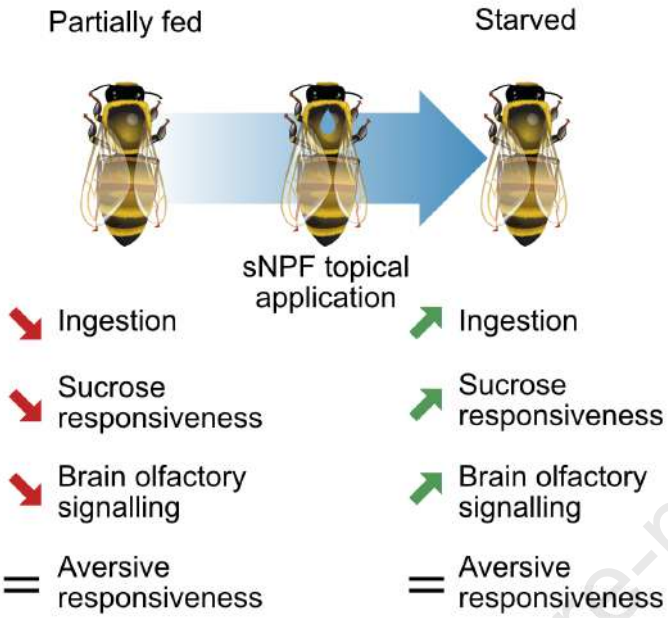

The short neuropeptide F regulates appetitive but not aversive responsiveness in a social insect

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- Abstract

The neuropeptide F (NPF) and its short version (sNPF) mediate food- and stress-related responses in solitary insects. In the honey bee, a social insect where food collection and defensive responses are socially regulated, only sNPF has an identified receptor. Here we increased artificially sNPF levels in honey bee foragers and studied the consequences of this manipulation in various forms of appetitive and aversive responsiveness. Increasing sNPF in partially-fed bees turned them into the equivalent of starved animals, enhancing both their food consumption and responsiveness to appetitive gustatory and olfactory stimuli. Neural activity in the olfactory circuits of fed animals was reduced and could be rescued by sNPF treatment to the level of starved bees. In contrast, sNPF had no effect on responsiveness to nociceptive stimuli. Our results thus identify sNPF as a key modulator of hunger and food-related responses in bees, which are at the core of their foraging activities.

Keywords

Short neuropeptide F, sNPF, feeding behavior, stress, honey bees, appetitive responsiveness, proboscis extension response, aversive responsiveness, sting extension response, calcium imaging, antennal lobe.

Introduction

Signaling *via* the neuropeptide Y (NPY) (Grundemar et al., 1993) plays and essential role for individual survival in vertebrates as it mediates both food- and stress-related responses (Smith and Grueter, 2021). Elevated NPY levels correlate with increased hunger and larger food intake (Loh et al., 2015; Tiesjema et al., 2009) while they also confer resilience to diverse stressors (Brumovsky et al., 2007; Tatemoto, 2004; Thorsell and Heilig, 2002; Villarroel et al., 2018). In invertebrates, two independent homologs of NPY have been identified (Nässel and Wegener, 2011): the neuropeptide F (NPF) (Brown et al., 1999; Feng et al., 2003; Hewes and Taghert, 2001) and the short neuropeptide F (sNPF) (Mertens et al., 2002; Spittaels et al., 1996; Vanden Broeck, 2001).

The effects of these peptides have been investigated in various species of non-social insects such as fruit flies, mosquitoes, cockroaches, silk moths and locusts, among others, focusing mostly on appetitive responses. Variable results have been reported with respect to the modulation of appetitive responses (Fadda et al., 2019) as NPF/sNPF may either promote or inhibit feeding and/or food search depending on the species considered. Fewer studies have analyzed the anti-nociceptive role of these peptides in insects. Yet, in the larva of the fruit fly, NPF promotes resilience to different forms of stress and aversive stimuli, thus paralleling the role of NPY for stress-related responses in vertebrates (Xu et al., 2010).

Surprisingly, studies investigating the impact of NPF/sNPF on appetitive and aversive responsiveness in the honey bee (*Apis mellifera*), a species which represents the pinnacle of sociality among insects, are scarce despite the model status of this insect for neuroscience research (Galizia et al., 2011). Two NPY-related genes, *npf* and short *npf (snpf),* and their corresponding peptides NPF and sNPF have been identified in honey bees. However, only a receptor gene for sNPF (*snpfR*) was found (Chen and Pietrantonio, 2006; Hauser et al., 2006), which advocates for a functional role of this peptide.

So far, no study has addressed the question of the potential link between sNPF signaling and aversive responsiveness in bees. Responses to stressors and nociceptive stimuli may be elicited at the individual level (Junca and Sandoz, 2015; Roussel et al., 2009), yet, they are also coordinated through alarm pheromones, which allow organizing the collective defense of the hive and its valuable resources (Nouvian et al., 2016). This scenario differs from that of non-social insects where aversive responsiveness is not under the control of such social cues. In regards to appetitive behavior, the honey bee also offers unique specificities, which make it different from the other non-social species studied so far. The appetitive behavior of bees is

mainly driven by social rather than by individual needs (Winston, 1991) as bees collect food to sustain a collective energy store rather than for individual consumption. Thus, studying the role of sNPF in both the aversive and the appetitive context may reveal if and how this modulatory pathway shapes sociality in insects.

A prior study addressing the role of NPF/sNPF signaling for appetitive behavior in bees (Ament et al., 2011) showed that the *npf* gene was upregulated in the brain of foragers compared to that of nurses, irrespective of their diet (Ament et al., 2011). However, injection of NPF into the foragers' brain did not influence their sugar syrup intake (Ament et al., 2011); *snpf* and its receptor gene *snpfR* were partially upregulated in the brain of foragers; yet, only the latter increased its expression when colonies were food deprived (Ament et al., 2011). No upregulation of *snpfR* was observed in the brain of foragers from well-fed colonies (Ament et al., 2011). Furthermore, when levels of sNPF were compared between nectar and pollen foragers arriving at an artificial feeder, significantly higher levels were found in nectar foragers (Brockmann et al., 2009), thus suggesting a dependency of sNPF on food type.

While these results suggest a link between sNPF and the nutritional state and foraging behavior of bees, studies evaluating the impact of this peptide on sensory processes preceding food ingestion are missing. These processes may include the subjective evaluation and responsiveness to sucrose solutions (Pankiw and Page, 1999; Scheiner et al., 2004) and odorants with intrinsic appetitive value (Nouvian et al., 2015), and are thus crucial to determine a bee's decision to initiate the ingestion of a given food.

Here we asked whether sNPF levels modulate appetitive and aversive responsiveness in honey bees. We compared honey bee foragers differing in feeding status (starved *vs.* partially fed) and in sNPF levels, which were varied by subjecting fed bees to topical applications of sNPF on their thorax. Appetitive responding was quantified via both the individual consumption of sucrose solution and the proboscis extension response (PER) (Scheiner et al., 2004), an appetitive reflex elicited by antennal stimulation with sucrose solution. As sNPF may also affect the bees' propensity to accept unpalatable food (Desmedt et al., 2016), as shown in fruit flies (Inagaki et al., 2014; Wu et al., 2005), we also evaluated its effect on both PER and ingestion of sucrose solution spiked with salicin, a mixture that is unpleasant to bees (de Brito Sanchez et al., 2005; Desmedt et al., 2016). In addition, we studied if sNPF changes PER to odorants with intrinsic appetitive value (Nouvian et al., 2015) and modulates the neural activity of olfactory projection neurons in the antennal lobe, the primary olfactory center in the insect brain. We determined if the feeding state changes the activity of these neurons *per se* and the effect of sNPF on these changes. Finally, we evaluated if sNPF affects aversive responding,

quantified *via* the sting extension response (SER), an aversive reflex exhibited by honey bees in response to nociceptive stimuli such as electric and thermal shocks (Junca et al., 2019; Vergoz et al., 2007b).

Results

Forager bees captured at a feeder and enclosed in individual syringes were assigned to five groups. One group was kept deprived of food ('Starved'). The other four groups were fed via an Eppendorf tip inserted in the syringe hub. Bees were fed with 5 µl of a mixture of honey, pollen, sucrose and water plus15 µl of a 1.5 M sucrose solution. The volume of food provided corresponded to a third of a bee's crop capacity (Núñez, 1966) so that bees were considered as partially fed ('henceforth P-fed') in terms of volume ingested. After feeding, one of the P-fed groups was left untreated ('P-fed'). Two other fed groups received a topical application (Barron et al., 2007) of sNPF on the thorax. One group received a concentration of 1 µg/µl ('P-fed sNPF 1') and the other a concentration of 10 µg/µl ('P-fed sNPF 10'). The last fed group received a topical application of the solvent (DMSO/Acetone) used to dissolve sNPF ('P-fed solvent'). As sNPF is supposed to enhance appetitive responsiveness, it was not delivered to starved bees, which were already at a ceiling level regarding this trait. Starved bees were the positive controls for the physiological effects of sNPF and allowed establishing whether sNPF treatment turned fed bees into starved-like animals. Untreated and solvent-treated P-fed bees constituted the negative controls. Experiments started between 20 and 30 min after the first topical application.

Experiment 1: sNPF increases food ingestion of P-fed honey bee foragers

We measured the quantity of food (µl) ingested individually by bees enclosed within their respective syringes and presented with a pipette tip inserted in the syringe hub, which was filled with either 100 µl of a 0.6 M sucrose solution (Figures 1*a*,*b*) or with the same amount of 0.6 M sucrose solution spiked with 0.001 M salicin (Figures 1*c*,*d*) (Desmedt et al., 2016).

The feeding status and the treatments applied had a significant effect on food ingestion (Figures 1*a-b*, $F_{4,128} = 13.04$, *d.f.* = 4, $p = 6.12 \times 10^{-9}$; Figures 1*c-d*, $F_{4,138} = 6.1$ *d.f.* = 4, $p = 1.5 \times 10^{-4}$). As expected, starved bees ingested significantly more sucrose solution than bees of the control groups (Figures 1*a-b;* P-fed bees, $p = 3.64 \times 10^{-8}$, [8.54; 21.79] CI 95 %; P-fed solvent bees, $p = 5.37 \times 10^{-7}$, [7.15; 20.3] CI 95 %) and their consumption reached values corresponding to an average full crop capacity (around 60 µl (Huang and Seeley, 2003; Núñez, 1970)). A

comparable result was observed for starved bees presented with sucrose solution spiked with salicin, who ingested more than control P-fed groups (Figures 1*c*-*d*; P-fed bees, $p = 8.44 \times 10^{-3}$, [1.54; 15.55] CI 95 %; P-fed solvent bees, $p = 3.42 \times 10^{-3}$, [2.25; 16.37] CI 95 %), reaching, however, a smaller volume (around 40 µl) than in the case of a pure sucrose solution, owing to the less palatable nature of the mixture offered. In both cases, the volume of food ingested by the controls (untreated P-fed and P-fed solvent bees) did not differ (Figure 1*a*-*b*: $p = 0.97$, [-4.89; 7.77] CI 95%); Figure 1*c*-*d*: $p = 1$, [6.24; 7.77] CI 95 %).

In the case of the pure sucrose solution, P-fed bees treated with sNPF 1 µg/µl increased significantly their food consumption compared to that of untreated P-fed bees (Figures 1*a*-*b*, $p = 0.002$, [2.48; 16.02] CI 95 %) and solvent-treated P-fed bees (Figures 1*a*-*b*, $p = 0.01$, [1.09; 14.53] CI 95 %), reaching the ingestion level of starved bees (Figure 1*a*-*b*, $p = 0.14$, [-1.08; 12.91] CI 95 %). In the case of P-fed bees treated with sNPF 10 µg/µl, the consumption was intermediate between that of P-fed sNPF 1 bees (Figures 1*a*-*b*, $p = 0.71$, [-3.94; 10.65] CI 95 %) and that of untreated P-fed bees (Figures 1*a*-*b*, $p = 0.38$, [-2.44; 11.34] CI 95 %) and P-fed solvent bees (Figures 1*a*-*b*, $p = 0.38$, [-2.44; 11.34] CI 95 %).

In the case of the mixture of sucrose and salicin, P-fed bees treated with sNPF 1 µg/µl or 10 µg/µl also increased their food consumption, reaching values that were similar to those of starved bees (Figure 1*c*-*d*, P-fed sNPF 1 *vs.* Starved: $p = 0.93$, [-5.10; 9.15] CI 95 %; P-fed sNPF 10 *vs.* Starved: $p = 1$, [-6.55; 7.83] CI 95 %) and different from those of P-fed solvent bees (Figure 1*c*-*d*, P-fed sNPF 1 *vs.* P-fed solvent: $p = 0.04$, [0.16; 14.41] CI 95 %; P-fed sNPF 10 *vs.* P-Fed Solvent: $p = 9.6 \times 10^{-8}$ [1.48; 15.86] CI 95 %). P-fed bees treated with sNPF 1 µg/µl did not differ significantly from P-fed untreated bees (Figures 1*c*-*d*, $p = 0.09$, [-0.54; 13.59] CI 95c %).

Overall, these results indicate that treating P-fed bees with sNPF renders them as responsive as starved bees: the lowest concentration of sNPF increased their consumption of pure sucrose solution while the highest concentration of sNPF increased their consumption of the less palatable mixture of sucrose solution and salicin relative to fed bees treated with solvent.

Experiment 2: sNPF increases gustatory responsiveness of P-fed honey bee foragers

Next, we studied if sNPF enhanced sucrose responsiveness, which was quantified by measuring PER to increasing concentrations of sucrose solution (Pankiw and Page, 1999; Scheiner et al., 2004) (0.1, 0.3, 1, 3, 10, and 30 % w/w). Sucrose solution was delivered to the antennae of harnessed bees by means of a toothpick. A score of 1 corresponds to a bee responding only to

196 the highest sucrose concentration (30 % or 0.9 M) while a score of 6 corresponds to a bee
197 responding to all six concentrations assayed.

198 Figure 2*a* shows the population responses (% of bees responding to a given
199 concentration) to the different concentrations of pure sucrose solution. PER increased
200 significantly with sucrose concentrations (GLMM, $\chi^2 = 34.21$, *d.f.* = 1, $p = 4.95 \times 10^{-9}$) and
201 varied between treatments (GLMM, $\chi^2 = 24.86$, *d.f.* = 4, $p = 5.37 \times 10^{-5}$). When tested with 10
202 % and 30 % sucrose solution, starved bees had a higher responsiveness than both controls,
203 untreated P-fed bees (Figure 2*a*, 10 % sucrose: $p = 0.03$; 30 % sucrose $p = 0.03$) and P-fed
204 solvent bees (Figure 2*a*, 10 % sucrose: $p = 1.7 \times 10^{-3}$; 30 % sucrose: $p = 1.7 \times 10^{-3}$). Treatment
205 with sNPF (1 and 10 µg/µl) yielded responses that were similar to those of starved and untreated
206 P-fed bees for both 10 % and 30 % sucrose solution (Figure 2*a*: $p > 0.05$ for all comparisons
207 between starved, untreated P-fed and sNPF-treated bees). Yet, bees treated with sNPF exhibited
208 a significantly higher responsiveness than P-fed solvent bees for both sucrose concentrations
209 and irrespective of the dose of sNPF used (Figure 2*a*: $p < 0.05$ for all comparisons between
210 sNPF treated and solvent-treated fed bees), which shows the enhancing effect of sNPF on
211 sucrose responsiveness with respect to the solvent control.

212 Figure 2*c* shows a similar trend for bees stimulated with increasing concentrations of
213 sucrose solution spiked with salicin. In this case, PER also varied significantly with the
214 concentration of sucrose solution (GLMM, $\chi^2 = 45.89$, *d.f.* = 1, $p = 1.25 \times 10^{-11}$) and with the
215 treatments (GLMM, $\chi^2 = 12.26$, *d.f.* = 4, $p = 0.01$). Responsiveness was again higher in starved
216 bees at the concentrations of 10 % and 30 % when compared to untreated P-fed bees and P-fed
217 solvent bees (Figure 2*c*, $p < 0.01$ for comparisons between starved *vs.* P-fed and starved *vs.* P-
218 fed solvent at both concentrations considered). For the same two concentrations, sNPF-treated
219 bees (1 and 10 µg/µl) had an intermediate level of responsiveness between that of starved bees
220 and that of both P-fed controls. Comparisons between PER levels of sNPF P-fed bees *vs.* starved
221 and P-fed controls were all non-significant (Fig. 2c; $p \geq 0.05$ for all comparisons at the
222 concentrations of 10% and 30%).

223 Individual responsiveness scores of bees stimulated with pure sucrose solution (Figure
224 2*b*, $H = 38.97$, *d.f.* = 4, $p = 7.07 \times 10^{-8}$) or with the mixture of sucrose and salicin (Figure 2*d*,
225 $H = 18.92$, *d.f.* = 4, $p = 8.13 \times 10^{-4}$) differed according to the treatment employed. In both cases,
226 starved bees had significantly higher sucrose response scores than both P-fed controls, untreated
227 P-fed (Figure 2*b*, pure sucrose solution; $W = 124$, $p = 7.46 \times 10^{-4}$; Figure 2*d*, sucrose solution
228 with salicin; $W = 233$, $p = 0.02$) and P-fed solvent (Figure 2*b*, pure sucrose solution; $W = 76$, p
229 $= 1.48 \times 10^{-5}$; Figure 2*d*, sucrose solution with salicin; $W = 235$, $p = 5.85 \times 10^{-3}$). sNPF1 P-fed

bees stimulated with pure sucrose solution had sucrose scores that were intermediate between those of starved bees (Figure 2*b*, W= 227.5, p = 1) and P-fed solvent bees (Figure 2*b*, W = 114, $p = 7.25 \times 10^{-4}$). Although the comparison between P-fed sNPF 1 bees and P-fed untreated bees was marginally non-significant (Figure 2*b*, W = 174.5, p = 0.05), a clear enhancement of appetitive responsiveness was detected in P-fed sNPF 10 bees. In this case, sucrose scores increased significantly to a level comparable to that of starved bees (Figure 2*b*, W = 241.5, p = 1), so that they differed significantly from those of both P-fed controls, untreated P-fed (Figure 2*b*, W = 150, p = 0.01) and P-fed solvent (Figure 2*b*, W = 479.5, $p = 2.48 \times 10^{-4}$).

When P-fed bees treated with sNPF (either 1 µg/µl or 10 µg/µl) were stimulated with sucrose solution spiked with salicin, no enhancing effect was detected in their sucrose scores (Figure 2*d*), which were intermediate between those of starved bees and those of both P-fed controls (Figure 2*d*, $p > 0.05$ for all comparisons).

Taken together, these results indicate that both the feeding status and sNPF, in particular at the highest dose assayed, have a significant effect on responsiveness to pure sucrose solutions. Starved bees responded more to sucrose stimulation than P-fed bees; however, treatment with the high dose of sNPF increased sucrose responsiveness of P-fed bees to levels that were similar to those of starved bees. These effects were less clear for the mixture of sucrose and salicin, probably because of its lower palatability.

Experiment 3: sNPF increases responsiveness of P-fed honey bee foragers to odorants with an intrinsic appetitive value

We then quantified PER to linalool and 2-phenylethanol, two floral odorants that elicit spontaneous appetitive responses in harnessed bees (Nouvian et al., 2015). Each bee was tested with both odorants in a random sequence; PER responses to both stimuli were pooled within each group as no significant odorant effect was found (see Supplementary Information, Figure S1). Olfactory responsiveness varied according to the treatment employed (Figure 3*a*, Fisher's exact test, $p = 1.69 \times 10^{-7}$). Starved bees were significantly more responsive to appetitive odorants than untreated P-fed bees (Figure 3*a-b*, $p = 1.03 \times 10^{-14}$, OsR = 12.63 [6.04;28.7] CI 95 %), P-fed solvent bees (Figure 3*a-b*, $p = 2.12 \times 10^{-11}$, OsR = 0.12, [0.06;0.23] CI 95 %) and bees treated with sNPF (Figure 3*a-b*, Starved *vs.* P-fed sNPF 1: $p = 6.99 \times 10^{-5}$, OsR = 0.31, [0.17;0.54] CI 95 %; Starved *vs.* P-fed sNPF 10 µg/µl: $p = 7 \times 10^{-8}$, OsR = 0.2, [0.11;0.36] CI 95 %;). P-fed bees treated with sNPF 1 µg/µl increased significantly their appetitive responses to both odorants with respect to both P-fed controls, untreated P-fed (Figure 3*a-b*, $p = 8 \times 10^{-4}$, OsR = 3.89, [1.81;8.99] CI 95 %) and P-fed solvent (Figure 3*a-b*, p = 0.03, OsR = 2.57,

[1.27;5.41] CI 95 %). P-Fed sNPF 10 bees did not differ from untreated P-fed bees (Figure 3*a-b*, $p = 0.07$, OsR = 0.15, [1.13;6.03] CI 95 %) and P-fed solvent bees (Figure 3*a-b*, $p = 0.49$, OsR = 1.67, [0.79;3.64] CI 95 %).

Overall, these results show that the lowest dose of sNPF enhanced olfactory responsiveness of P-Fed bees to appetitive odorants, yet not to the extent reached by their starved counterparts. This result indicates a modulatory role of sNPF in olfactory perception.

Experiment 4: sNPF increases the neural activity of projection neurons in the antennal lobe of P-Fed honey bee foragers

To analyze the neural bases of this modulation, we focused on the antennal lobe (AL), the primary olfactory center in the insect brain. The AL is a bilateral neuropil constituted by glomeruli, which are interaction sites between afferent olfactory receptor neurons located on the antennae, local interneurons, and projection neurons (PN). The latter convey the olfactory information reshaped by the AL network to higher-order brain centers (Galizia and Rössler, 2010). Odorants are encoded in the AL as specific glomerular maps, which can be visualized using *in vivo* calcium imaging (Paoli and Galizia, 2021; Sandoz, 2011). Using the fluorescent calcium-sensitive dye Fura-2, we recorded PN activity in the AL by means of two-photon fluorescence microscopy. Starved bees prepared for imaging were stimulated with the two appetitive odorants linalool and 2-phenylethanol, and with the neutral odorant 1-nonanal. Thereafter, all starved bees were fed with the same mixture used in the previous experiments to establish the same feeding status as in the behavioral experiments. P-fed bees received then a topical application of either sNPF 10 µg/µl or solvent (DMSO/ acetone). Thirty min later, responses to the three odorants were measured again in these two groups of fed bees (sNPF 10 and P-fed solvent).

A comparison of the response of odorant-activated glomeruli in starved *vs.* P-fed bees shows that feeding decreased PN response intensity both for the appetitive and the neutral odorants (Figure 4*a-c*, top row). Yet, this decrease was rescued by the topical application of sNPF 10 µg/µl (Figure 4*a-c*, bottom row). The difference in response intensity of individual glomeruli before and after the treatment (*i.e.* feeding plus topical application of solvent or feedings plus topical application of sNPF) was higher than zero in P-fed solvent bees, which indicates a significant decrease of signals across the glomerular population after feeding. Conversely, glomerular responses of P-fed sNPF bees were similar to those measured during the starved stage (Figure 4*d*) (Student's *t*-test: linalool/solvent: $n = 64$ responsive glomeruli, $p = 5 \times 10^{-5}$; linalool/sNPF 10: $n = 33$ glomeruli, $p = 0.96$; 1-nonanal/solvent: $n = 40$ glomeruli,

p = 0.024; 1-nonanal/sNPF 10: n = 20 glomeruli, p = 0.74; 2-phenyethanol/solvent: n = 55 glomeruli, p = 0.0004; 2-phenyethanol/sNPF 10: n = 16 glomeruli, p = 0.27). Finally, an analysis of the overall distribution of response changes (starved *minus* fed response intensity) across all glomeruli and odorants shows that response changes in solvent-treated and sNPF-treated bees partially overlapped but had different probability distributions (Figure 4*e*), with glomeruli from sNPF-treated bees showing significantly less change in response intensity after feeding (Student *t*-test, p = 0.002, solvent-treated group n = 159 glomeruli; sNPF 10-treated group n = 69 glomeruli). Overall, these results show that feeding decreases the neural activity of olfactory circuits in the bee brain and that sNPF rescues neural responses of P-Fed bees to the level exhibited by starved bees.

Experiment 5: sNPF does not affect aversive shock responsiveness of fed honey bee foragers

To determine if sNPF affects not only appetitive but also aversive responsiveness, increasing resilience to nociceptive stimuli, we assessed its effect on SER elicited by a series of electric shocks of increasing voltages (Roussel et al., 2009; Tedjakumala et al., 2014) delivered 30 min after the end of the topical-application phase. The voltages used were 0.25, 0.5, 1, 2, 4 and 7 V (Roussel et al., 2009). A score of 1 corresponds to a bee responding only to the highest voltage (7 V) while a score of 6 corresponds to a bee responding to all six voltages assayed. In this experiment, there were neither starved nor P-fed bees to avoid large differences in body conductivity associated with the presence of an empty ('Starved') *vs.* a partially filled crop ('P-fed') as in prior experiments. Thus, all bees received 5 µl of 1 M sucrose solution to ensure survival and four groups were then established: untreated bees ('Untreated'), bees topically exposed with the solvent ('Solvent') and bees topically dosed with either 1 µg/µl ('sNPF1') or 10 µg/µl of sNPF ('sNPF10'). Untreated bees acted as positive controls displaying normal shock responsiveness against which the effect of sNPF could be tested.

At the population level (% of bees responding with SER to a given voltage), shock responsiveness did not differ between groups, thus showing that sNPF did not affect aversive responsiveness (Figure 5*a*, GLMM, χ^2 = 0.28, *d.f.* = 3, p = 0.96). The analysis of individual responsiveness scores revealed that the solvent increased shock responsiveness *per se* as solvent-treated fed bees had higher scores than untreated fed bees (Figure 5*b*, W = 645, p = 0.009). This suggests that the solvent but not the sNPF increased the bees' sensitivity to the electric shock. No effect of sNPF *per se* on shock responsiveness scores was detected (Fig. 5*b*, $p > 0.05$ for all comparisons between sNPF fed bees and solvent fed bees).

Experiment 6: sNPF does not affect aversive thermal responsiveness of fed honey bee foragers

To verify that sNPF has no effect on aversive responsiveness, we subjected bees to a different form of aversive stimulation, namely antennal contact with a heated probe, which also elicits SER (Junca and Sandoz, 2015). As the previous experiment revealed an effect of the solvent (DMSO/Acetone) on aversive responsiveness, we replaced it by dimethylformamide (DMF). A control experiment (see Supplementary Information, Figure S4) excluded any influence of this solvent on feeding behavior.

We subjected bees differing in feeding status (Starved vs. P-fed) and sNPF levels to an increasing series of aversive temperatures delivered by means of a heating probe contacting the antennae. At the population level (% of bees responding with SER to a given temperature), there were no significant differences between the groups subjected to stimulation with the increasing temperatures (Figure 5*c*, GLMM, $\chi^2 = 0.033$, *d.f.* = 4, $p = 0.99$). Furthermore, focusing on individual thermal-responsiveness scores did not reveal significant differences between groups (Figure 5*d*, $H = 0.13$, *d.f.* = 4, $p = 1$). Thus, aversive responsiveness to thermal stimuli was unaffected both by the feeding status and sNPF levels.

Discussion

By manipulating sNPF levels in fed bees, we demonstrated that this neuropeptide increases feeding, sucrose responsiveness and spontaneous responses to appetitive odors but has no influence on aversive responsiveness to nociceptive stimuli. We showed in addition that sNPF acts on the olfactory network of the AL and rescues the decrease of neural activity induced by feeding to the level observed in starved animals. Overall, the consequence of sNPF treatment was that fed bees behaved like starved bees despite their feeding status. Thus, in the honey bee, sNPF facilitates responsiveness to appetitive, food-related stimuli, but seems to be dispensable for responding to aversive stimuli.

sNPF enhances food intake in honey bee foragers

The involvement of sNPF in feeding processes has been studied in solitary insect species, where variable effects were found (see Fadda et al., 2019 for review). For instance, in adult fruit flies, a facilitating effect, similar to that found in our work, has been reported. Gain-of-function sNPF

mutants show increased food intake, whereas loss-of-function mutants display suppressed food intake (Lee et al., 2004). Similarly, neurogenetic activation of NPF neurons promotes food intake in adult flies (Hergarden et al., 2012), while in starved larvae NPF signaling promotes the intake of noxious food and inhibits the aversive response that such food normally elicits (Wu et al., 2005). This effect is mediated by a sNPF-driven cascade that leads to the GABAergic inhibition of gustatory receptors that normally sense bitter compounds (Inagaki et al., 2014). This facilitating effect of sNPF was also found in cockroaches *Periplaneta americana* where starvation increases the number of sNPF immunoreactive cells both in the brain subesophageal zone and in the midgut, while feeding decreases this immunoreactivity (Mikani et al., 2015, 2012). Furthermore, sNPF injection at the level of the midgut increases locomotion, crucial for food search, to a level comparable to that of starved cockroaches, thus showing the stimulating effect of sNPF for appetitive searching behavior (Mikani et al., 2015). Yet, in other insect species, sNPF has an opposite effect on feeding and food search behavior. For instance, in the desert locust (*Schistocerca gregaria*), injection of sNPF in the abdomen causes food-uptake inhibition, while knocking down the sNPF receptor (sNPFR) increases total food uptake (Dillen et al., 2013). Similarly, in the silkworm *Bombyx mori*, starvation decreases both the transcriptional levels of sNPFR and sNPF levels in the brain, which increase upon refeeding (Nagata et al., 2012).

In honey bees, sNPF increased food ingestion of pure sucrose solution and of a less palatable mixture of sucrose and salicin to levels comparable to those observed under starvation. These findings are consistent with the demonstration that sNPF receptor transcription is upregulated in the brain of foragers searching for food compared to nurses, in particular when colonies are food-deprived (Ament et al., 2011). This foraging-associated upregulation of the sNPF system may depend on the type of food searched given that levels of sNPF were significantly higher in nectar foragers than in pollen foragers arriving at a food source (Brockmann et al., 2009).

sNPF enhances gustatory and olfactory processes in an appetitive context

Topical application of sNPF on fed bees increased their PER to increasing concentrations of sucrose solution and to attractive odorants, thus showing that sNPF modulates appetitive gustatory and olfactory processes prior to ingestion.

As sNPF-treated fed foragers exhibited higher PER to lower sucrose concentrations than control bees, sNPF may modulate either the sensitivity of sweet-sensing GRNs, changing thereby the threshold for responding to sucrose solutions of lower quality, and/or central

processing of tastes in the bee brain. In *D. melanogaster*, starvation drives an overexpression of sNPF, which activates GABAergic neurons that in turn inhibit bitter-taste GRNs (Inagaki et al., 2014). In honey bees, there is no clear evidence of a dedicated bitter-sensing GRN, but bitter compounds mixed with sucrose inhibit the response of sweet-sensing GRNs to sucrose (Bestea et al., 2021; de Brito Sanchez et al., 2005). The increased ingestion of the mixture of 0.6 M sucrose and 0.001 M salicin (Fig. 1*b*) found upon sNPF treatment contrasts with the lack of a clear effect of this treatment on responsiveness to the same mixture (Fig. 2*d*). This difference may indicate that rather than acting peripherally on sweet-sensing GRNs, the ingestion effect observed for the mixture of sucrose and salicin resulted from a modulation of food consumption by sNPF.

This neuropeptide is expressed in 4-6 pairs of lateral neurosecretory cells in the brain of foragers (Ament et al., 2011) and in the mid-gut (Christie, 2020), suggesting a possible role as an internal energy sensor, similarly to the *Drosophila* receptor DmGr43a, which is tuned to fructose and is expressed not only in gustatory organs but also in the digestive tract, uterus, and in the central brain where it senses energy levels (Miyamoto et al., 2012). It has been suggested that the NPF neurons either act as energy sensors or modulate a different subset of neurons or tissues acting as energy sensors themselves such as the DmGr43a neurons (Itskov and Ribeiro, 2013). In honey bees, the orthologue of DmGr43a is the fructose receptor AmGr3, which is also expressed in the gut and which could also act as an internal energy sensor (Takada et al., 2018). Thus, sNPF neurons could either signal increases in energy needs, promoting food intake, or they could modulate the activity of AmGr3-expressing neurons, which would achieve this signaling. Whether sNPFR is expressed in crop cells expressing AmGr3 remains unknown (Takada et al., 2018).

sNPF also modulated PER to appetitive odorants such as linalool and 2-phenlyethanol (Nouvian et al., 2015). Starved foragers were more sensitive to appetitive odorants than fed individuals, which is consistent with work on *D. melanogaster*, in which hunger promoted expression of sNPFR1 in olfactory receptor neurons (Ko et al., 2015), increasing thereby sNPF signaling. This signaling induced presynaptic facilitation of these neurons and potentiation of glomerular responses in the antennal lobe, leading to enhanced food-seeking behavior (Root et al., 2011). A comparable result was found in the larva of *Drosophila*, where certain odorants repel well-fed animals but attract food-deprived animals and the feeding state changes *per se* the neural activity of the AL upon olfactory stimulation (Vogt et al., 2021). In adult flies, NPF modulates the responses of a specific population of olfactory sensory neurons (OSNs), the so-called ab3A neurons, which respond to several food-derived esters (Lee et al., 2017). Knock-

down of NPF in NPF neurons or loss of its receptor (NPFR) in ab3A neurons reduces the response of these neurons and disrupts the ability of the flies to locate food (Lee et al., 2017). In our study, topical application of sNPF on the thorax of fed bees enhanced significantly PER to odors but not to the extent observed in starved bees. This suggests that sNPF may act in concert with other neurotransmitters and neuropeptides related to nutritional status at the level of the olfactory circuit. One candidate could be insulin, which increases spontaneous olfactory responsiveness of naive bees when injected into their brain compared to non-injected bees independently of their age (Goñalons et al., 2016). In the brain of foragers, *npf*, *snpfR* and *ilp1* (insulin like peptide dominantly expressed in the bee's brain) are upregulated with respect to the levels found in nurse brains (Ament et al., 2011). It was thus suggested that upregulation of NPF- and insulin signaling could make foragers more sensitive to hunger and satiety cues, contributing to their increased responses to nutritional stimuli (Root et al., 2011). To date, no functional studies have explored the interaction between sNPF and insulin signaling in bee nurses and foragers.

Although the modulatory effect of sNPF on odorant responses could also occur at the periphery, *i.e*. acting directly on OSNs, our results show that sNPF modulates the activity of PNs, which convey the olfactory message from the AL to higher brain centers; sNPF rescued the activity depressed by feeding, revealing therefore a central role of this peptide for olfactory perception. In fruit flies, appetitive odorants promote feeding by activating NPFR expressed in a subclass of dopaminergic interneurons in the lateral horn (DL2-LH neurons) (Wang et al., 2013). Food odorants also excite NPF neurons, which are necessary to drive attraction to food while activating genetically enhanced NPF neurons promotes attraction to aversive odorants (Beshel and Zhong, 2013). Future research on honey bees should aim at uncovering the neurons providing the sNPF signal both to the AL and to gustatory centers in the brain.

sNPF does not affect aversive responsiveness

Neither the feeding status nor sNPF had any effect on aversive responsiveness of honey bees to thermal and electric shocks. This result differs from previous findings on NPY and NPF signaling in both mice and flies, respectively, where enhancement of NPY/NPF signaling renders animals more attracted to food but also more resilient to aversive stressors (Flood and Morley, 1991; Jewett et al., 1995; Lingo et al., 2007; Wu et al., 2005). In flies, NPF/NPFR1 signaling has an anti-nociceptive function, which reduces responsiveness to diverse stressors *via* attenuation of the neuronal excitation induced by TRP (transient receptor potential) family channels (Xu et al., 2010). In honey bees, the TRP channel AmHsTRPA modulates thermal

responsiveness (Junca and Sandoz, 2015), possibly with other TRP channels (Kohno et al., 2010). Yet, our results indicate that it is not under the control of sNPF-signaling. This specificity might be related to the social life style of honey bees, in which nociception and resistance to stressors like electric shocks are under control of defensive pheromones, which activate an opioid like system (Balderrama et al., 2002; Núñez et al., 1997), probably via serotonin and dopamine signaling (Nouvian et al., 2018). This activation renders bees more tolerant to stressors, which is adaptive in the framework of colony defense as honey bee guards may sacrifice their lives upon stinging. Social control of nociception via alarm pheromones is a specific trait of bees that is not found in solitary insects and may explain the lack of effect of sNPF on aversive responsiveness.

sNPF delivery via topical application on the thorax of honey bees

The method chosen to deliver sNPF has been repeatedly used in many insect species to determine the effect of neurotransmitters and bioactive substances such as pesticides and other molecules of interest (Copijn et al., 1977; Killiny et al., 2014; Motta et al., 2020; Paes-de-Oliveira et al., 2008; Park and Smith, 2021; Sierras and Schal, 2017; Tozetto et al., 1997). In the honey bee, the efficiency of three delivery methods - oral delivery, topical delivery on the thorax and injection into the brain - was compared in the case of 3H-radiolabelled octopamine (Barron et al., 2007). Only lower levels of neurotransmitter were detected in the nervous system after oral delivery. On the contrary, injection into the brain via the ocellar tract resulted in higher neurotransmitter levels but it damaged the animals and diminished the possibility of studying their behavior. The topical application on the thorax constituted a good compromise as it resulted in higher levels of octopamine in the nervous system and preserved the animals for behavioral studies. Further works focusing on other biogenic amines (e.g. serotonin, dopamine, 6,7-ADTN) and on the antagonists of their corresponding receptors have used successfully the thoracic topical exposure to determine their effects on defensive responses (Nouvian et al., 2018) and on social interactions within the hive (Hewlett et al., 2018), without quantifying the amount of neuroactive substance that reached the insect nervous system, due to the difficulty of the task, and the significant effects observed at the level of the behaviors studied. These effects showed that in all cases the substances applied affected the insect nervous system. The same can be said in the case of our work. Despite the fact that membrane-bound peptidases may degrade neuropeptides with different speed (depending on the type of neuropeptide), the effects observed on appetitive behaviors indicate that the amounts delivered were large enough to induce significant changes in behavior and physiology and/or that the peptide triggered a

receptor-mediated response that is long lasting such as that shown in other insect species (Liu and Kubli, 2003). Further studies should aim at quantifying the amounts of sNPF that can reach the central nervous system using this methodology.

Overall, our results allow understanding the mechanisms underlying food consumption by foragers when they collect food for the colony. They uncover how nutritionally related pathways drive the emergence of food attraction and appetitive responses and they underline the dispensability of these pathways for resistance to aversive stimuli, which in a social insect may be driven by social cues mediating the defense of the colony and its vital resources.

Limitations of Study

We used a topical delivery of sNPF to the thorax to increase sNPF levels. This method is commonly used in many insect species to determine the effect of neurotransmitters, neuropeptides and bioactive substances such as pesticides and other molecules of interest (e.g. Barron et al., 2007; Copijn et al., 1977; de Brito Sanchez et al., 2021; Hewlett et al., 2018; Killiny et al., 2014; Motta et al., 2020; Nouvian et al., 2018; Pankiw and Page, 2003; Park and Smith, 2021; Sierras and Schal, 2017). It has the advantage of preserving insects intact for behavioral analyzes, which is not the case when injections (e.g. into the brain) are used. Yet, we did not quantify the amount of sNPF acting in the nervous system of the bees treated in this way. Quantification of circulating neuropeptides is technically difficult, yet needs to be attempted. In spite of this limitation, the results obtained were clear and showed consistent effects of sNPF treatment, different from those obtained upon topical application of the solvent alone.

Another limitation refers to the lack of a loss-of effect approach showing the opposite effect to that of sNPF increase via the topical application. This could be achieved via a RNAi approach targeting the sNPF receptor identified in the honey bee. In this way, sNPF signaling would be decreased, leading theoretically to a decrease of appetitive responses. Although this approach is technically feasible, molecular genetics in honey bees are not straightforward (i.e. difficulties for generating mutants given the complexity of the life cycle and life style requiring the hive environment and exposure to the environment). We are currently working in the development of this RNAi approach.

STAR Methods

RESOURCES AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Maria Gabriela de Brito Sanchez (maria.de-brito-sanchez@univ-tlse3.fr).

Materials availability

This study did not generate new unique reagents.

Data and code availability

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials.

All data have been deposited at DOI: https://doi.org/10.6084/m9.figshare.15134859 and are publicly available as of the date of publication. DOIs are listed in the key resources table.

Original codes have been deposited at DOI: https://doi.org/10.6084/m9.figshare.16864408 and are publicly available as of the date of publication. DOIs are listed in the key resources table.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Insects

Honey bee foragers from a colony located in the apiary of the Research Centre on Animal Cognition (Toulouse, France) were collected in the morning at an artificial feeder to which they were previously trained. Bees were trained using the traditional von Frisch's method, i.e. moving them progressively from the hive entrance to the site of the experimental feeder (von Frisch, 1967). Since sNPF brain levels can vary depending on crop filling (Brockmann et al., 2009), empty foragers were caught upon landing on the feeder, just before they started feeding. They were then enclosed individually into syringes with an open hub to allow for respiration.

Although we did not detect intruders from different colonies at the feeder, which typically leads to biting and other forms of interindividual aggression by bees 'owning' the place, genetical homogeneity cannot be ensured in natural honey bee colonies as a honey bee queen mates with several drones in a nuptial flight, thus resulting in various patrilines co-

existing within a colony (Mattila and Seeley, 2007). Thus, the variability existing in our data is intrinsic to natural colonies.

Experimental groups and pharmacological treatments

Bees were divided into five groups. One group was kept deprived of food ('Starved'). The other four groups were fed by fitting within the open hub of the syringe an Eppendorf tip so that the enclosed bee could feed from it. Bees were presented with a first tip containing 5 µl of a mixture of honey, pollen, sucrose and water, and then with a second tip containing 15 µl of a 1.5 M sucrose solution, (*partially fed bees;* henceforth P-fed bees). Feeding lasted between 15 and 30 min, depending on the number of bees assigned to an experiment. After feeding, the syringes with all the bees were placed in ice during 5 min. This allowed to take out the cold-narcotized bees and proceed to the topical-application phase. One of the fed groups was left untreated ('P-fed'). Two other fed groups received a topical application (Barron et al., 2007) of 1 µl of sNPF solution on the thorax. One group received sNPF at a concentration of 1 µg/µl ('P-fed sNPF 1') and the other at a concentration of 10 µg/µl ('P-fed sNPF 10'). The fourth fed group received a topical application of the solvent used to dissolve sNPF ('P-fed solvent'). The solvent was a mixture of 20 % dimethyl sulfoxide (DMSO) and 80 % acetone (DMSO/Acetone) in all experiments except in the aversive thermal responsiveness test in which dimethylformamide (DMF, 100%) was used (see results for explanations). As sNPF is supposed to enhance appetitive responsiveness, it was not delivered to starved bees, which were already at a ceiling level regarding appetitive responsiveness. Starved bees constituted therefore a positive control for the physiological effects of sNPF on appetitive responses and allowed establishing whether sNPF treatment turned fed bees into starved-like animals. The group of fed bees treated with the solvent and the untreated fed bees constituted the negative controls for the sNPF treatment.

Each bee was replaced within its individual syringe after topical treatment where it recovered from the cold treatment. Experiments started between 20 and 30 min after the first topical application. Sucrose and solvents were purchased from Sigma-Aldrich (Steinheim, Germany) while honey bee sNPF was purchased from NovoPro (Shanghai, China; *Apis mellifera* sequence: SDPHLSILSKPMSAIPSYKFDD (Boerjan et al., 2010)).

METHOD DETAILS

Experiment 1: the effect of sNPF on food ingestion

We developed a procedure to measure individual ingestion by inserting a pipette tip in the hub of the enclosing syringe. The tip was filled up with either 100 µl of a 0.6 M sucrose solution or with the same amount of 0.6 M sucrose solution spiked with 0.001 M salicin (Desmedt et al., 2016). The quantity of food (µl) ingested by each bee of the five groups described above was measured one hour later with a 200 µl pipette. Salicin was purchased from Sigma-Aldrich. Sample sizes were as follows: *Ingestion of 0.6 M sucrose;* $n_{P\text{-fed}} = 30$; $n_{Starved} = 26$; $n_{P\text{-fed solvent}} = 31$; $n_{P\text{-fed sNPF 1}} = 24$; $n_{P\text{-fed sNPF 10}} = 22$. *Ingestion of 0.6 M sucrose solution spiked with 0.001 M salicin*: $n_{P\text{-fed}} = 30$; $n_{Starved} = 29$; $n_{P\text{-fed solvent}} = 29$; $n_{P\text{-fed sNPF 1}} = 28$; $n_{P\text{-fed sNPF 10}} = 27$.

Experiment 2: the effect of sNPF on appetitive sucrose responsiveness

After the topical application and while bees were still cold-narcotized, they were harnessed individually in vertical tubes to quantify sucrose responsiveness. They were then fed *ad libitum* with water delivered to the proboscis. Sucrose responsiveness was quantified 30 min after the end of the topical-application phase by measuring PER to increasing concentrations of sucrose solution in the five experimental groups of bees. We used a standard protocol (Pankiw and Page, 1999), in which concentrations of 0.1, 0.3, 1, 3, 10, and 30 % (w/w) (*i.e.* 0.003, 0.009, 0.03, 0.09, 0.3 and 0.9 M) were delivered to the antennae of harnessed bees by means of a toothpick (Page et al., 1998; Pankiw et al., 2001; Scheiner et al., 2004). Trials in which distilled water was used to stimulate the antennae were interspersed between sucrose trials as controls and to avoid sensitization. The percentage of animals responding to a given stimulation was calculated (population response) as well as the individual sucrose responsiveness score of each bee (the number of sucrose concentrations to which a bee responded). A score of 1 corresponds to a bee responding only to the highest sucrose concentration (0.9 M) while a score of 6 corresponds to a bee responding to all six concentrations. At the end of the stimulation sequence, bees were stimulated with a 1.5 M sucrose solution to check for PER integrity. Bees were discarded from the experiment if they responded with PER to water stimulation, if they exhibited inconsistent responses (*e.g.* PER to 0.009, 0.03, 0.09, 0,9 but not to 0.3 M) (Baracchi et al., 2017), and if they did not show PER upon stimulation with a 1.5 M sucrose solution. Excluded bees represented a minor percentage of the individuals both for the stimulation with pure sucrose solution (5.1 %; 8 out of 156 bees) and for the stimulation with sucrose solutions altered with 0.001 M of salicin (1.1 %; 2 out of 181 bees). Bees not responding to the 1.5 M

sucrose solution represented 17.3 % (27 out of 156) and 17.7 % (32 out of 181) of the bees stimulated with pure sucrose solution and with sucrose solution spiked with salicin, respectively. Sample sizes were as follows: *Pure sucrose solutions*; $n_{P\text{-}fed} = 26$; $n_{Starved} = 25$; $n_{P\text{-}fed\ solvent} = 25$; $n_{P\text{-}fed\ sNPF\ 1} = 24$; $n_{P\text{-}fed\ sNPF\ 10} = 23$. *Sucrose solutions spiked with 0.001 M salicin*; $n_{P\text{-}fed} = 26$; $n_{Starved} = 33$; $n_{P\text{-}fed\ solvent} = 28$; $n_{P\text{-}fed\ sNPF\ 1} = 29$; $n_{P\text{-}fed\ sNPF\ 10} = 30$.

Experiment 3: the effect of sNPF on appetitive olfactory responsiveness

After the topical application and while the bees were still cold-narcotized, they were harnessed individually in vertical tubes. Appetitive olfactory responsiveness was then quantified by measuring PER to linalool and 2-phenlyethanol, two floral odorants known for eliciting spontaneous PER (Goñalons et al., 2016; Laloi et al., 2001; Nouvian et al., 2015; Sandoz et al., 1995). Odorants were obtained from Sigma-Aldrich. Recordings were made 30 min after topical application. To achieve the olfactory stimulation, the bee was placed between an olfactory stimulator that delivered a continuous clean airflow to the antennae and an air extractor (Raiser et al., 2017). The stimulator allowed sending odorant pulses of controlled duration to the bee; the air extractor prevented odor accumulation. During the test, bees received initially 16 s of continuous clean airflow, then 6 s of odorant stimulation and finally 23 s of continuous clean air flow. Odorants were delivered by activating valves that redirected air towards a syringe containing 4 µl of the pure odorant impregnated on a 30 × 3 mm filter paper. Occurrence of spontaneous PER during olfactory stimulation was recorded as 1 when elicited and as 0 when not. Both odorants were presented to each bee in a randomized sequence. The interval between the two stimulations was 35 min. Bees were discarded from the experiment if they did not respond with PER to antennal stimulation with 1.5 M sucrose solution offered after the olfactory test (62 from 376 bees did not respond to 1.5 M sucrose solution, e.g. 16.48 %). None of the bees tested responded to air flow alone. Sample sizes were as follow: $n_{P\text{-}fed} = 65$; $n_{Starved} = 60$; $n_{P\text{-}fed\ solvent} = 61$; $n_{P\text{-}fed\ sNPF\ 1} = 63$; $n_{P\text{-}fed\ sNPF\ 10} = 64$.

Experiment 4: the effect of sNPF on neural activity in the antennal lobe

a) Projection neuron (PN) staining

Honey bees were collected in the morning at an artificial feeder to which they were previously trained and brought back to the laboratory for PN staining (Paoli et al., 2017; Sachse and Galizia, 2002). They were then briefly immobilized on ice and placed on a custom 3D-printed plastic holder. Their head was stabilized with a drop of wax and the antennae were immobilized in a forward-facing position by a drop of eicosane (Sigma-Aldrich, CAS 112-95-8). A

rectangular window was open on the head cuticle to access the brain. Glands and tracheas covering the mushroom body were gently removed, and a glass capillary bearing a crystal of Fura-2 Dextran 10 kDa (ThermoFisher Scientific, CAS 108964-32-5) was injected between the mushroom body calices. Two injections were performed in the right brain hemisphere to increase staining success. Thereafter, the head capsule was closed and sealed with eicosane. Bees were then fed with 5 µl of 50% sugar/water solution and left in the dark at 20°C for a minimum of five hours for the dextran to stain efficiently the PNs. After five hours, the bee brain was re-exposed and covered with a transparent two-component silicon (Kwik-Sil, WPI) for calcium imaging analysis.

b) Calcium-imaging experimental design and signal processing

Undiluted solutions of linalool, 1-nonanal and 2-phenylethanol were delivered to the bees using the same automated olfactometer (Guerrieri et al., 2005) used for the behavioral experiments (Experiment 3). Odorants were alternated and presented ten times on a 1/9 second ON/OFF configuration. To compare glomerular activity across starved and fed animals and the effect of sNPF 10 µg/µl on olfactory coding, we used a procedure that reproduced the rationale and dynamics of our behavioral experiments, yet using a repeated measurement design to improve signal-to-noise ratio of stimulus-elicited signals. First, starved honeybees were imaged following the stimulation protocol described above. Then, these bees, still harnessed, were fed as in the behavioral experiments, *i.e.* with 5 µl of a mixture of honey, pollen, sucrose and water, and 15 µl of a 1.5 M sucrose solution. Bees were then either topically exposed to the solvent solution (DMSO/Acetone) or to sNPF 10 µg/µl. After 30 min, bees were imaged again following the same stimulation protocol. Sample sizes in the experimental groups were as follows: $n_{\text{Starved/P-fed solvent}} = 9$; $n_{\text{Starved/P-fed sNPF10}} = 6$.

Calcium imaging recordings were conducted with a straight Leica SP8 scanning microscope (Leica Microsystems, Germany) equipped with a SpectraPhysics InSight X3 multiphoton laser tuned at 780 nm for Fura-2 excitation. All images were acquired with a water immersion 16x objective (Leica HC FLUOTAR 16x/0.6 IMM CORR, Leica Microsystems, Germany), at 64x64 pixel resolution and 127 Hz.

Experiment 5: the effect of sNPF on aversive electric-shock responsiveness

After the topical application and while still cold-narcotized, the bees were harnessed individually between two brass plates so that it built a bridge between them. In this way, a 2 s

electric shock passed, through the bee when it was delivered to the plates (Carcaud et al., 2009; Roussel et al., 2009; Vergoz et al., 2007a). Occurrence of the sting extension response (SER) upon a series of electrical stimulation was recorded as 1 when elicited and as 0 when not. (Carcaud et al., 2009; Roussel et al., 2009; Vergoz et al., 2007a). The voltages used were 0.25, 0.5, 1, 2, 4 and 7 V (Roussel et al., 2009). An air extractor placed behind the holder prevented the potential accumulation of alarm pheromone released by the bee upon electric shock stimulation. Recordings were made 30 min after the topical application. Placement trials in which a bee was placed in the shock delivery setup but without shock stimulation were interspersed between shock trials as controls. Bees were discarded from the experiment if they produced inconsistent responses (*i.e.* responding with SER to a given voltage but not to higher subsequent ones; 34 from 239 bees, 14 %). No bee responded to placement trials.

The percentage of animals responding with SER to a given stimulation was calculated (population response) as well as the individual shock responsiveness score of each bee (the number of electric shocks to which a bee responded). A score of 1 corresponds to a bee responding only to the highest voltage (7 V) while a score of 6 corresponds to a bee responding to all six voltages. Neither starved nor P-Fed bees participated in this experiment to avoid differences in body conductivity between empty and loaded bees given the low conductance of sucrose solution. All bees were fed with 5 µl of 1 M sucrose solution to ensure their survival and they were assigned to one of our groups: untreated bees, bees treated with the solvent (DMSO/Acetone), and bees that received the topical application of either 1 µg/µl or 10 µg/µl of sNPF. Sample sizes in the experimental groups were as follows: $n_{Untreated} = 49$; $n_{Solvent} = 42$; $n_{sNPF\ 1} = 39$; $n_{sNPF\ 10} = 44$.

Because the feeding state and crop volume of these bees differed from those of P-fed bees used in the previous experiments (fed with a mixture of 5 µl of honey/pollen/sucrose/water and 15 µl of 1.5 M sucrose solution), we performed control experiments to assess whether this difference influenced the shock responsiveness recorded. No differences were found according to these feeding treatments (see Supplementary Materials, Figures S2 and S3).

Experiment 6: the effect of sNPF on thermal responsiveness

After topical application and while the bees were still cold-narcotized, they were harnessed individually in the same horizontal supports used in the electric-shock experiment. SER upon antennal contact with a heated probe was quantified 30 min after topical application (Junca and Sandoz, 2015). The same five groups as in the appetitive experiments were used. Each bee was stimulated with a series of six increasing temperatures: ambient temperature (~25°C), 35, 45,

55, 65 and 75°C. Stimulation temperatures were established by means of a resistance (3 x 1.5 mm) mounted within the far tip of the pen-like probe touching the antennae of the bee during 1 s. Temperature was controlled by an NTC thermistor (MICRO-BETACHIP - MCD) glued to the resistance. An air extractor placed behind the holder prevented the potential accumulation of alarm pheromone released by the bee upon thermal stimulation. Trials with tactile stimulation with a glass rod at ambient temperature were interspersed between thermal trials as controls. Tactile stimulations were applied on the antennae as controls, to ensure that SER was a consequence of thermal stimulation and not of the mechanic contact with the antennae. For each bee, whether the first stimulation was tactile or thermal was determined randomly prior to starting the experiment. Stimulations were performed at 15 min intervals. A thermal responsiveness score was calculated for each bee as the number of SER to the different thermal stimuli assayed. Bees were discarded from the experiment if they responded to all tactile stimulations (1 bee), or if they produced inconsistent responses (*i.e.* responding with SER to a given temperature but not to higher subsequent ones; 25 among 212 bees, 11.79 %). Sample sizes in the experimental groups were as follows: $n_{P\text{-}fed} = 38$; $n_{Starved} = 33$; $n_{P\text{-}fed\ solvent} = 37$; $n_{P\text{-}fed\ sNPF\ 1} = 40$; $n_{P\text{-}fed\ sNPF\ 10} = 38$.

In this experiment, dimethylformamide (DMF) was used as solvent for sNPF instead of the mixture of dimethyl-sulfoxide (DMSO) and acetone (20/80) used in prior experiments, as we noticed that the latter increased the sensitivity to electric shocks. We thus performed a control experiment to ensure that DMF had no effect on food intake and compared ingestion of a 0.6 M sucrose solution in DMSO/Acetone-treated P-Fed bees and in DMF-treated P-Fed bees using the same procedure as in Experiment 1. Both groups behaved similarly, thus excluding any influence of the solvent on feeding behavior (see Supplementary Information, Figure S4).

QUANTIFICATION AND STATISTICAL ANALYSIS

Data were analyzed and plotted using R software (R Core Team, 2019) and MatLab (The MathWorks, Inc.) custom-made scripts. In all cases, data met the assumption of the tests used. Effects of treatments on food intake were analyzed with a one-factor ANOVA followed by a Tukey HSD *post hoc* test. Responsiveness scores were analyzed with a Kruskal-Wallis rank test followed by multiple pairwise Wilcoxon comparisons (Holm *p*-value adjustment method). For the ingestion experiment, the difference between two treatments was assumed to be statistically significant when the *p*-value was below 0.05 and the Confidence Intervals – size effect statistic - (CIs) 95% did not contain 0. While a *p*-value informs on the risk of not refuting

the null hypothesis (here, that there was no difference between groups), it does not inform on size effect (*i.e.* on the amplitude of the difference) (Halsey et al., 2015; Nieuwenhuis et al., 2011; van Helden, 2016). We chose the 95 % confidence intervals (CI 95 %) of the difference between group means as a size-effect statistic. We obtained these CI 95 % from the Tukey HSD *post hoc* tests. They represent a set of values calculated from sample observations that likely contain the true estimate (*i.e.* true mean difference between two groups). Therefore, if CI 95 % includes a 0 value, there is no significant difference between the means of the two groups compared as there is a chance that 0 is the true mean difference. This method provides: i) a simple visual assessment (*i.e.* whether CI 95% includes 0 or not); and ii) information about if and how the difference between two groups is likely to be based on a reliable sampling (*i.e.* large confidence intervals indicate low confidence in the sampling).

Appetitive (PER-based) and aversive (SER-based) population responses were fitted with general linear mixed models (GLMM) using the *glmer* function of the *lme4* package (Bates et al., 2018). PER/SER served as a binary-response variable (binomial family, 'logit' link), while treatments and stimuli were entered as fixed effects. Individual identity was entered as a random effect. Only factors with a minimum of one responding bee per group were considered for the analysis. ANOVA (package *car*) was performed on GLMMs and *post hoc* multiple comparisons were used when necessary (Tukey *p*-value adjustment method, R package *emmeans*).

For the olfactory responsiveness experiment, the effect of treatment was analysed with an exact Fisher's test for proportions, followed by *post hoc* multiple comparisons (Holm *p*-value adjustment method, R package *rstatix*). In addition, CIs 95% of Odds Ratio (OsR) of the pairwise comparisons were plotted and used as an effect size statistic. The odds ratio of an event (here PER) is the ratio between the frequency (or likelihood) of event occurrence and the frequency (or likelihood) of its non-occurrence (Bland and Altman, 2000). CI 95 % estimate the precision of the OsR (here the OsR is the estimate) (see above, Experiment 1). If a CI 95 % does not include 1, the calculated odds ratio is considered statistically significant. In our case, it means that the odds of PER is significantly different between two groups.

For calcium imaging data analysis, baseline signal was calculated as the mean fluorescence in the 5 seconds before stimulus onset. Such baseline activity was used to calculate baseline-subtracted and normalized stimulus-induced glomerular activity ($\Delta F/F$). Normalized activity was multiplied by -1 to display excitatory/inhibitory responses as positive/negative changes ($-\Delta F/F$). Elicited activity was then averaged across 10 stimulations with the same odorant. For the analysis of glomerular responses in starved and fed animals topically exposed

to the solvent or sNPF 10 µg/µl, only responsive glomeruli were selected. Responsive glomeruli were defined in an unsupervised way as those glomeruli, in which the mean activity during the 0.6-1s interval after stimulus onset (*i.e.* when odorant-elicited activity was strongest) was greater than the baseline activity + standard deviation. Any glomerulus labelled as "responsive", either before or after feeding, was kept in the analysis. Selected glomeruli from nine control bees (solvent treated) and from six sNPF-treated bees were pooled together for the analysis. Analysis of the difference in glomerular responses before and after the treatment was conducted by subtracting the responses of active glomeruli in fed bees from the response in the same glomerulus in the starved condition. Student's paired *t*-tests were employed to assess if the glomerular activity before and after feeding (and treatment) were significantly different (*i.e.* if the distribution of starved-fed differential activity was different from 0).

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Contributions

L.B performed the behavioral experiments and M.P performed the calcium imaging experiments with the assistance of B.R. P.A. provided technical assistance for the experiments on aversive responsiveness. Statistical analyses on behavioral and imaging data were performed by L.B and M.P., respectively. Results and conclusions were discussed by L.B., M.P., R.V., J.-C.S., J.C. M.G. and G. de B.S. The manuscript was written by L.B., M.G. and G. de B.S. All experiments were supervised by M.G. and G. de B.S. Funding was obtained by M.G., G. de B.S. and J.-C.S. All authors reviewed and approved the final version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1. The effect of feeding status and sNPF on food consumption. (a) Consumption of bees (n = 133) offered with 100 µl of a 0.6 M pure sucrose solution. **(b)** Tukey post-hoc CI 95% represents the effect size (difference of means – black dot) between groups of bees that ingested a 0.6 M sucrose solution. **(c)** Consumption of bees (n = 143) offered with 100 µl of a 0.6 M sucrose solution spiked with a 0.001 M salicin. **(d)** Tukey *post hoc* CI 95% represents the effect size (difference of means – black dot) between groups of bees that ingested a 0.6 M sucrose solution spiked with 0.001 M salicin. In **(a)** and **(c)**, scattered plots show individual consumption values; each box extends from the 25th to the 75th percentiles; the line in the middle of the box shows the median. Letters on top of box plots represent statistical differences (Tukey *post hoc* test). Sample sizes are indicated for each group in parentheses below box plots. In **(b)** and **(d)**, if an interval does not contain zero, the corresponding means are significantly different. Sample sizes for the experimental groups were as follows: *Ingestion of 0.6 M sucrose;* $n_{\text{P-fed}}$ = 30; n_{Starved} = 26; $n_{\text{P-fed solvent}}$ = 31; $n_{\text{P-fed sNPF 1}}$ = 24; $n_{\text{P-fed sNPF 10}}$ = 22. *Ingestion of 0.6 M sucrose solution spiked with 0.001 M salicin*: $n_{\text{P-fed}}$ = 30; n_{Starved} = 29; $n_{\text{P-fed solvent}}$ = 29; $n_{\text{P-fed sNPF 1}}$ = 28; $n_{\text{P-fed sNPF 10}}$ = 27.

Figure 2. The effect of feeding status and sNPF on sucrose responsiveness. (a) Population response (% of bees, n = 121) responding with proboscis extension response (PER) to a series of six increasing concentrations of sucrose solution. **(b)** Individual sucrose-responsiveness scores calculated on the basis of the number of concentrations to which a bee responded with PER during the stimulation series. A high score indicates that the bee was responsive to most of the sucrose concentrations assayed while a low score indicates that the bee responded only to the highest sucrose concentrations. **(c)** Population response (% of bees, n = 147) responding with PER to a series of six increasing concentrations of sucrose solutions spiked with 0.001 M salicin. **(d)** Individual sucrose-responsiveness scores of bees calculated on the basis of the number of concentrations to which a bee responded with PER during the stimulation series. In **(a)** and **(c)**, error bars represent the 95 % bootstrapped confidence intervals. In **(b)** and **(d),** boxes with different letters differed significantly (Wilcoxon sum-rank pairwise test, Holm p adjustment method). Each boxplot extends from the 25th to the 75th percentiles; the line in the middle of the box shows the median. Sample sizes are indicated in parentheses below each box.

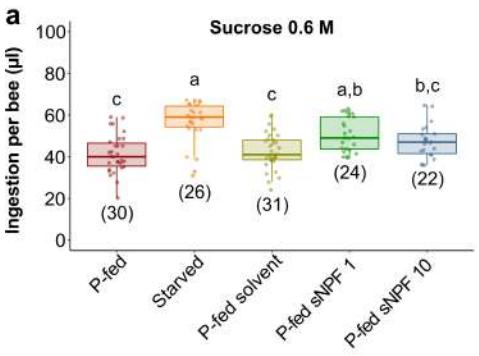
They were as follows: *Pure sucrose solutions*; $n_{P\text{-fed}} = 26$; $n_{Starved} = 25$; $n_{P\text{-fed solvent}} = 25$; $n_{P\text{-fed sNPF 1}} = 24$; $n_{P\text{-fed sNPF 10}} = 23$. *Sucrose solutions spiked with 0.001 M salicin*; $n_{P\text{-fed}} = 26$; $n_{Starved} = 33$; $n_{P\text{-fed solvent}} = 28$; $n_{P\text{-fed sNPF 1}} = 29$; $n_{P\text{-fed sNPF 10}} = 30$.

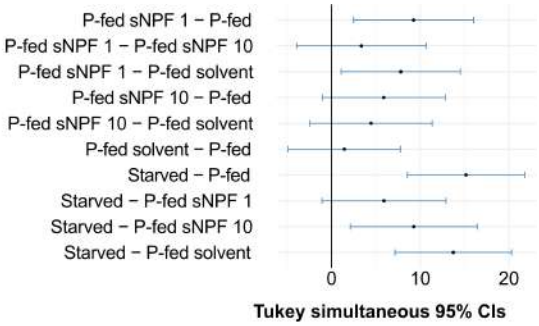
Figure 3. The effect of feeding status and sNPF on appetitive olfactory responsiveness. Spontaneous PER to two appetitive odorants (linalool and 2-phenlyethanol) in bees differing in feeding status and sNPF levels. Sample sizes were as follow: $n_{P\text{-fed}} = 65$; $n_{Starved} = 60$; $n_{P\text{-fed solvent}} = 61$; $n_{P\text{-fed sNPF 1}} = 63$; $n_{P\text{-fed sNPF 10}} = 64$. **(a)** Percentage of bees responding with PER to both odorants according to treatment ($n = 313$; responses pooled for both odorants – see text). Letters above the bars indicate significant statistical differences (Fisher's multiple pairwise comparisons, Holm *p* adjustment method). Error bars represent the 95% bootstrapped confidence intervals. Values in parentheses below bars indicate group sample sizes. **(b)** Odds ratio and CI 95% estimated by Fisher's multiple pairwise comparisons (Holm *p* adjustment method); if an interval does not contain 1, the corresponding odds ratio are significantly different.

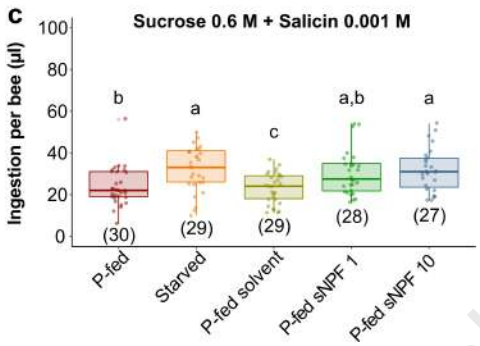
Figure 4. The effect of feeding status and sNPF on glomerular activity in the antennal lobe. (a) Glomerular responses to linalool before (left) and after feeding + topical application of solvent (top middle) or sNPF 10 µg/µl (bottom middle). On the right, mean temporal profiles (± s.e.m.) of responsive glomeruli before and after feeding + treatment with solvent (top right) or sNPF 10 µg/µl (bottom right). Olfactory stimulation occurs between 0 and 1 s. **(b,c)** Glomerular responses to 1-nonanal **(b)** and 2-phenylethanol **(c)** according to the same scheme described for **(a)** (linalool solvent: $n = 64$ responsive glomeruli from 9 bees; linalool sNPF 10: $n = 33$ glomeruli from 6 bees; 1-nonanal solvent: $n = 40$ glomeruli from 9 bees; 1-nonanal sNPF 10: $n = 20$ glomeruli from 6 bees; 2-phenyethanol solvent: $n = 55$ glomeruli from 9 bees; 2-phenyethanol sNPF 10: $n = 16$ glomeruli from 6 bees. (**d**) Mean difference (± s.e.m.) between response intensity of glomeruli before and after treatment (i.e. feeding and solvent or sNPF topic application) calculated for each odorant and treatment. For each odorant, the solvent-treated group shows a significant decrease in response after feeding, whereas sNPF-treated animals do not show such decrease. Student's *t*-test: * = $p < 0.05$; ns = non-significant. (**e**) Comparison of the probability histograms of glomerular response differences before and after

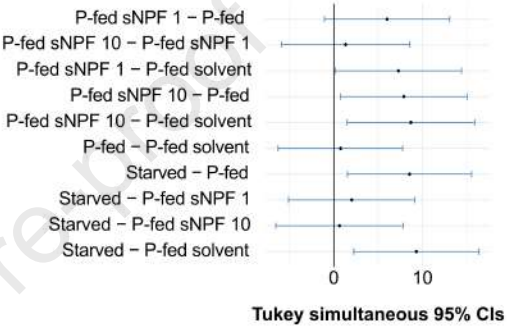
feeding in solvent- and sNPF-treated animals (solvent-treated group *n* = 159 glomeruli from 9 bees; sNPF-treated group = 69 glomeruli from 6 bees).

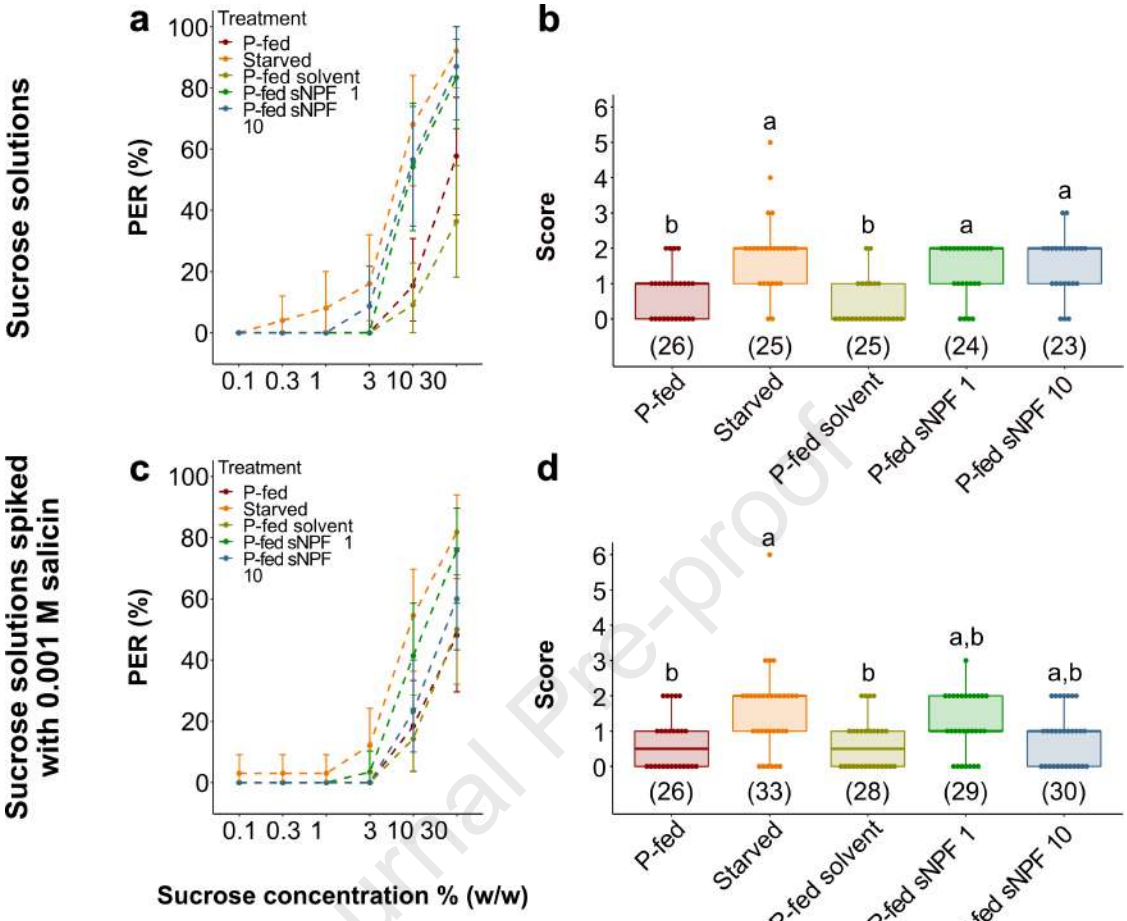
Figure 5. The effect of feeding status and sNPF on responsiveness to nociceptive stimuli. (a, b) Responsiveness to electric shocks. Sample sizes were as follows: $n_{Untreated} = 49$; $n_{Solvent} = 42$; $n_{sNPF\ 1} = 39$; $n_{sNPF\ 10} = 44$. **(b, d) Responsiveness to thermal stimuli.** Sample sizes were as follows: $n_{P\text{-}fed} = 38$; $n_{Starved} = 33$; $n_{P\text{-}fed\ solvent} = 37$; $n_{P\text{-}fed\ sNPF\ 1} = 40$; $n_{P\text{-}fed\ sNPF\ 10} = 38$. **(a)** Population response (% of bees, *n* = 174) responding with Sting Extension Response (SER) to increasing voltages. **(b)** Individual scores of bees were calculated from the number of SER elicited by bees along the electric shock stimuli. A high score indicates that the bee was responsive to low voltages, conversely a low score indicates that the bee responded only to high voltages. **(c)** Population response (% of bees, *n* = 186) responding with a Sting Extension Response (or SER) to increasing temperatures. **(d)** Individual scores were calculated from the number of SER elicited by each bee along the thermal stimuli. A high score indicates that the bee was responsive to low temperatures, conversely a low score indicates that a bee responded only to high temperatures. In **(b, d),** scattered plots represent individual values. Sample sizes are indicated in parentheses below each box. Boxes with different letters differed significantly (Wilcoxon sum-rank pairwise test, Holm *p* adjustment method), ns = non-significant. Error bars represent the 95 % bootstrapped confidence intervals.

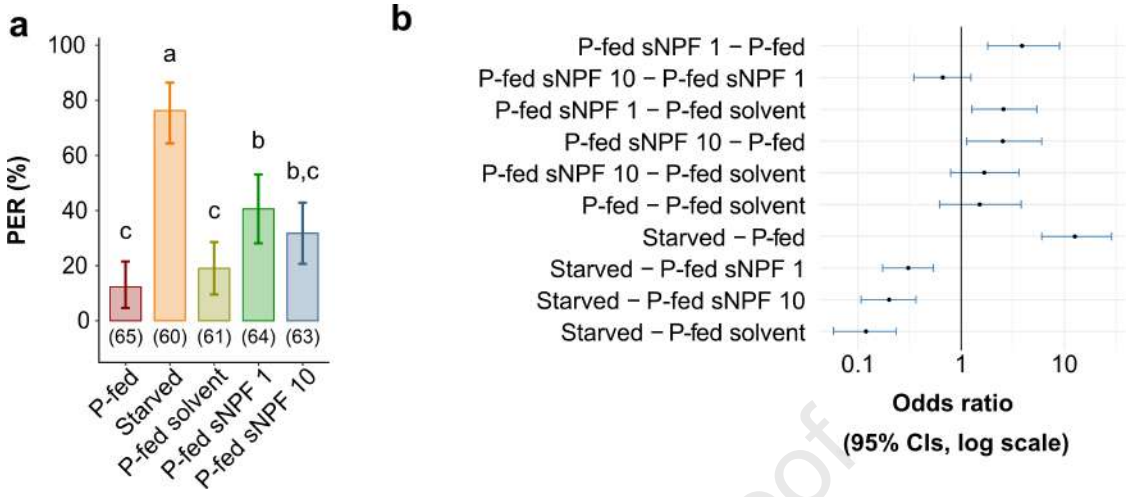


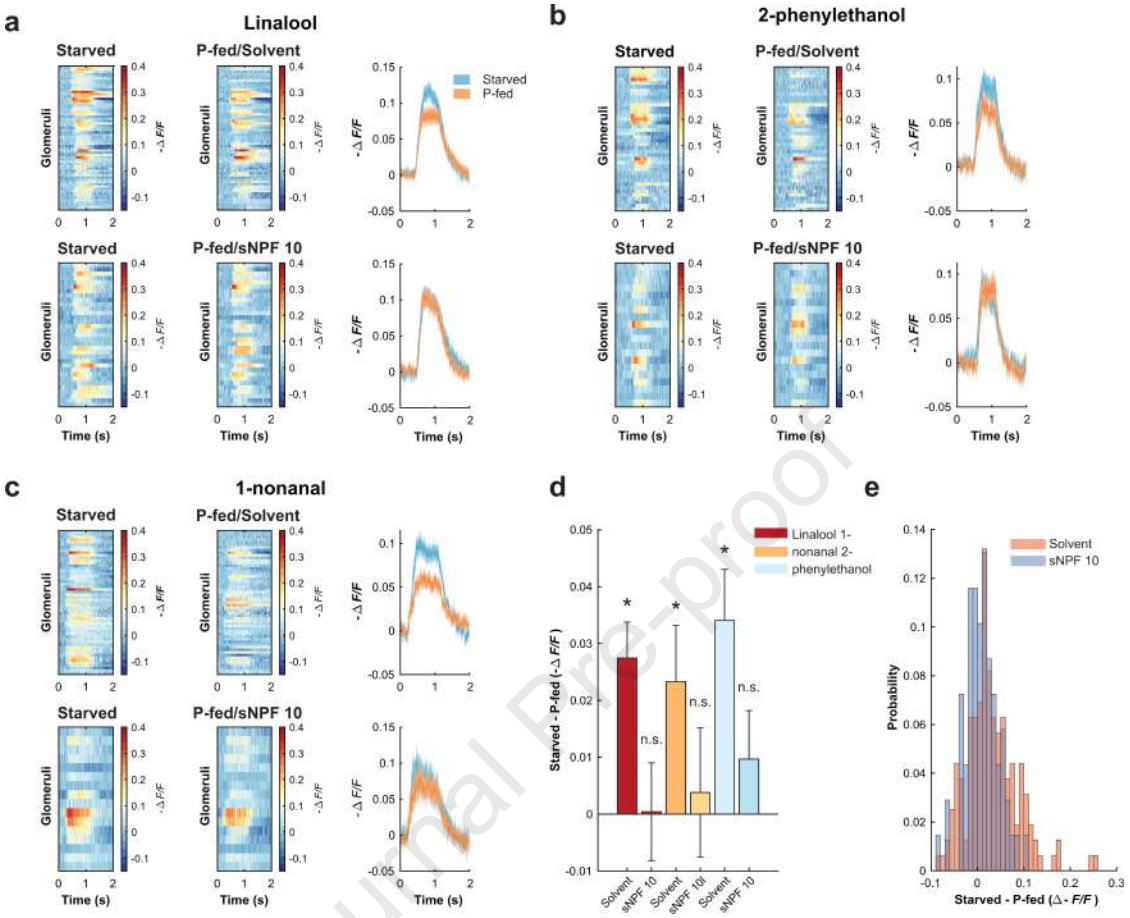


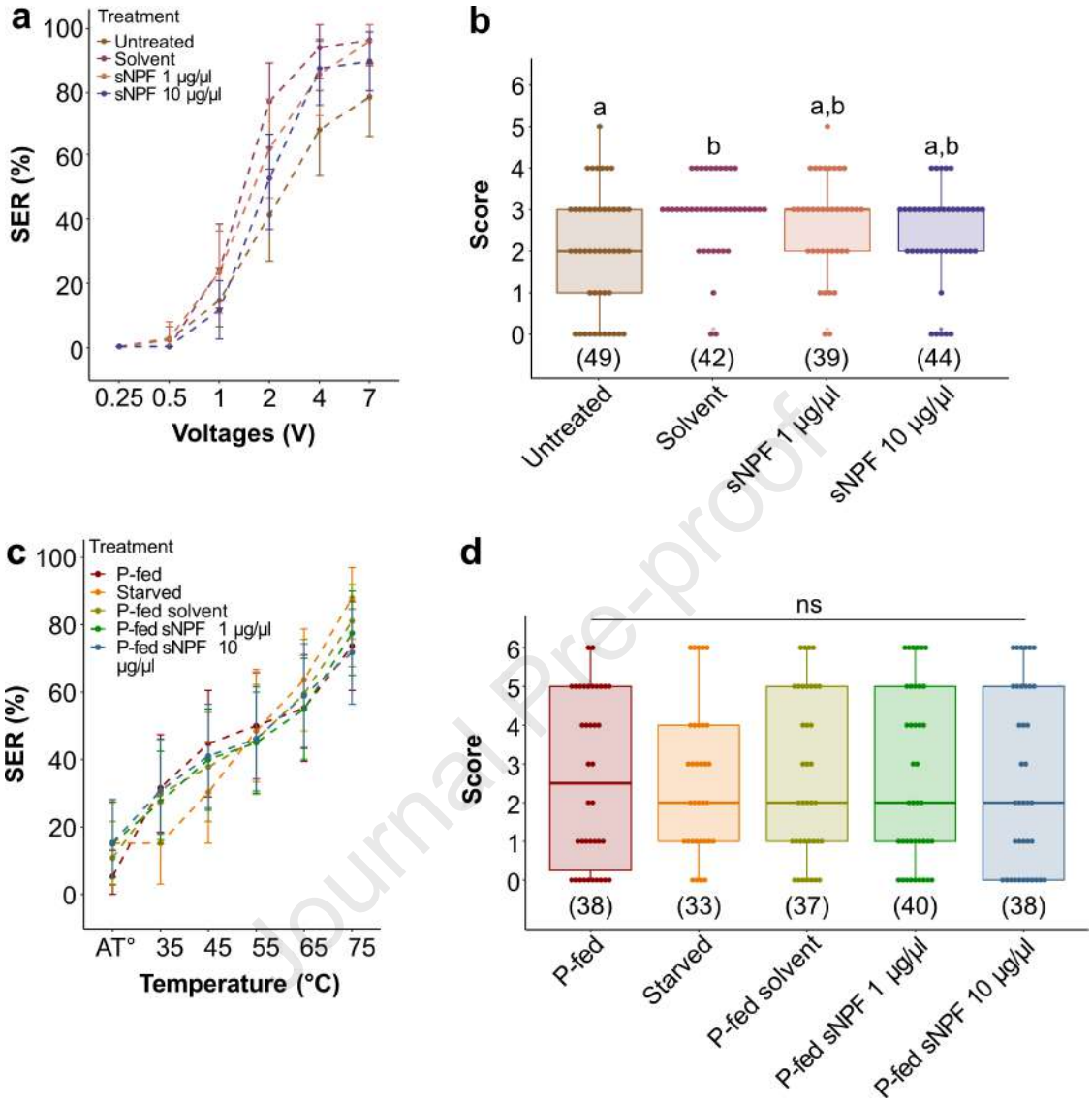












Highlights

- sNPF increases food consumption and appetitive responsiveness of honey bees.
- Feeding reduces neural activity in odor circuits; sNPF restores it to the starved level.
- sNPF has no effect on responsiveness to nociceptive stimuli in honey bees.
- sNPF is a key modulator of hunger and food-related responses in bees.

Chapter 2

Short neuropeptide F (sNPF) promotes the acquisition and formation of visual memories in honey bees

Louise Bestea, Emmanuelle Briard, Julie Carcaud, Jean-Christophe Sandoz, Rodrigo Velarde, Martin Giurfa and Maria Gabriela de Brito Sanchez

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Abstract

Appetitive motivation drives both foraging and appetitive learning and memory formation. In insects, appetitive motivation is modulated by multiple signalling mechanisms among which the neuropeptide F (NPF) and its short variant (sNPF) play an essential role. In honey bees (*Apis mellifera*), both peptides exist but only sNPF has an associated receptor, thus advocating for a functional role of the short peptide. Honey bees learn and memorize visual cues in the context of their foraging activities but the underlying motivational mechanisms are poorly known. Here we manipulated the feeding state of honey bee foragers and studied if sNPF modulates the acquisition and formation of colour memories when free-flying bees were trained to discriminate a blue from a yellow target within a miniature maze. Artificially increasing of sNPF levels in partially fed foragers with a reduced motivation to learn colours resulted in significant colour learning and memory above the levels exhibited by starved foragers. Our results thus identify sNPF as a critical component of motivational processes underlying learning and memory formation in bees and thus, of their foraging activities, which rely on these capacities.

Introduction

A crucial requisite for appetitive associative learning is the presence of the appropriate motivation to respond to the appetitive reinforcement [134]. Motivation, defined as the energizing of behaviour in pursuit of a goal [3], is modulated by both environmental factors and internal state. This modulation occurs via the action of signalling mechanisms, which act at diverse circuit levels and allow integrating multiple sources of information in decision-making processes. Two key molecules regulating food-related behaviours in invertebrates are the neuropeptide F (NPF) and its short variant (sNPF) [49, 35]. This regulation may extend to appetitive cognitive performances driven by food rewards, although little is known about the impact of these peptides on learning and memory formation. In the fruit fly *Drosophila melanogaster*, NPF can either modulate the retrieval of an appetitive olfactory memory based on the hunger state of the fly [56], or impede ethanol and high sugar concentrations to act as reward during training in adults [135] and larvae respectively [95]. In addition, downregulation of sNPF levels or knockdown of sNPF receptors in the fly brain impairs olfactory memory [99].

Equivalent studies do not exist in the case of the honey bee, an insect that has a model status for studies on the neurobiology of learning and memory [136, 137]. Honey bees are well known for their capacity to associate multiple sensory cues with appetitive nectar, which in the laboratory can be replaced by a drop of sucrose solution [137]. The feeding state of the trained bees is crucial as it determines their willingness to either learn or express a learned response [92, 138]. However, the neural signalling mediating this appetitive motivation remains poorly known.

The genes *npf* and *snpf* and their corresponding peptides NPF and sNPF have been identified in honey bees. However, only a receptor gene for sNPF (*snpfR*) was found in the honey bee genome [43, 48], which advocates for a functional role of the short peptide. Here we focused on visual learning and memory of honey bee foragers [139] and analysed if and how sNPF levels modulate their capacity to learn and memorize colours. To this end, we increased artificially sNPF levels in partially-fed, free-flying honey bees, with a reduced motivation to learn and memorize colours, and trained them to discriminate colours within a miniature Y-maze. Our results show that enhancing sNPF levels leads to a significant improvement of colour learning and retention, above the levels exhibited by starved foragers. These results thus demonstrate a promoting effect of sNPF on motivational and attentional processes underlying colour learning and memory in honey bees.

Material and Methods

Pre-training and set up

Free-flying honey bee foragers were trained to visit a miniature PVC Y-maze [140] covered with a transparent plastic cover (fig. 2.1*a*). Each arm measured 10 × 5.5 × 4 cm with a detachable end section (5.5 × 4 cm) that allowed replacing the focal bee at the starting position of the maze after each choice. The back walls displayed a grey HKS-92N colour paper. Bees were pretrained to collect 50 % (w/w) sucrose solution provided alternately between the left and right arms by means of 1 µl Eppendorf tips inserted in the middle of the back walls. A bee was selected for further use if it had visited at least twice both the left and the right arm. In this case, it was marked with a colour spot on the thorax to allow its identification.

Experimental groups and pharmacological treatments

Upon return to the maze, the selected bee was moved to a small compartment (5.5 × 5.5 × 4 cm) offering an Eppendorf tip which could be filled either with distilled water (starved bees) or with 5 µl of a mixture of honey, pollen, sucrose and water and then with 15 µl of a 1.5 M sucrose solution (partially fed bees; henceforth P-fed bees) [236]. The volume of food provided corresponded to a third of a bee's crop capacity [142] so that bees may decide to continue foraging. After feeding, or after 5 min in the case of the starved bees, each bee was placed in a container with ice during 5 min to immobilize it and then either left unhandled or, in the case of P-fed bees, topically exposed to one of two doses of sNPF applied on the thorax: 1 µg/µl (hence 'P-fed sNPF 1') or 10 µg/µl (hence 'P-fed sNPF 10'). Another group of P-fed bees received the solvent used to dissolve sNPF (hence 'P-fed solvent'), which was a mixture of 20 % dimethyl sulfoxide and 80 % acetone. Each bee was then placed in an individual small cage where it recovered during 30 min. As sNPF is supposed to enhance appetitive responsiveness, it was not delivered to starved bees, which were presumed to be at a ceiling level regarding this trait. Starved bees constituted a control to establish whether sNPF improved learning and memory of P-fed bees. Overall, five groups of bees were established ('P-fed', 'P-fed sNPF 1', 'P-fed sNPF 10', 'P-fed solvent' and 'starved'). Sucrose and solvent drugs were purchased from Sigma-Aldrich (Steinheim, Germany) while honey bee sNPF was purchased from NovoPro (Shanghai, China; sequence: SDPHLSILSKPMSAIPSYKFDD [237]).

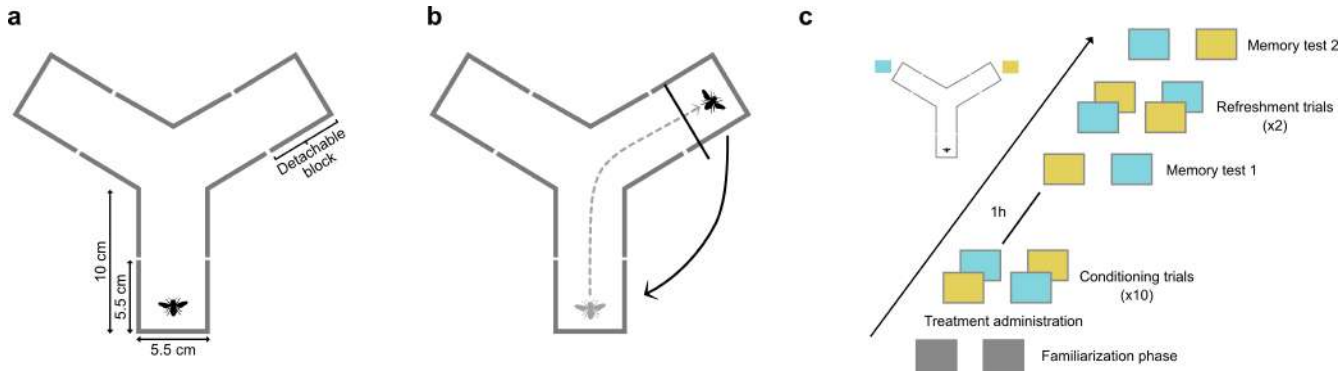

Figure 2.1: **Experimental setup and differential colour conditioning.** **(a)** Marked were individually trained in a miniature Y-maze whose end compartments could be closed by a sliding door after each choice and **(b)** translocated to the maze entrance for another trial. **(c) Conditioning procedure.** Each bee was trained to discriminate a yellow from a blue colour along ten consecutive trials. One colour was reinforced with sucrose and the other with water. One hour after conditioning, bees were tested twice for memory retention in the absence of reinforcement, with two refreshment trials interspersed between memory tests.

Differential visual conditioning

After recovery, the bee was released in a maze identical to the one in which it had been pre-trained (fig. 2.1*ab*) where it was conditioned during ten trials to discriminate a blue (B, HKS-47N) from a yellow (Y, HKS-2N) cardboard displayed on the back walls. Colours (fig. 2.3*a*) were well distinguishable from each other but had different chromatic contrasts against the grey H toKS-92N background (fig. 2.3*bc*) with yellow (Y) being more salient than blue (B). The visual angle subtended by each cardboard to the decision point of the maze was 31°, which ensured that bees were guided by the chromatic properties of the stimuli [143]. A 1 µl Eppendorf tip was inserted in the middle of each colour cardboard. One of them offered a 50 % (w/w) sucrose solution and the other distilled water. For each treatment, two subgroups with reversed contingencies (Y+ vs. B- and Y- vs. B+, '+' indicating sucrose reward and '-' distilled water) were conditioned in parallel. Colours were swapped pseudo-randomly between the arms of the maze (fig. 2.1*c*). A choice was recorded when the bee entered one of the two arms and contacted the tip in the middle of the colour stimulus. The bee was then translocated to the entrance compartment and a new choice was presented to it (fig. 2.1*b*). The latency of each choice was also recorded. After completing the ten choices, the focal bee was captured and kept in the dark for 1 h in order to be tested for memory retention in the absence of reward in a 1st memory test. Thereafter, two refreshment trials with reinforcement were allowed and a 2nd memory test with the colours swapped between maze arms was performed to finalize the experiment.

Data analysis

Data were analysed and plotted using R software [242]. Learning curves are presented in Supplementary Information. Responses in the last trial were analysed with a Generalized Linear Model (GLM) and choice latency during learning trials was analysed using a Linear Mixed Model (LMM) (R package *nlme*) followed by ANOVA (R package *car*) and Tukey *post hoc* tests when necessary. Latency served as a quantitative variable while treatments, trials and the colour conditioned served as fixed effect. Bee's identity was entered as a random effect. Cumulative learning scores were calculated for each bee by attributing a score of +1 upon each correct choice and -1 upon each incorrect choice. The cumulative score of each bee was obtained by summing the scores of all trials (see Supplementary Information) and its distribution was analysed using pairwise Kolmogorov Smirnov tests with Bonferroni correction for p-values. The proportion of correct choices in the last trial and in both retention tests was compared to a theoretical value of 50 % using Wilcoxon signed-rank test.

Results

To evaluate learning success in each group, we focused on the last conditioning trial as successful learning should lead to correct discrimination at the end of training (fig. 2.2, 1st bar in each panel). Neither the treatment nor the colour conditioned had any effect on colour discrimination in that trial (Treatment, GLM, $\chi^2 = 3.93$, d.f. $= 4$, $p = 0.42$; Colour conditioned, GLM, $\chi^2 = 0.53$, d.f. $= 1$, $p = 0.47$). Only P-fed sNPF 10 bees showed a proportion of correct choices that differed significantly from chance level when rewarded on yellow (fig. 2.2*e*; V $= 104$, $p = 4.98 \times 10^{-3}$). The learning curves of the different groups (fig. 2.4) did not show a consistent trend, which may have been caused by the impossibility to return to the hive. It was thus difficult to decide if learning took place.

The choice latency of bees was not influenced by the colour conditioned (fig. 2.4 right: LMM, $\chi^2 = 0.08$, d.f. $= 1$, $p = 0.77$) but decreased significantly along trials (fig. 2.4right: LMM, $\chi^2 = 62.87$, d.f. $= 9$, $p = 2.2 \times 10^{-15}$) and varied with treatment (fig. 2.4 right: LMM, $\chi^2 = 14.33$, d.f. $= 4$, $p = 6.31 \times 10^{-3}$) as starved bees chose faster than P-fed bees treated with sNPF 1 µg/µl ($p = 0.01$) and P-fed solvent bees ($p = 0.01$) when pooling colours. The distribution of cumulative scores did not differ between treatments when bees were conditioned on yellow (fig. 2.5 right: all tests $p > 0.05$) or blue (fig. 2.5 left: all tests $p > 0.05$). Overall, these data did not provide clear evidence for learning although P-fed sNPF 10 bees trained on yellow exhibited a significant preference for yellow in the last conditioning trial and choices became faster along conditioning.

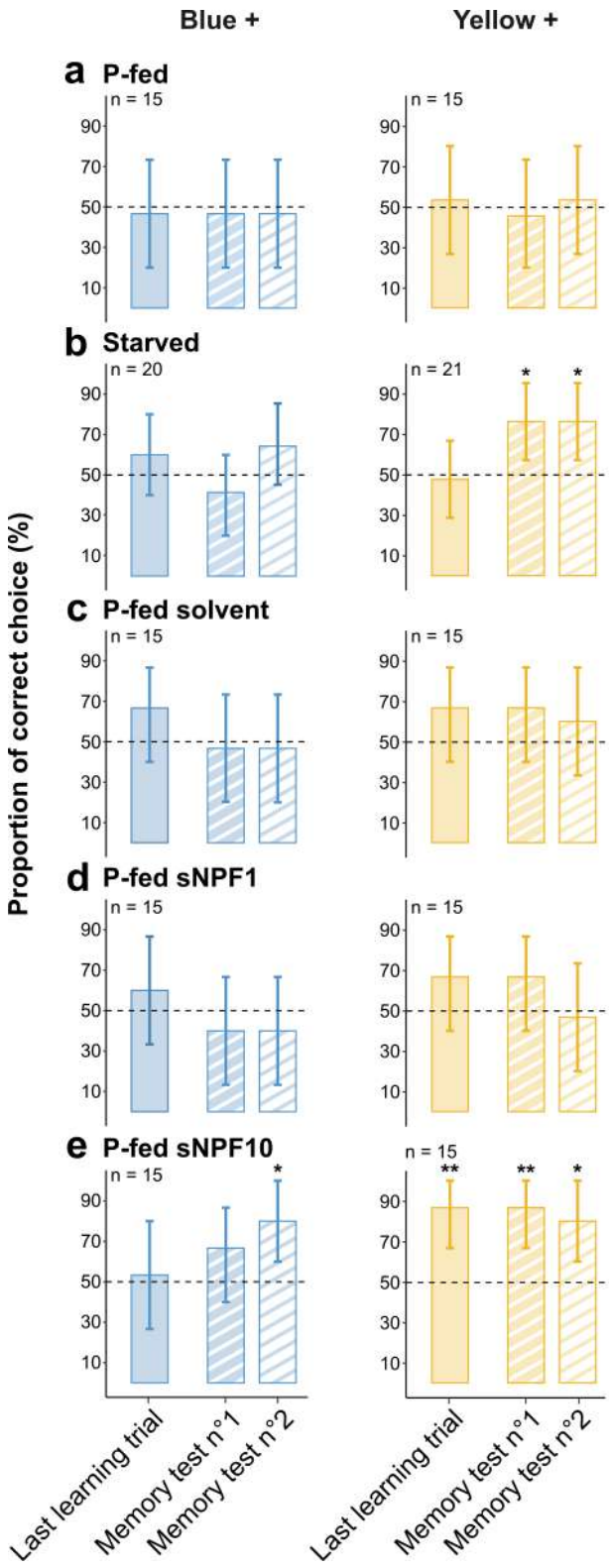

Figure 2.2: **Learning and retention performances following differential colour conditioning.** Proportion of correct choices in the last trial of the visual conditioning (1st full bar in each panel) and in the two retention tests (2nd and 3rd hatched bars in each panel). P-fed, blue-trained: n = 15, yellow-trained: n = 15; starved bees, blue-trained: n = 20, yellow-trained: n = 21; P-fed solvent, blue-trained: n = 15, yellow-trained: n = 15; P-fed sNPF 1, blue-trained: n = 15, yellow-trained: n = 15; P-fed sNPF 10, blue-trained: n = 15, yellow-trained: n = 15. Error bars represent the 95 % boostrapped confidence interval. *: p-value < 0.05; **: **p**-value < 0.01.

fig. 2.2 also shows the retention performances in the 1st and 2nd memory tests performed 1 h after training (2nd and 3rd bars in each panel). Starved bees exhibited a significant preference for yellow (fig. 2.2*b*; test 1: V = 176, $p = 0.02$; test 2: V = 176, $p = 0.02$), thus showing that they had learned the colour-sucrose association at the end of the conditioning, even if they did not express it, and that the time elapsed since training stabilized and rendered visible the information learned. In the case of blue training, starved bees exhibited a non-significant percentage of choices (40 %) in the first test (fig. 2.2*b* left; test 1: V = 84, $p = 0.38$), which increased in favour of the trained blue colour (65 %) in the second test without reaching significance (V = 136.5, $p = 0.09$). Differences in performances between the starved animals trained on blue and yellow reflect the different chromatic salience of these colours.

Bees of the P-fed, P-fed solvent and P-fed sNPF 1 groups, which showed no sign of learning during training, also showed non-significant retention during the memory tests, both after yellow and blue training (fig. 2.2*a-cd*; all analyses: $p > 0.05$). Thus, the time elapsed between the last training trial and the memory tests did not improve *per se* the performance of P-fed bees. However, P-fed sNPF 10 bees exhibited significant retention in both memory tests when the trained colour was yellow (test 1: V = 104, $p = 4.98 \times 10^{-3}$; test 2: V = 96, $p = 0.02$), in which case performance was similar to that of the starved group, and in the second memory test when it was blue (V = 96, $p = 0.02$), in which case performance was better than that of the starved group. Thus, P-fed bees treated with the highest dose of sNPF had learned their colour associations and expressed it during the memory tests.

Discussion

Our results show that treating bees with the higher dose of sNPF promotes associative colour learning and the expression of colour memories during retention tests performed 1 h after training. Performance of P-fed sNPF 10 bees was better than that of starved bees, which exhibited learning and retention only in the case of the highly salient yellow colour (fig. 2.2*de*). Such a colour-dependent bias was partially overcome in P-fed sNPF 10 bees, which exhibited significant retention for both yellow and blue, despite their differences in chromatic contrast. For blue, this preference was only visible in the 2nd memory test, showing that colour saliency also affected their memory. Remarkably, the memory performance of starved bees after blue training followed a similar tendency as in P-fed sNPF 10 bees with the difference that it did not reach significance in the 2nd test (compare left panels of fig. 2.2*b-e*).

Honey bees satiated before being subjected to olfactory conditioning exhibit impaired learning and memory, thus suggesting that satiation interferes with the process of associative learning itself

[92]. This was not the case in our visual conditioning experiments: although learning performances were not stable, P-fed sNPF 10 bees exhibited significant memory performances one hour after training. This suggests that the 1-h period between the last training trial and the retention tests contributed to consolidate the colour memories in these animals. Satiated bees in which basal phosphokinase A (PKA) was increased before olfactory conditioning via injection of the PKA activator Br-cAMP exhibited a performance similar to that of P-fed sNPF 10 bees subjected to visual conditioning: their learning was impaired but their memory was enhanced in a time dependent manner [92]. It is thus possible that our free flying bees had higher levels of PKA and that a functional link exists between sNPF and PKA signalling, which affects memory expression. The fact that the same lapse of time had no influence on memory expression in the other P-fed groups indicates that these animals did not learn the task and that their feeding and motivational state were inappropriate for learning the colour information. The highest dose of sNPF was able to revert this state and induce an enhanced appetitive state compatible with learning and memory formation.

In our experiments, returning to the hive was not possible after each colour trial as bees were translocated to the maze entrance for additional choices. Proceeding in this way was important because returning to the hive and unloading the food gathered would change the crop contents and eventually sNPF levels, which can vary according to the foraging phase [235]. This would render our experimental treatments useless. The fact that this impediment did not prevent P-fed sNPF 10 bees from establishing colour memories suggests that sNPF has a crucial role in regulating the individual appetitive motivation and perceptual and attentional processes referred to food-related cues. This is particularly evident in the case of the blue colour, for which significant retention was only found under the highest dose of sNPF (2nd test), even above the level of starved bees, which showed nevertheless the same retention trend, without reaching significance.

The role of sNPF in insect visual performances has only been studied in the desert locust (*Schistocerca gregaria*) [78, 100]. In this insect, starvation reduces the sNPF precursor transcript level in the optic lobes [101]. Yet, knocking down sNPF to mimic starvation state had no influence on the appetitive visual learning and memory of locusts [100]. This lack of effect contrasts with results obtained in odor-trained fruit flies where starvation increases sNPF levels and knock down of the sNPF precursor to mimic a fed state leads to a reduction of olfactory memory [99]. Accordingly, stimulation of NPF neurons in fed flies before a memory test promoted olfactory memory retrieval, in a situation in which it should not have been expressed [56]. Our results are in line with these findings obtained in fruit flies in the framework of olfactory learning and show that sNPF has the capacity to improve learning and retention performances, thus providing a neural basis for motivational processes that are necessary to sustain both cognitive tasks in honey bees and foraging behavior, which are crucial for colony survival.

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Contributions

L.B. conceptualization, data analysis, writing and editing of the manuscript. E.B. data collection. J.C editing of the manuscript. J.-C.S. funding acquisition and editing of the manuscript. R.V. editing of the manuscript. M.G. conceptualization, data collection, funding acquisition, writing and editing of the manuscript. M.G.B.S. conceptualization, funding acquisition, writing and editing of the manuscript.

Supplementary information

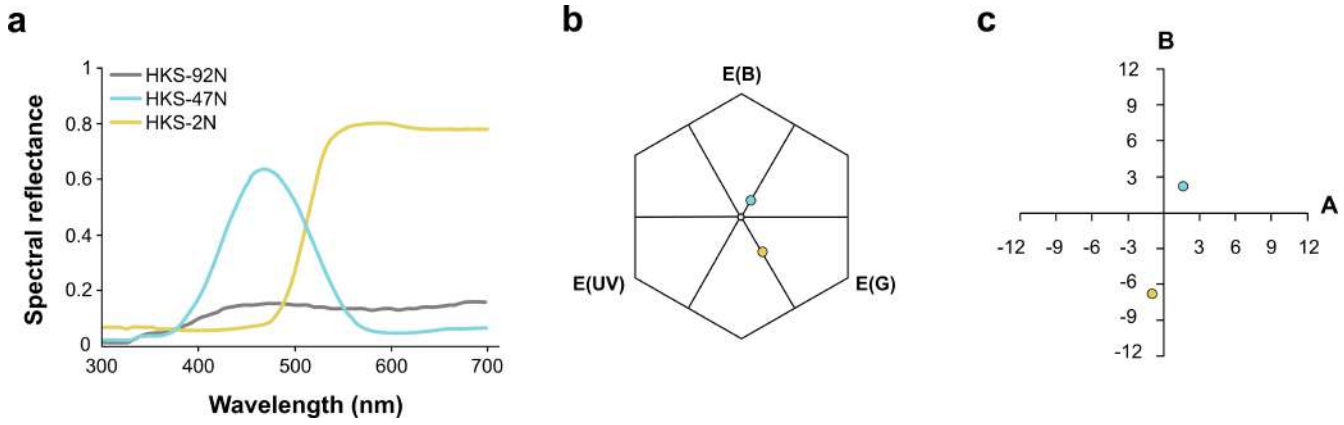

Figure 2.3: **Chromatic properties of the colours used.** **(a)** Spectral reflectance curves of yellow (HKS-2 N), blue (HKS-47 N) and grey (HKS-92 N). **(b)** Colour loci of the stimuli used in the colour hexagon of the honeybee [145]. The diagram represents the loci of the colour stimuli after coding by two generic colour opponent coding processes and provides a measure of colours as perceived by bees using the grey HKS-92N as adaptation background. **(c)** Colour loci of the stimuli used in the colour opponent coding (COC) space of the honeybee [146]. The diagram represents the loci of the colour stimuli after coding by two kinds of specific colour opponent coding processes (UV *vs.* B/G and B *vs.* U/G) and provides a measure of colours as perceived by bees using the grey HKS-92N as adaptation background. Both spaces provide similar results: the yellow colour HKS-2N had a larger chromatic distance to the background than the blue HKS-47N and was therefore more salient in perceptual terms.

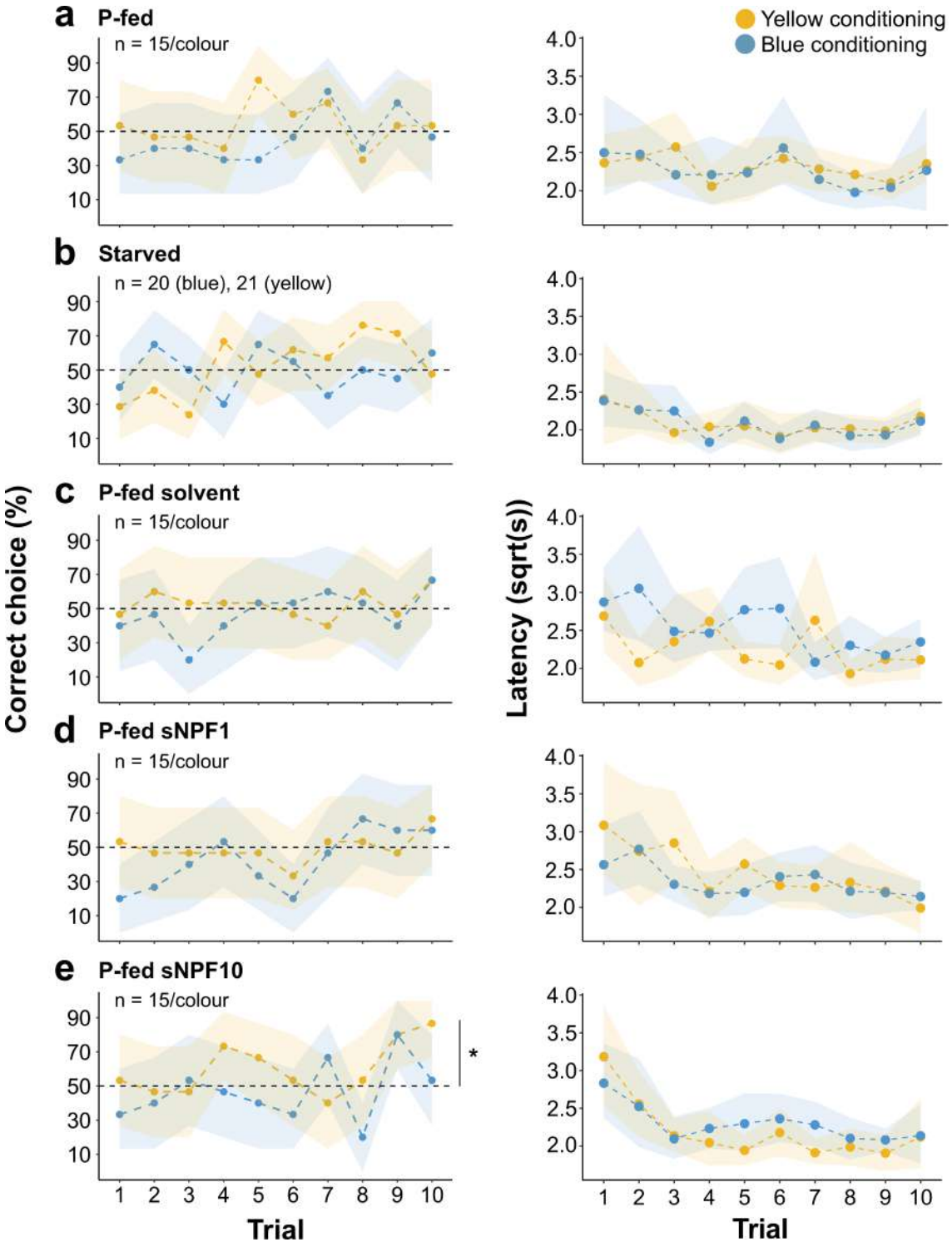

Figure 2.4: **Learning curves representing percentage of correct choices (left) and the choice latency of bees (right) during differential visual conditioning.** Square root transformation of latencies was used for better visualizing group performances based on a common scale. Yellow and blue areas around the curves represent the 95 % boostrapped confidence interval of CS+ choices. *: p-value < 0.05.

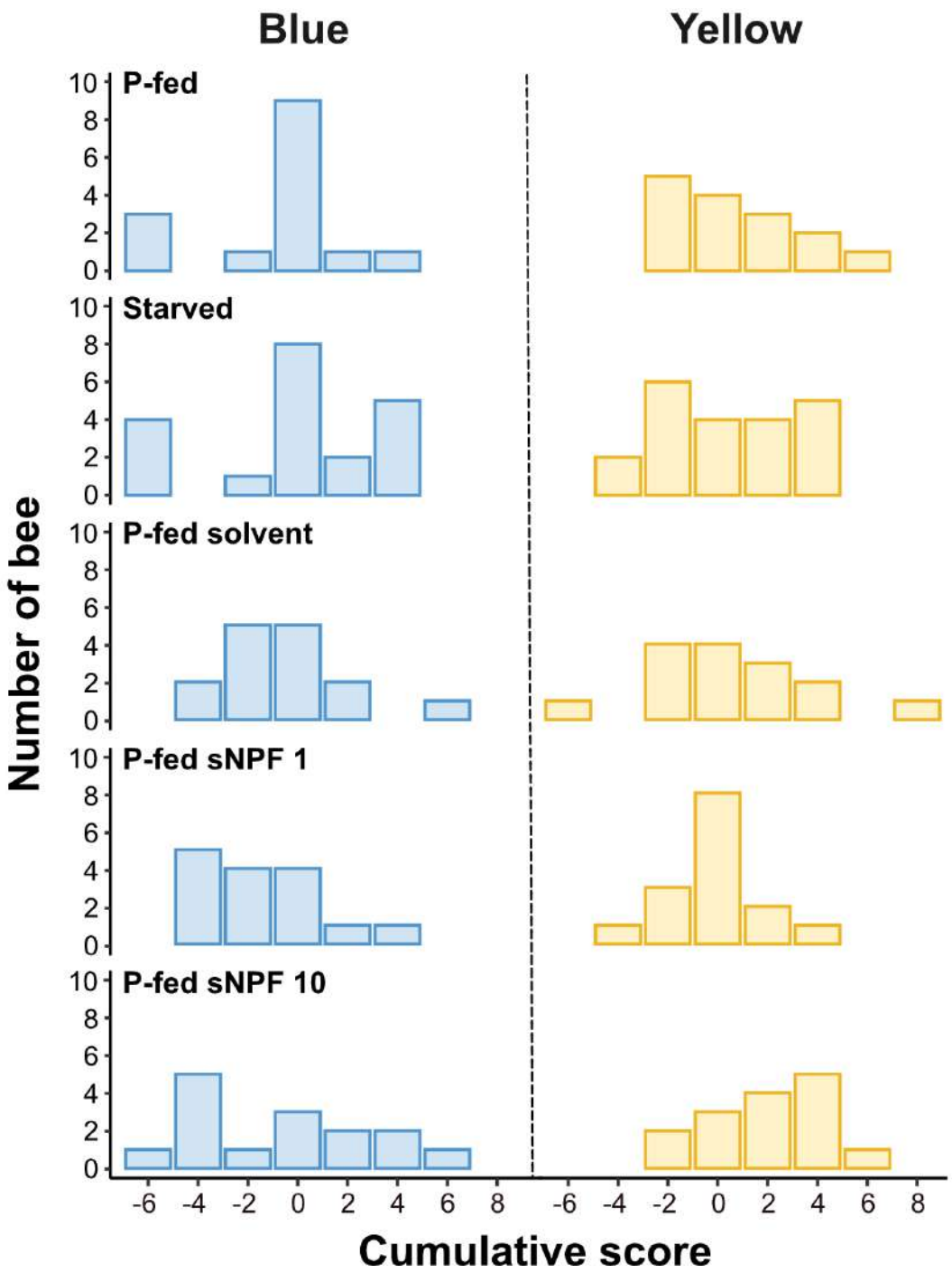

Figure 2.5: **Distribution of cumulative learning scores of bees rewarded on blue (left) and on yellow (right).**

Chapter 3

Effect of the neuropeptide sNPF on appetitive and aversive learning in honey bees

Louise Bestea, Caroline Marchand, Lou Frehring, Martin Giurfa and Maria Gabriela de Brito Sanchez

This chapter is not yet ready for publication.

Introduction

Associative learning in both appetitive and aversive contexts is fundamental for animals to increase chances of survival in a dynamic environment. Being able to seek and find food is as crucial as avoiding nociceptive stimuli than endanger an individual's survival. Animals are able to efficiently associate these contexts with features, such as odorants, tastants or visual stimuli. Such is the case of the honey bee, which learns, both in nature and in the laboratory, to associate multiple sensory cues with either appetitive reinforcements (typically sucrose solution used as replacement for nectar) or aversive reinforcements (typically electric or thermal shocks) [147, 124, 148, 129]. In both the appetitive and aversive domains, learning is constrained by sensory responsiveness to the reinforcement used for conditioning. For instance, in the appetitive domain, responsiveness is evaluated through the proboscis extension response (PER) exhibited to a series of increasing concentrations of sucrose solution [149] (see Chapter 1, **????**; [150]). Forager bees with a high sucrose responsiveness (i.e. highly responsive to various sucrose concentrations) are better learners than those with low sucrose responsiveness [149, 151] as their subjective evaluation of reward is higher during odour conditioning of PER, a protocol in which harnessed bees learn the association between a neutral odorant (the conditioned stimulus or CS) and the sucrose reward (the unconditioned stimulus or US) [148, 152]. A similar relationship was observed within the aversive domain. In this case, aversive responsiveness is quantified via the sting extension response (SER) exhibited to aversive stimuli such as electric shocks [147, 124, 148, 125] or thermal shocks [127, 153]. Animals with high aversive responsiveness learn better the association between odorants (CS) and the aversive reinforcement (US) triggering SER [147, 154].

A neuronal mechanism integrating the internal state of hunger and appetitive memory has been identified in the fruit fly *Drosophila melanogaster* and involves the neuropeptide F (NPF) [56], an orthologue of the vertebrates NPY [40, 155, 46], known to play a key role in the regulation of food-related behaviours [49]. NPF signalling via its associated receptor NPFR promotes appetitive memory performance by suppressing the inhibitory activity of dopamine neurons innervating the mushroom bodies [13], which are high-order brain regions of the insect brain involved in associative learning and memory formation and retrieval [60, 61]. NPF also plays a role in aversive but not appetitive olfactory learning in the nematode *Caenorhabditis elegans* [106]. Yet, the underlying mechanisms relating these peptides and learning circuitries are still poorly understood, in particular in social insects, where food decision-making is mainly driven by the colony needs rather than individual ones, although both factors might be related.

Two NPY-related genes, *npf* and short *npf* (*snpf*), and their corresponding peptides NPF and sNPF have been identified in honey bees. However, only a receptor gene for sNPF (*snpfR*) was found [43, 48], which justifies a special focus on this short peptide. A prior study addressing the

role of NPF/sNPF signaling for appetitive behavior in bees [234] showed that the snpf gene and its receptor gene *snpfR* were partially upregulated in the brain of foragers; yet, only the latter increased its expression when colonies were food deprived [234]. No upregulation of *snpfR* was observed in the brain of foragers from well-fed colonies [234].

In chapter 1 we demonstrated that artificial increase of sNPF in partially fed foragers, with a reduced appetitive motivation, increases both their sucrose responsiveness and their responsiveness to odorants (Chapter 1, **????**). Multiphoton *in vivo* recordings of neural activity of antennal lobes, the primary olfactory centre in the bee's brain, revealed that partially fed bees exhibit a decreased responsiveness to appetitive and neutral odours that is rescued by treatment with sNPF to the level exhibited by starved bees (Chapter 1, **??**). However, the enhancing effect of sNPF was totally absent when assessing responsiveness to aversive stimuli (Chapter 1). Based on these results, it could be expected that sNPF has a positive impact on appetitive olfactory learning and memory (*via* its double impact, both on sucrose responsiveness and on odour processing). On the contrary, it can be predicted that sNPF should have no effect on aversive learning and memory as it is unable to modulate responsiveness to aversive reinforcements.

In a previous chapter (see Chapter 2), we explored the role of sNPF in appetitive visual learning of free-flying honey bee foragers trained to discriminate a blue from a yellow target. We showed that increasing artificially sNPF levels in partially fed foragers, with a reduced motivation to learn colours, resulted in significant colour learning and memory above the levels exhibited by starved foragers (Chapter 2). This finding confirmed the hypothesis mentioned above relating appetitive responsiveness, sNPF levels and learning and memory but requires further testing using conditioning protocols addressing different sensory modalities. Moreover, studying the effect of sNPF in a form of aversive conditioning is also missing to confirm the dispensability of this peptide for aversive learning and memory. Here we subjected bees to the olfactory conditioning of PER (see above) and to the aversive gustatory conditioning of SER [129] to determine if sNPF is necessary for the former but not for the latter. In the first protocol, bees learned an association between a single odorant and sucrose (absolute conditioning) and their memory was tested 24 h later. In the second protocol, harnessed bees learned to discriminate two tastants associated with different outcomes (differential conditioning). Tastants were delivered to opposite antennae, one of them being paired with an electric shock (CS+) and the other with the absence of shock (CS-). Bees should thus learn the association between taste and shock; their aversive gustatory memory was tested 1 h later. In both cases, we used partially fed bees treated with sNPF, to determine the impact of this neuropeptide on olfactory appetitive learning and memory and on gustatory aversive learning and memory, respectively. Our results show that a tendency towards a facilitation of appetitive learning and memory existed, yet further replications would be needed to determine whether this trend can become significant or not. On the contrary, no effect of sNPF

on aversive gustatory learning and memory could be detected, thus suggesting that the effect of sNPF for experience-dependent behavior is restricted to the appetitive modality.

Material and Methods

Insects

Honey bee foragers from colonies located in the apiary of the Research Centre on Animal Cognition (Toulouse, France) were collected in the morning at an artificial feeder they were previously trained to visit. Since sNPF brain levels can vary depending on crop filling [235], foragers were caught upon landing on the feeder, just before they started feeding, and placed individually into syringes with an open hub to allow for respiration. They were then brought to the laboratory for further use.

Experimental groups and pharmacological treatments

Bees were divided into five groups. One group was kept deprived of food ('starved'). The other four groups were fed by fitting within the open hub of the syringe an Eppendorf tip so that the enclosed bee could feed from it. Bees got a first tip containing 5 µl of a mixture of honey, pollen, sucrose and water, and then a second tip containing 15 µl of a 1.5 M sucrose solution in which case the feeding state was labelled as 'partially fed' (henceforth P-fed). Feeding lasted between 15 and 30 min, depending on the number of bees assigned to an experiment. After feeding, the syringes containing the bees were placed in ice during 5 min. This allowed to take out the cold-narcotized bees and proceed to the topical-application phase. One of the fed groups was left untreated ('P-fed'). Two other fed groups received a topical application [156] of sNPF on the thorax. One group received a concentration of 1 µg/µl ('P-fed sNPF1') and the other a concentration of 10 µg/µl ('P-fed sNPF10'). The last P-fed group received a topical application of the solvent used to dissolve sNPF ('P-fed solvent'), which was a mixture of 20 % dimethyl sulfoxide (DMSO) and 80 % acetone (DMSO/AC). As sNPF is supposed to enhance appetitive responsiveness, it was not delivered to starved bees, which were already at a ceiling level regarding appetitive responsiveness. Starved bees constituted therefore a control for the physiological effects of sNPF and allowed establishing whether sNPF treatment turned fed bees into starved-like animals. The topical-application phase lasted typically between 20 and 30 min depending on the number of bees assigned to an experiment. Each bee was replaced within its individual syringe after topication where it recovered from the cold treatment. Conditioning experiments started between 20 and 30 min

after the first topical application. Sucrose and solvent drugs were purchased from Sigma-Aldrich (Steinheim, Germany) while honey bee sNPF was purchased from NovoPro (Shanghai, China; sequence: SDPHLSILSKPMSAIPSYKFDD [237]).

Absolute olfactory conditioning

After receiving the treatment, bees were harnessed in individual metal tubes so they could only move their antennae and mouthparts [118]. The conditioning session consisted of three conditioning trials spaced by 15 min in which a single odorant (i.e. absolute conditioning) was paired with a 50 % sucrose solution (weight/weight) [3]. The odorant (conditioned stimulus – CS) was delivered through an automated odour-releasing machine (olfactometer) controlled by a microcomputer (Arduino® Uno) [157] while the sucrose solution (unconditioned stimulus – US) was delivered by means of a toothpick dipped into the sucrose solution and contacting first the antennae and then the extended proboscis. The harnessed bee was placed in front of the olfactometer, which released a continuous flow of clean air (3300 ml/min) to the bee antennae [157]. Fifteen seconds after the onset of the training, the airflow was diverged upstream through the vial containing the odorant serving as the CS during 6 s. An air extractor was placed behind the bee to prevent odorant accumulation. The US was delivered during 3 s using a toothpick, 4 s after CS onset. The CS and the US had an overlap of 2 s. After US offset, the bee was left in front of the clean air flow for additional 28 s, so that the training trial lasted 50 s in total. The definition of a conditioned response was the full extension or the proboscis (PER) at the onset of the odour delivery prior to the antennal stimulation with the US. The P-fed bees that did not respond to the sugar stimulation by extending their proboscis were still kept for the experiment as they might learn without displaying PER due to their partially satiated state [138, 158].

Four odorants were used as CS in these experiments: 2-hexanol and nonanal, in one experiment, and limonene and eugenol in another experiment. The two odorants used per experiment allowed testing for odorant generalization in a retention test (see below). Olfactory discrimination as quantified in prior studies guaranteed that odorants within each pair were perceived as dissimilar by bees [159, 160]. All odorants were purchased in Sigma-Aldrich, France. Groups of 15 bees were trained one after the other so that the intertrial interval was 15 min. In this way, 15 min were required for the bees to complete each trial and to move to the next trial fig. 3.1.

Retention tests were performed 24 h after the training to assess long-term memory [61]. The CS was presented without reward; in addition, a novel odorant (NOd) was also presented in order to assess generalization. The Nod was the odorant of the pair that was not used for conditioning (for instance when the CS was 2-hexanol, nonanal was the NOd and vice versa). Based on these responses, the percentage of bees exhibiting CS-specific memory (i.e. responding to the CS and not

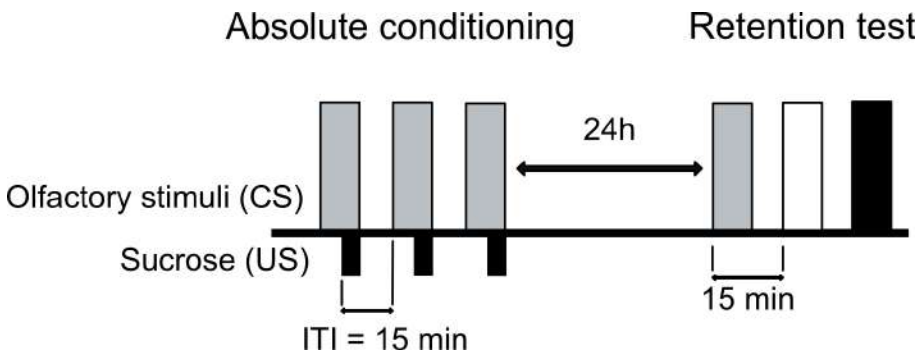

Figure 3.1: **Absolute olfactory conditioning of the Proboscis Extension Response (PER)**. Experimental schedule for absolute olfactory conditioning. An odorant is delivered through an automated odor-releasing machine (olfactometer). Bees were trained to associate one odorant (CS) (grey bars) paired 3 times with a 50 % sucrose reward (black bars, US). The intertrial interval (ITI) was 15 min. 24 hours after the end of conditioning a retention test was performed in which the learnt odorant (grey bar) and a new neutral odorant (white bar) were delivered without the sucrose reward. At the end of the test, the sucrose was delivered alone (black bar) to check the integrity of PER.

to the NOd) could be established. Test odorants were presented in a sequence that was randomized from bee to bee. Retention tests followed the same dynamics of conditioning trials but with no reward delivery: the bee was placed in front of the air-flow for 15 s followed by 3 s of odour presentation, and then by 42 s without odour stimulation. The proboscis extension response to the odorant stimulation was then measured. The interval between the two olfactory tests was 15 min. Each bee was tested in a single retention test, so that different groups of bees were used for the different retention tests. At the end of tests, bees were stimulated with 50 % sucrose solution on their antennae to verify the integrity of PER and were discarded from analyses if they did not respond.

Aversive differential gustatory conditioning

The same five groups as in the previous experiments were established. To ensure the same conductance for electric shock between P-fed and starved bees, 'starved' bees were given 20 µl of a diluted sucrose solution (7.45 % w/w). Such treatment still ensures two nutritional states as 'starved' bees consume more sucrose solution than our typical P-fed bees (see Supplementary Information Chapter 1).

After receiving their corresponding feeding/sNPF/solvent treatment, bees were harnessed on individual holders designed for aversive stimulation via delivery of an electric shock of 7 V [147] and kept in the dark for 1 h before the experiment. Holders consisted of two brass plates fixed to a Plexiglas plate. Brass plates were connected to the output of the stimulator (50Hz–AC current).

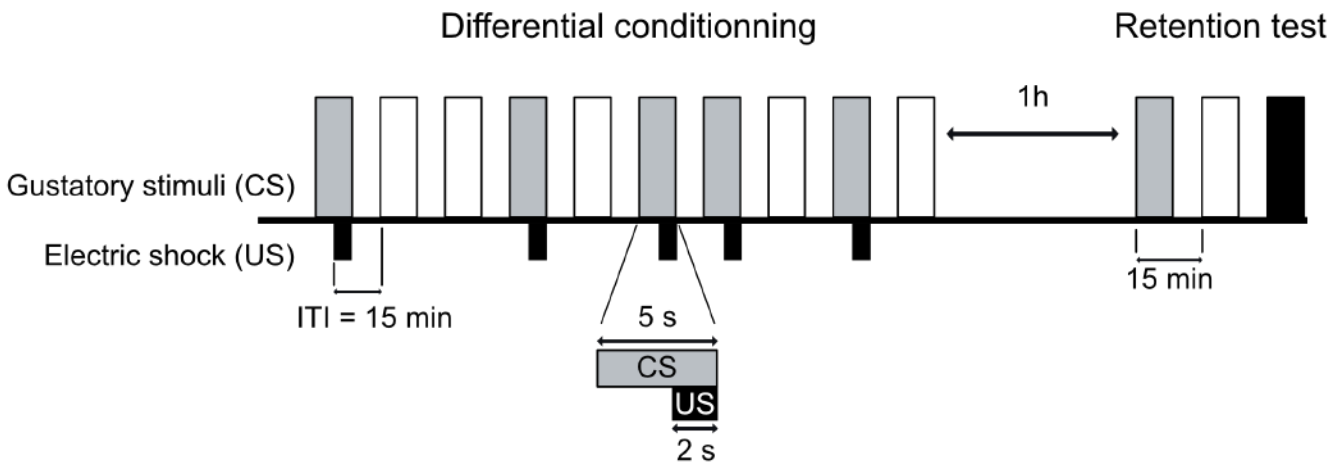

Figure 3.2: **Differential gustatory conditioning of the Sting Extension Reflex (SER).** Experimental schedule for differential gustatory conditioning of SER. A tastant is delivered by a toothpick contacting the antennae. Bees were trained to discriminate two tastants (CS), one (grey bars) paired 5 times with shock delivery (black bars, US) and the other presented 5 times without shock punishment (white bars). The intertrial interval (ITI) was 15 min. One hour after the end of conditioning a retention test was performed in which the two CSs were delivered without shock (white and grey bars). At the end of the test, the shock was delivered alone (black bar) to check the integrity of SER. Non-punished trials were similar except that no shock was delivered.

Conductance gel was applied below the thorax and the abdomen to ensure efficient shock delivery. Bees were then trained to discriminate 1 M sucrose from 3 M NaCl, two solutions that bees easily discriminate [129]. One tastant was paired with electric shock (CS+) while the other remained unpunished (CS). Bees had to learn to extend the sting only to the punished tastant. Four groups were conditioned to balance the contingencies between gustatory stimulus, antennal side and reinforcement.

Appetitive responsiveness to both tastants was first recorded for each bee by quantifying PER upon antennal stimulation. Then, aversive responsiveness to a single electric shock of 7 V was tested. Only bees that showed SER to the electric shock were used for gustatory conditioning.

Following the protocol established by Guiraud et al. [129], each conditioning trial lasted 1min. The bee was placed in the stimulation site in front of the air extractor to avoid contamination by alarm pheromones and left for 20 s before being exposed to the tastant paired with the electric shock. A toothpick soaked in the tastant solution was used to deliver the tastants (CS+ and CS-) to the antennae during 5 s. The electric shock (US) lasted 2 s; it started 3 s after onset of the gustatory stimulus and finished with it fig. 3.2. The bee was left in the setup for 35 s and then removed. Groups of 15 bees were trained one after the other so that the intertrial interval was 15 min. In this way, 15 min were required for the bees to complete each trial and to move to the next trial.

Gustatory stimuli were presented in a pseudo-random sequence of five reinforced and five non-reinforced trials (e.g. ABBABAABABBA) starting with stimulus A or B in a balanced manner. Each gustatory stimulus (A, B) was delivered to a single antenna, left (L) or right (R), so that the experiment involved four subgroups of animals to achieve balance between antennal sides and reinforcement contingencies: AL+ vs. BR, AL vs. BR+, AR+ vs. BL and AR vs. BL+. We quantified the occurrence of SER and PER to both tastants during conditioning trials and retention tests. Quantifying PER allows to assess a potential devaluation of the value of the tastant during the experiment and the influence of the conditioning on this process. Retention tests were performed 1h after the last conditioning trial and consisted of a presentation of the gustatory stimuli without punishment, using the same timing as in the conditioning trials. The sequence of tastant presentation (A, B) varied randomly from bee to bee during the tests. Once the tests were finished, the bees' response to a single electric shock was measured to verify the integrity of the unconditioned response. Only bees that consistently reacted to the electric shock were taken into consideration for the analyses.

Data analysis

Data were analysed and plotted using R software [242]. In all cases, data met the assumption of the tests used. For differential aversive gustatory conditioning only the last trial of each stimulation was analysed. As several factors needed to be considered, we built a Generalized Linear Model (GLM, R package *lmer*) where SER served as a binary-response variable (binomial family, 'logit' link), while treatments, tastants, antennal side and configuration (A-B+/A+B-) were entered as fixed effects. ANOVA (R package *car*) was performed on GLMs and post hoc multiple comparisons were used when necessary (Tukey p-value adjustment method, R package *emmeans*).

During aversive gustatory conditioning, proportions of spontaneous PER were quantified for each tastant. They were compared between the first and last trial of the conditioning within each treatment using McNemar tests for paired proportions. The same analysis was conducted on PER proportions between the beginning of the conditioning and the retention test to consider the whole experiment schedule.

For absolute olfactory conditioning, only the last conditioning trial was analysed in order to compare learning success achieved at the end of training in the different groups. Proportions of PER of the last trial were compared between treatments with multiple Fisher's exact tests (R package *rstatix*, Holmes p-value adjustment method).

CS-specific memory (the percentage of bees that responded only to the CS+ and not to the NOd during the retention test) was quantified after both differential and absolute conditioning.

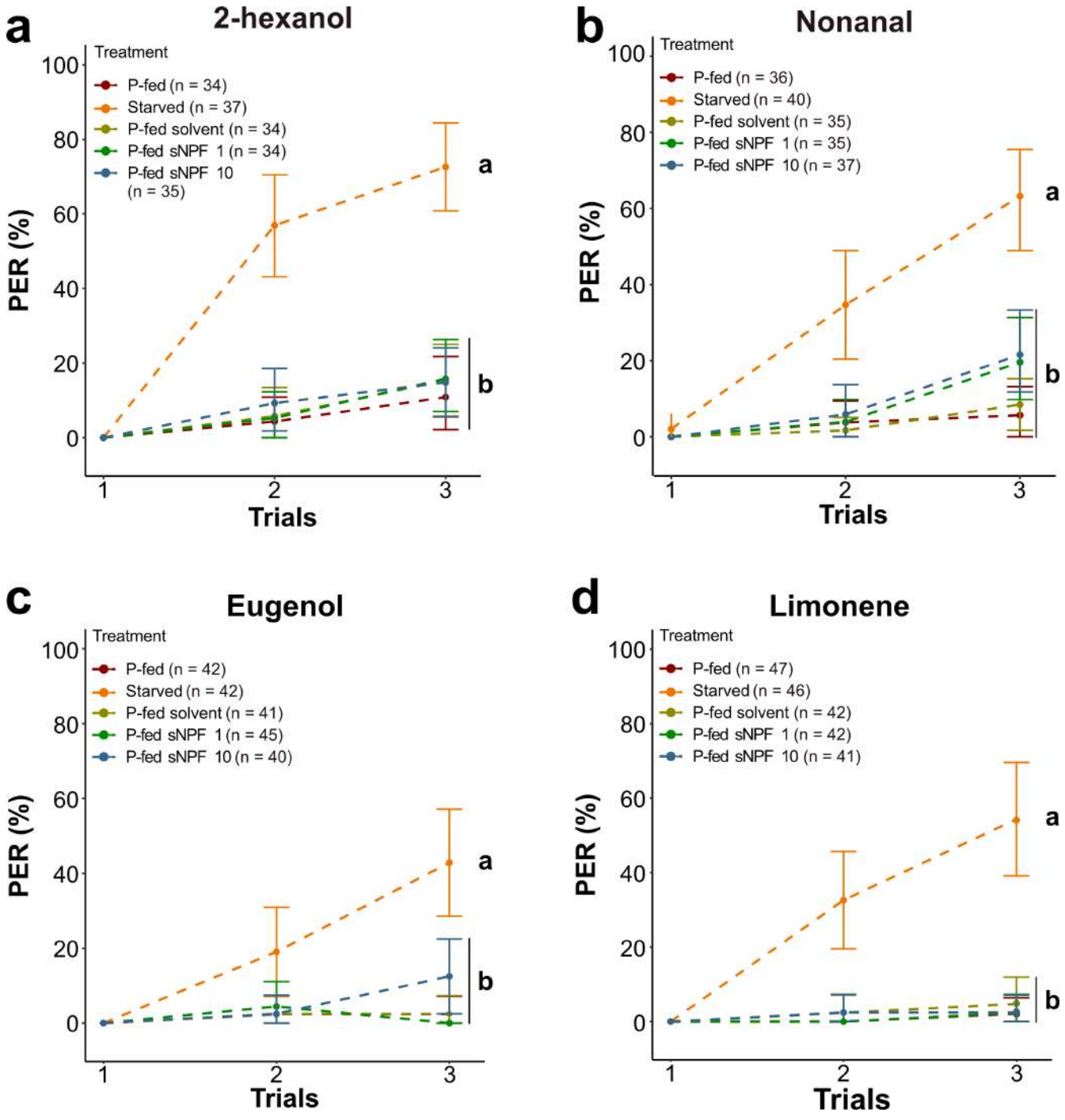

Figure 3.3: **Learning assays in absolute olfactory conditioning.** Bees were either trained to associate a sucrose reward with 2-hexanol or nonanal **(a,b)**. In a second conditioning, bees were trained to pair a sucrose reward with eugenol or limonene **(c,d)**. Error bars represent the 95 % boostrapped confidence interval of percentage of PER. Values in parentheses indicate group sample size.

Percentage of bees responding with PER to odorants were compared between treatments using multiple Fisher's exact tests (R package *rstatix*, Holmes p-value adjustment method).

Results

Effect of sNPF on appetitive olfactory learning and memory.

The effects of sNPF on olfactory learning and long-term (24 h) memory were first assessed in spring 2021 by conditioning the five groups of bees (starved, P-fed, P-fed solvent, P-fed sNPF 1 and P-fed sNPF 10) with either 2-hexanol or nonanal. In summer 2021 the same experiment was conducted again using limonene and eugenol as conditioned odorants.

In both experiments, the treatment had a significant effect on the proportion of bees exhibiting PER to the conditioned odorant during the last trial (fig. 3.3: Fisher's exact test, 2-hexanol: p = 1.28 × 10-4; nonanal: $p = 2.47 \times 10^{-8}$; eugenol: $p = 5.75 \times 10^{-10}$; limonene: p = 1.07 × 10-14). For each odorant conditioned, this effect was provoked by starved bees which responded significantly more to the conditioned odorant than the remaining four groups (fig. 3.3: Fisher's exact test performed on the last conditioning trial: all comparisons between starved bees and other treatments, $p < 0.05$). This result was expected given the higher appetitive motivation of starved bees. P-fed bees treated with either dose of sNPF did not show a better learning compared to control groups (P-fed and P-fed solvent bees) (fig. 3.3, all comparisons $p > 0.05$). For nonanal and eugenol, a non-significant tendency to PER increase was observed either for the two sNPF groups (nonanal) or for the higher dose of sNPF (eugenol) in the last conditioning trial.

Performance in the retention tests allowed to focus on the percentage of bees exhibiting CS-specific memory, i.e. the bees that responded only to the CS+ and not to the NOd during the retention test. Figure 4, shows that the treatment had again a significant effect on CS-specific memory for three out of four conditioned odorants (fig. 3.4a: Fisher's exact test, 2-hexanol; p = 4.69 × 10^{-7}; fig. 3.4b: nonanal; $p = 2.01 \times 10^{-6}$; fig. 3.4d: limonene; $p = 4.01 \times 10^{-4}$). In the first experiment, in which 2-hexanol and nonanal were used, significant differences were again introduced by starved bees, which showed a significant higher specific memory than the remaining P-fed groups when conditioned with 2-hexanol (fig. 3.4a: all comparisons between starved bees and the others treatments, $p < 0.01$). The P-fed groups conditioned with 2-hexanol behaved similarly (fig. 3.4a: all comparisons between P-fed groups, $p > 0.05$). When bees were conditioned with nonanal, the percentage of bees with CS-specific memory was also significantly higher in starved bees than in P-fed and P-fed solvent bees (fig. 3.4b: $p = 6.8 \times 10^{-5}$ and $p = 6.8 \times 10^{-5}$,

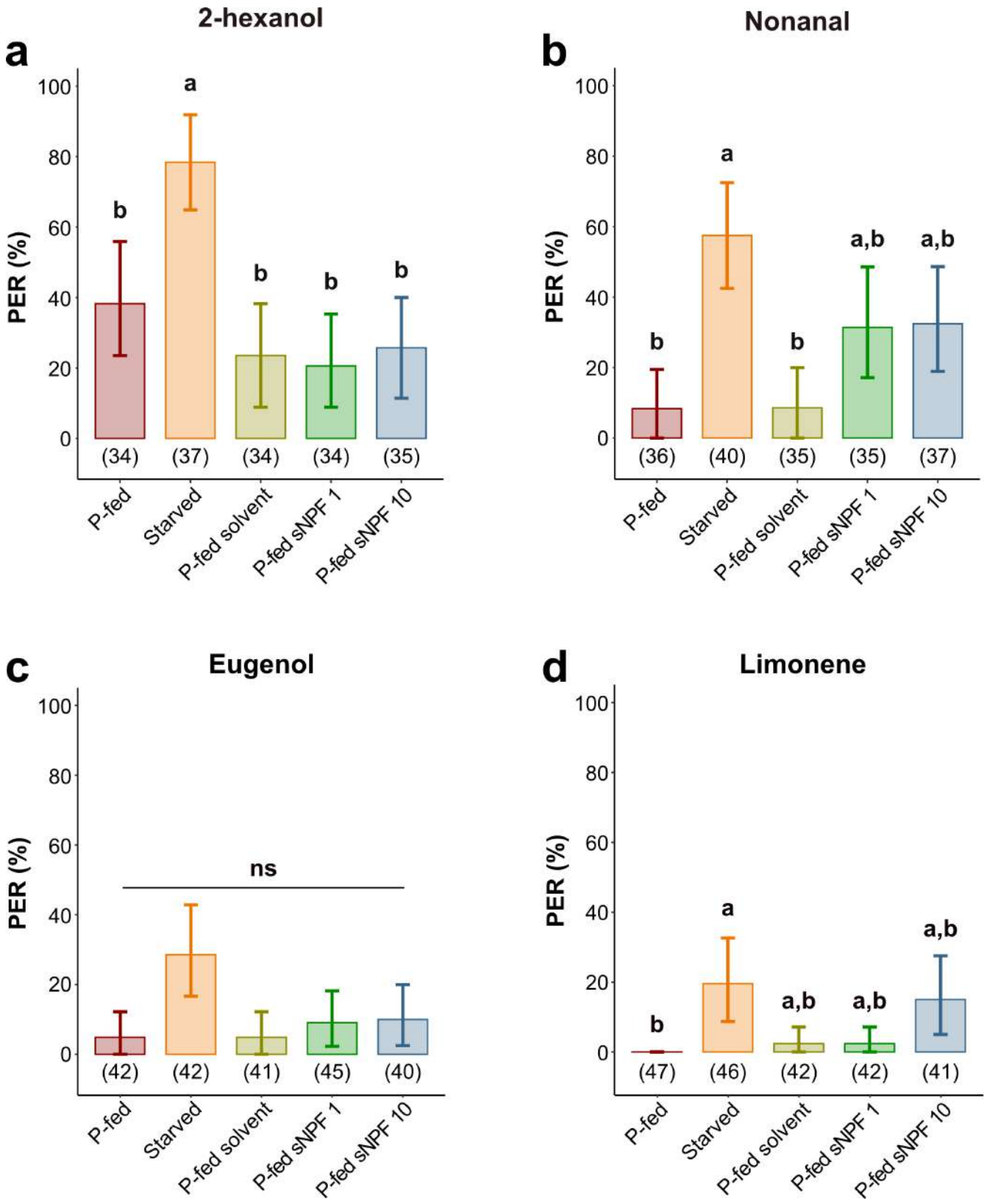

Figure 3.4: **Specific long-term memory after absolute olfactory conditioning.** A retention test was performed 24 h after the olfactory conditioning where bees were again stimulated with the learnt odorant and a new neutral odorant without reinforcement. Letters represent significant differences. Error bars represent the 95 % boostrapped confidence interval of correct choice percentage. Values in parentheses indicate group sample size.

respectively). Interestingly, P-fed bees treated with either dose of sNPF showed a tendency to increase the percentage of bees with CS specific memory but this percentage remained intermediate between that of starved bees and that of P-fed controls (fig. 3.4*b*: all comparisons, $p > 0.05$).

In the second experiment, in which eugenol and limonene were used, treatment had a significant effect on CS-specific memory when bees were conditioned with eugenol (fig. 3.4*c*: Fisher's exact test, $p = 8.81 \times 10^{-3}$). Yet, multiple comparisons between treatments with p-value adjustment method (Holmes) only showed a tendency towards significance between starved bees and both P-fed bees (fig. 3.4*c*: $p = 0.07$) and P-fed solvent bees (fig. 3.4*c*: $p = 0.07$). The remaining comparisons were not significant (fig. 3.4*c*: all remaining comparisons, $p > 0.05$). When limonene was the conditioned odorant, starved bees showed a significantly higher percentage of bees with CS-specific memory compared to P-fed bees (fig. 3.4*d*: $p = 0.03$). There was again a tendency for P-fed sNPF 10 bees to have a higher CS-specific memory than P-fed bees (fig. 3.4*d*: $p = 0.07$) but this tendency was absent in the comparison with P-fed solvent bees (fig. 3.4*d*: $p = 0.33$). The remaining comparisons did not show a significant difference (fig. 3.4*d*: all remaining comparisons, $p > 0.05$).

Besides quantifying the percentage of bees with CS-specific memory, we also quantified the percentage of bees that generalized between the CS and the Nod, thus responding to both odorants. In both experiments the percentage of generalizing bees was low within each group (less than 20 % of bees per group generalized, data not shown). Treatments had no effect on these bees after conditioning with 2-hexanol, nonanal or eugenol (Fisher's exact test: $p = 0.75$; $p = 0.39$; $p = 0.23$, respectively), i.e. when the NOd was nonanal, 2-hexanol and limonene, respectively. When limonene was used as CS+, treatment had an effect on generalization (Fisher's exact test: $p = 0.01$) but multiple post-hoc comparisons showed only a tendency towards significance when comparing starved and P-fed sNPF 1 bees ($p = 0.06$).

Overall, these results did not show a clear facilitatory effect of sNPF on olfactory learning or memory retention. Only non-significant tendencies towards an increase of the percentage of bees with CS-specific memory in some sNPF groups were observed, eventually overcoming the inhibitory effect of satiation on memory retention.

Effect of sNPF on aversive gustatory conditioning and memory.

Bees were trained under a differential conditioning regime in which they had to learn to distinguish a punished tastant (CS+) from an unpunished tastant (CS-). This protocol differs from the absolute conditioning used in the previous section as performances in SER conditioning are typically lower than in appetitive conditioning and differential conditioning increases learning success by

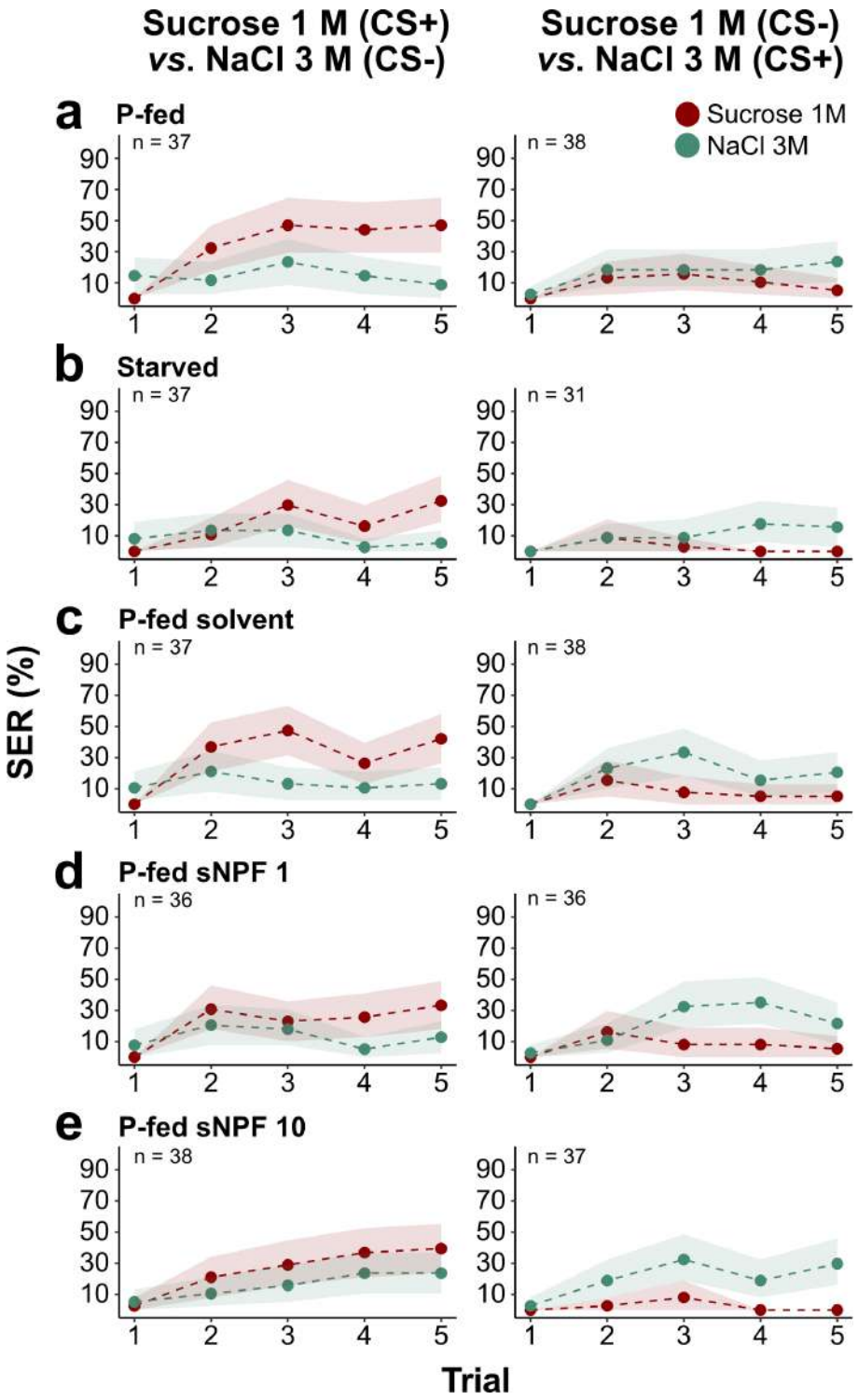

Figure 3.5: **Learning assays in differential aversive gustatory conditioning.** Differential conditioning of 1 M sucrose *vs.* 3 M NaCl. Conditioned responses (% SER) of a groups of bees subjected to five CS+ and five CS trials (n = 366). The red and green areas around the curves represent the 95 % boostrapped confidence interval of SER proportions. Values in parentheses indicate group sample size.

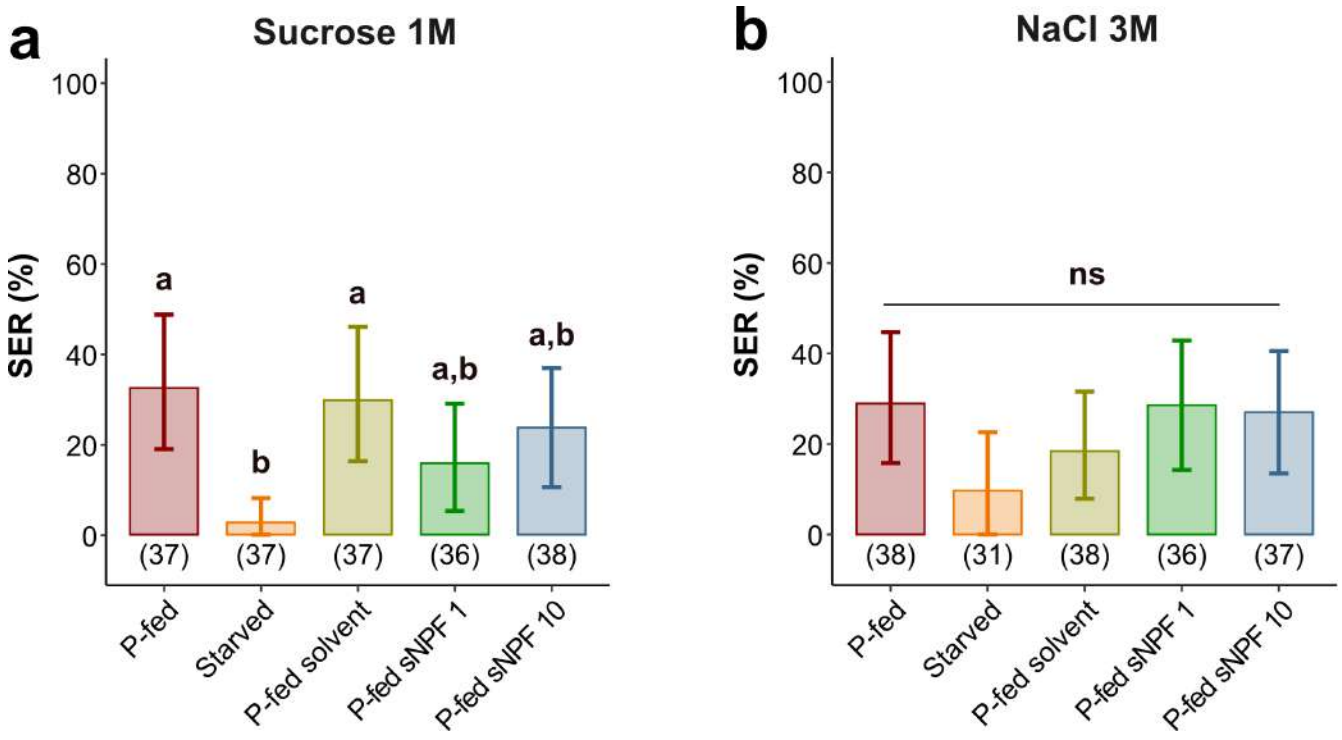

Figure 3.6: **Specific mid-term memory after differential aversive gustatory conditioning.** A retention test was performed 1 h after the gustatory conditioning where bees were again stimulated with both tastant without reinforcement. Red and green bars show the specific memory of bees conditioned on the 1 M sucrose **(a)** and 3 M NaCl **(b)** respectively. Letters represent significant differences, ns = non-significant. Error bars represent the 95 % boostrapped confidence interval of correct choice percentage. Values in parentheses indicate group sample size.

enhancing the attention the bees have to pay to each odorant to achieve the discrimination [124].

Antennal side had no effect on gustatory learning of a given CS (GLM: $\chi^2 = 0.41$, d.f. $= 1$, $p = 0.52$) so that responses of bees stimulated on different antennae with the CS+ or the CS- were pooled according to the type of CS and represented as a CS+ vs. CS- discrimination (fig. 3.5). SER responses for CS+ in the last trial were similar across treatments (fig. 3.5: GLM, $\chi^2 = 6.08$, d.f. $= 4$, $p = 0.19$) and not associated with tastant contingency (A+ vs. B- or A- vs. B+) (fig. 3.5: GLM, $\chi^2 = 2.01$, d.f. $= 4$, $p = 0.73$). Yet, there was a significant interaction between tastant and the differential-conditioning regime (1 M sucrose CS+ *vs.*3 M NaCl CS- or 1 M sucrose CS- *vs.* 3 M NaCl CS+) (fig. 3.5: GLM, $\chi^2 = 67.66$, d.f. $= 1$, $p = 2.2 \times 10^{-16}$). Overall, bees responded more to the conditioned tastant when 1 M sucrose was the CS+ (fig. 3.5 *left*: $p = 2.59 \times 10^{-7}$) whereas proportions of SER were similar between tastants when 3 M NaCl was the CS+ (fig. 3.5 *right*: $p = 1$). These results show that neither the nutritional state nor sNPF treatment affected aversive gustatory learning of bees but that they overall learned better 1 M sucrose than 3 M NaCl as CS+.

In the retention test one hour after conditioning, the performance of bees conditioned to as-

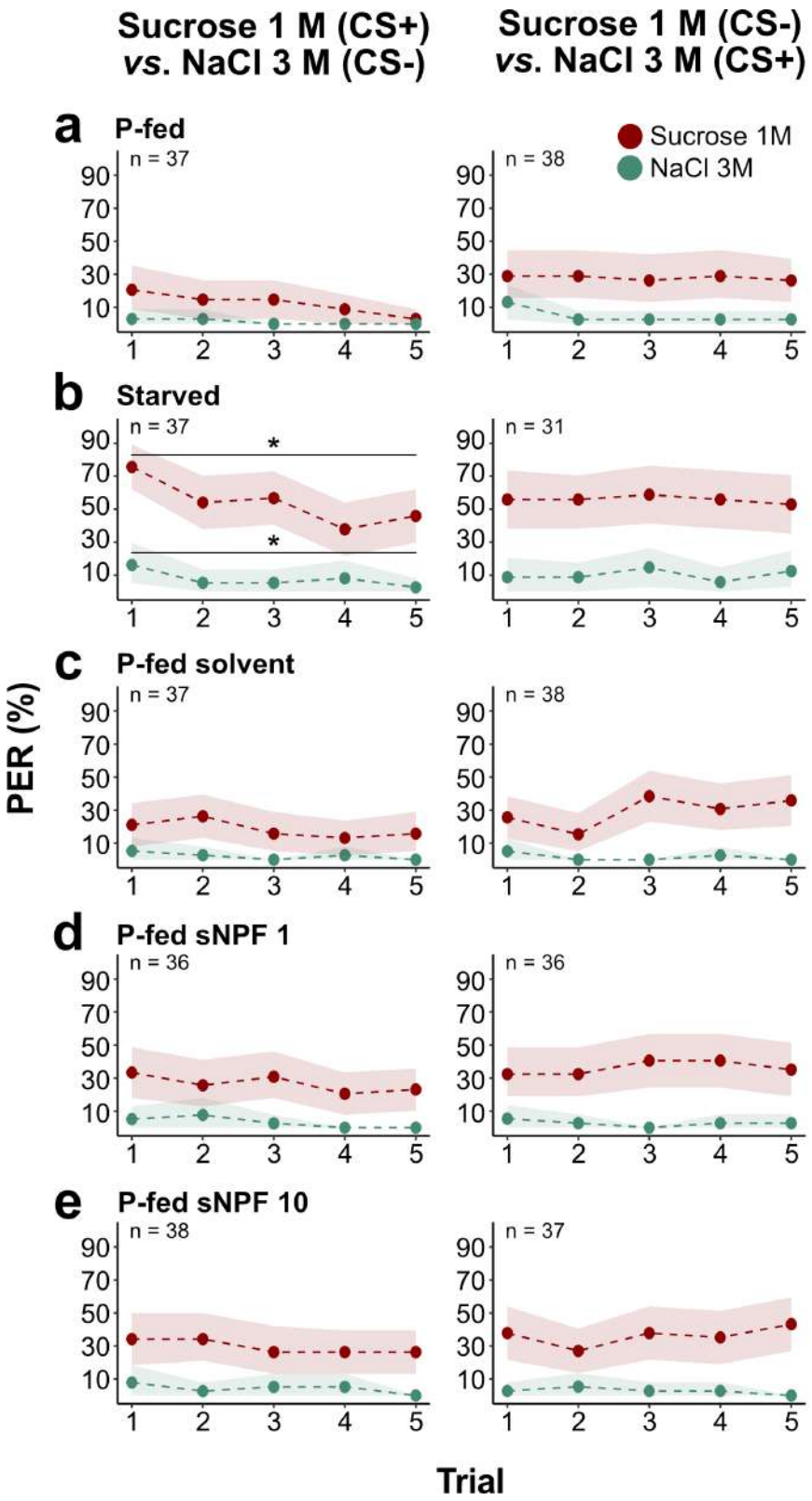

Figure 3.7: **Proboscis Extension Response (PER) during aversive gustatory conditioning.** The red and green areas around the curves represent the 95 % boostrapped confidence interval of PER proportions. Values in parentheses indicate group sample size. *: $p<0.05$

sociate 1 M sucrose with an electric shock and 3M NaCl with the absence of shock (fig. 3.6*a*) showed that treatment had a significant effect on the percentage of bees exhibiting CS-specific gustatory memory (i.e. responding to the CS+ with SER and not to the CS-) (Fisher's exact test, $p = 4.49 \times 10^{-3}$). This significant difference was introduced by starved bees, which had a significantly lower percentage of bees with CS-specific memory than untreated P-fed bees (fig. 3.6*a*: $p = 0.01$) and P-fed bees treated with solvent (fig. 3.6*a*: $p = 0.03$). Treating bees with sNPF did not improve the percentage of bees with CS-specific memory in the group in which 1M Sucrose was the CS+ (fig. 3.6*a*: all comparisons between P-fed groups: $p > 0.05$). In the retention test of bees conditioned to associate 3 M NaCl with an electric shock and 1M Sucrose with the absence of shock (fig. 3.6*b*), no significant differences were detected between groups; all of them exhibited a similar percentage of bees with CS-specific memory (Fisher's exact test, $p = 0.23$) even if the starved group tended to have a lower yet non-significant percentage (all comparisons between the five groups: $p > 0.05$).

Proportion of PER (fig. 3.7) between the first and the last conditioning trial were compared to assess whether the intrinsic value of the tastant (hedonic or non-hedonic) changed during aversive gustatory conditioning, i.e. to determine if the pairing of a tastant with a shock modifies the responsiveness to that tastant. Proportions of PER significantly decrease between the first and last trials only in the case of starved bees when stimulated with Sucrose 1 M CS+ or NaCl 3 M CS- (fig. 3.7*b*: $\chi^2 = 5.1$, $p = 0.02$), thus suggesting that tastant devaluation depended on the tastant and was not caused by the conditioning itself.

We next compared the proportions of spontaneous PER before the beginning of the conditioning and during the retention test to assess whether tastant evaluation varied over the whole experiment (conditioning + retention test) (fig. 3.8). Overall, we obtained mitigate results. Only P-fed bees showed a significant decrease in PER to 1 M sucrose when it was paired with the shock (fig. 3.8*a*: $\chi^2 = 5.88$, $p = 0.02$), which is consistent with aversive learning. A similar tendency was observed in starved bees (fig. 3.8*a*: $\chi^2 = 3.76$, $p = 0.05$) but the remaining groups (P-fed solvent, P-fed sNPF 1, P-fed sNPF 10) did not change their behavioural response (fig. 3.8*a*: all Mc Nemar tests, $p > 0.05$). The PER percentage to 3M NaCl as CS- was the same before and after the conditioning for P-fed bees (fig. 3.8*b*: $\chi^2 = 2.29$, $p = 0.13$) but there was a decrease in the four remaining groups (fig. 3.8*b*: all Mc Nemar tests, $p < 0.05$). In other words, the PER to the non-appetitive tastant NaCl decreased in four out of five groups but the PER to the appetitive sucrose was maintained in four out of five groups, thus showing that despite the shock association sucrose did not lose its appetitive nature.

This conclusion was reinforced in the case of the group for which sucrose was the CS- and was therefore never associated with electric shock (fig. 3.8*c*). In this case, the percentage of PER of all groups did not change between the start of conditioning and the retention test (fig. 3.8*c*: all

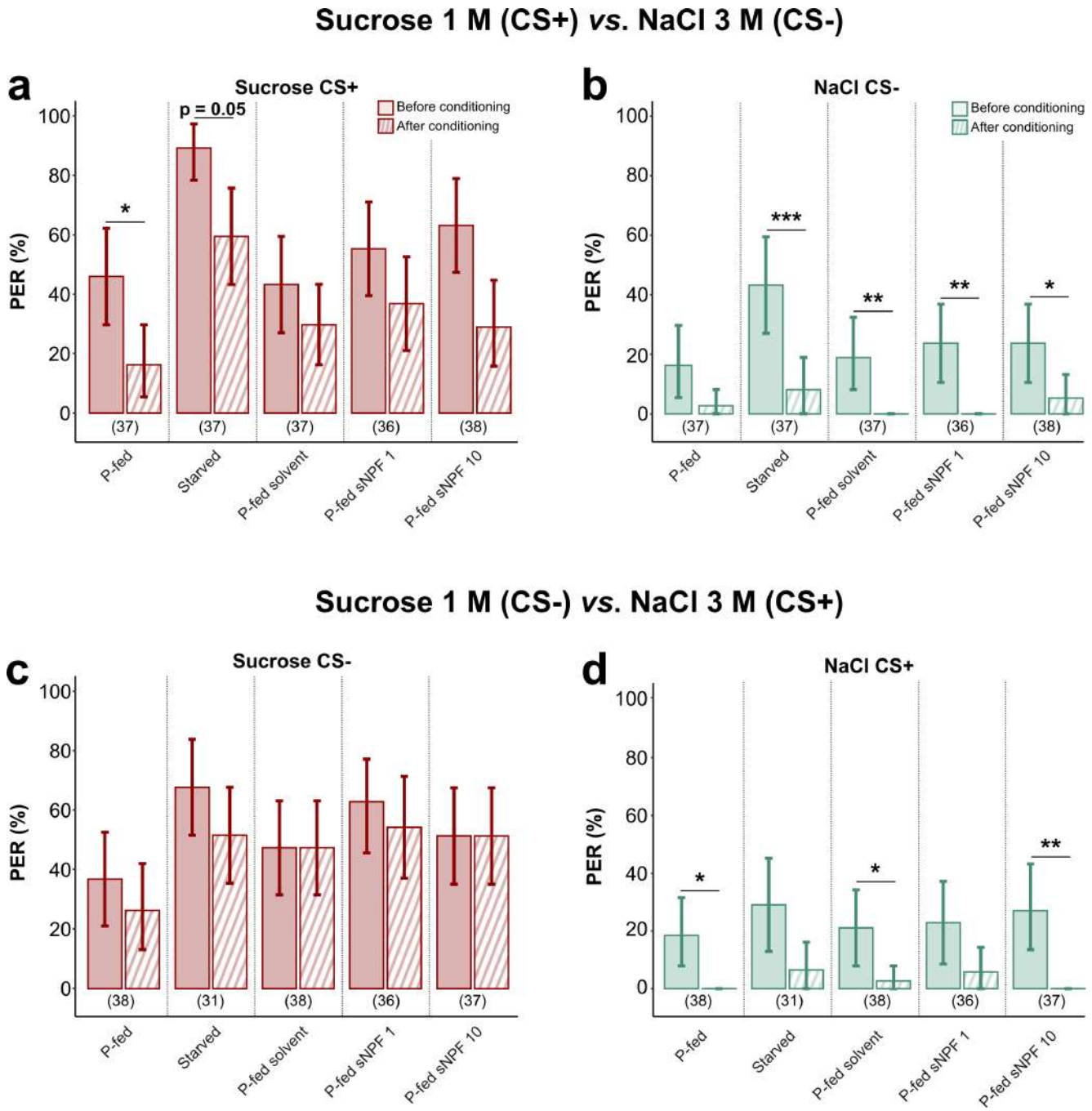

Figure 3.8: **Proboscis Extension Response (PER) before and after the aversive gustatory conditioning.** PER to tastants was recorded before the aversive gustatory conditioning and during the retention test one hour after the last trial. Values in parentheses indicate group sample size. *: $p<0.05$; **: $p<0.01$: ***: $p<0.001$.

Mc Nemar tests, $p > 0.05$), i.e. in the absence of punishment, sucrose kept its appetitive nature intact. In the case of 3 M NaCl paired with shock (fig. 3.8*d*), the percentage of PER to this tastant decreased between the start of conditioning and the retention tests in three out of five groups: P-fed solvent (fig. 3.8*d*: $\chi^2 = 4$, $p = 0.04$), P-fed bees ($\chi^2 = 5.14$, $p = 0.02$) and P-fed sNPF 10 bees ($\chi^2 = 8.1$, $p = 4 \times 10^{-3}$). In the other two groups, a non-significant tendency in the same direction was observed (starved: $\chi^2 = 3.27$, $p = 0.07$; P-fed sNPF 1: $\chi^2 = 3.12$, $p = 0.07$).

Discussion

We analysed the effect of sNPF on learning and memory formation after two different forms of conditioning: an appetitive absolute olfactory conditioning of PER, which used sucrose solution as positive reinforcement, and an aversive differential gustatory conditioning of SER, which used electric shock as negative reinforcement. Based on our previous results showing an enhancement of sucrose and olfactory responsiveness upon sNPF treatment (at least with the higher dose of this peptide; see Chapter 1), we expected a facilitation of olfactory learning and memory in the sNPF 10 group of bees. This expectation was reinforced by the results on appetitive visual learning and memory formation in free-flying bees (see Chapter 2). On the contrary, as we had not detected any effect of sNPF on two different forms of aversive responsiveness (see Chapter 1), we expected no effect of this peptide on aversive gustatory conditioning of SER.

Our expectations were only partially filled. Although no effect on aversive gustatory conditioning (neither at the level of the acquisition nor of retention) was detected, the effect on appetitive olfactory conditioning was either non-existent (acquisition) or reduced to the level of non-significant tendencies the case of memory retention. During aversive conditioning with 1 M sucrose as CS+, the specific memory of starved bees was very low. It is possible that this experiment was too long (4 h) for bees in such starving state so that hunger overrode aversive conditioning. Overall, partially fed bees, treated with sNPF or not, showed aversive specific memory to both tastants, meaning that sNPF did not interfere with the pathways recruited by gustatory aversive conditioning (mainly dopaminergic signalling required to mediate electric-shock representation) [129]. Given the absence of effect on aversive responsiveness by sNPF topical application (see Chapter 1), these outcomes were not surprising.

Topical application of sNPF did not increase appetitive olfactory learning over the three conditioning trials, in the same way it did not improve acquisition of a visual discrimination in the case of free-flying bees (see Chapter 2). It thus seems that the effects of sNPF, if any, are rather visible at the level of retention performances quantified after conditioning. Yet, if retention performances change, this is because the processes leading to memory formation, i.e. learning, are also modi-

fied by sNPF. This suggests that time is required to visualize the effects of sNPF. After olfactory conditioning of PER, a non-significant enhancement of CS-specific memory was observed in some cases, 24 h after training. In visual conditioning, however, significant improvements of memory were observed already 1 h after conditioning. This difference could be due to the experimental conditions of the two experiments: while in visual conditioning, free-flying bees were active and flew/walked within the maze, in olfactory conditioning bees were harnessed. It may thus be that the activity deployed by the free-flying bees accelerated the incorporation of sNPF and rendered its effect visible 1 h after training. The same period was, nevertheless, enough to visualize sNPF effects in harnessed bees tested for spontaneous sucrose and odorant responsiveness (see Chapter 1). Overall, this experiment could be repeated properly, comparing the retention performances 1 h and 24 h after olfactory appetitive conditioning.

We could discuss whether topical application of sNPF was the most accurate method to provoke an effect on appetitive olfactory memory. Although this non-invasive method proved to be efficient to affect both behavioural responsiveness and olfactory processing (see Chapter 1), injection of drugs directly to the median ocellus is more precisely aimed at the central nervous system [156]. Thus, experiments could be conducted again using this technique to determine whether increasing the incidence of sNPF renders the tendencies observed with P-fed sNPF 10 bees. Alternatively, if the target of sNPF action resides in the fat body, an organ that is essential for regulating metabolic needs in insects [161], injections could also be applied at this level (i.e. in the abdomen).

The role of NPF and sNPF signalling in memory formation have been studied in *Drosophila*. In adults, sNPF has been identified as a functional neuromodulator in Kenyon cells, the constitutive neurons of the mushroom bodies (MBs). The target cells of sNPF are likely MB extrinsic neurons, rather than Kenyon cells themselves [99]. sNPF knockdown in MBs impaired olfactory appetitive memory in fruit flies [99] but the underlying mechanisms are still not well studied. However, mechanisms underlying the role of NPF in appetitive memory retrieval are better understood. NPF signalling through NPFR promotes appetitive memory performance by suppressing the inhibitory activity of specific dopaminergic neurons innervating the MBs [56]. These MB-MP neurons - named according to the regions of the mushroom bodies (MB) that they innervate, the medial lobe and the pedunculus (MP) - are thought to gate MBs output via dopamine release. Stimulation of NPF neurons in fed flies mimics the hunger state by disinhibiting MB-MP neurons through the action of NPFR1. Therefore, when hungry flies are stimulated with the conditioned odorant during retention test, the relevant MB neurons are activated and the signal propagates to downstream neurons, leading to expression of the conditioned behaviour. On the contrary, knockdown of npfr prevents hungry flies from expressing hunger-induced appetitive memory [56].

In *Drosophila* larvae, three pair of NPF neurons that specifically modulate sugar reinforcement were identified and overlap with dopaminergic neurons [95], that form the most downstream

element of the sugar reinforcing subcircuit [96]. The artificial activation of NPF neurons inhibits its appetitive olfactory learning by modulating the sugar reward signal during acquisition, which changes the conditioned response from attraction to avoidance [95]. This effect is only present when using low fructose concentrations. No effect is detectable for the retrieval of an established appetitive olfactory memory.

In *Caenorhabditis elegans*, another model organism in neuroscience research, NPF receptor, NPR-11, mediates aversive but not appetitive olfactory learning through the signal of serotoninergic neurons [106]. Serotonin signalling in turn inhibits specific interneurons that are known for playing a role as sensory integration centres involved in aversive learning [162].

Interestingly, NPY/NPF investigated so far mainly exert a inhibitory effect on their receptor-expressing neurons [163]. Overall, these results support the theory that continuously inhibitory processes in the insect brain control behavioural expression [164].

To our knowledge, this is the second study investigated the role of NPF-signalling in cognitive abilities in social insects (see Chapter 2 for the first study). The results presented in this chapter should be replicated with adjustments made to the protocol as suggested above, in order to unravel the effect of sNPF on olfactory memory formation. Aversive olfactory conditioning could be conducted as well to investigate whether the absence of effect in aversive conditioning was specific to the gustatory modality.

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We thank Nina Colombel and Loïc Sauvadet for their help in the preliminary experiments of olfactory conditioning which are not presented in this thesis.

Chapter 4

Peripheral taste detection in honey bees: what do taste receptors respond to?

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REVIEW ARTICLE

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Peripheral taste detection in honey bees: What do taste receptors respond to?

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Abstract

Understanding the neural principles governing taste perception in species that bear economic importance or serve as research models for other sensory modalities constitutes a strategic goal. Such is the case of the honey bee (*Apis mellifera*), which is environmentally and socioeconomically important, given its crucial role as pollinator agent in agricultural landscapes and which has served as a traditional model for visual and olfactory neurosciences and for research on communication, navigation, and learning and memory. Here we review the current knowledge on honey bee gustatory receptors to provide an integrative view of peripheral taste detection in this insect, highlighting specificities and commonalities with other insect species. We describe behavioral and electrophysiological responses to several tastant categories and relate these responses, whenever possible, to known molecular receptor mechanisms. Overall, we adopted an evolutionary and comparative perspective to understand the neural principles of honey bee taste and define key questions that should be answered in future gustatory research centered on this insect.

KEYWORDS

Apis mellifera, gustation, gustatory receptors, honey bee

Martin Giurfa and Maria Gabriela de Brito Sanchez senior authorship shared.

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1 | INTRODUCTION

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Taste, the sense by which chemical substances are perceived when they are brought into contact with gustatory chemoreceptors, allows discriminating edible from non-edible items based on multiple characteristics such as the nature of the taste experienced, its hedonic and nutritional value, and its toxicity, among others. It is, therefore, crucial for survival not only because it participates in the regulation of energy budgets through the consumption of food but also because it mediates other functions such as the regulation of pH and water-saline balance via electrolyte detection and consumption. In insects, taste perception occurs via the contact of gustatory substances with chemoreceptors, which are hosted within specialized hairs termed *sensilla* or bristles (Nayak & Singh, 1983; Stocker, 1994) located on chemosensory organs (see reviews in de Brito Sanchez & Giurfa, 2011; Liman et al., 2014; Scott, 2018; Stocker, 2004; Vosshall & Stocker, 2007). The organs related to taste perception are mainly the antennae, the mouthpieces, the tarsi, and the margins of the wings, although *sensilla* containing chemoreceptors may also be found in other regions of the body, such as the ovipositor or on the body surface itself, enhancing thereby the possibility of gustatory contact (Scott, 2018; Stocker, 1994; Figure 1a). These *sensilla* are cuticular evaginations with a characteristic aperture at their apex (Figure 1b). By being located on different body organs, gustatory *sensilla* may participate in several behavioral contexts, from food and oviposition-site detection (Sollai et al., 2018; Sollai & Crnjar, 2019) to nestmate and sexual recognition (Meunier et al., 2000; Starostina et al., 2012; Stoffolano et al., 1997), among others.

Chemoreceptor neurons—termed gustatory receptor neurons (GRNs)—are hosted within *sensilla* and are tuned to detect different types of tastants based on the different types of molecular receptors they may express in their dendritic membrane. Chemical substances enter into the *sensillum* through the pore and reach the dendrites of the GRNs, which bathe into a receptor hemolymph. This hemolymph is enclosed by surrounding cells and differs in electrolytic composition from the hemolymph circulating in the insect body (Kaissling & Thorson, 1980). In lepidopterans, the *sensilla* hosting GRNs are termed *sensilla styloconica* and are located on the maxilla, although other gustatory *sensilla* can be located on the ventral side of the labrum. *Sensilla styloconica* have four gustatory GRNs and one mechanosensory neuron (Agnihotri et al., 2016; Xu, 2020). GRNs respond with specific activity patterns to plant tastants such as sugar and amino acids, which

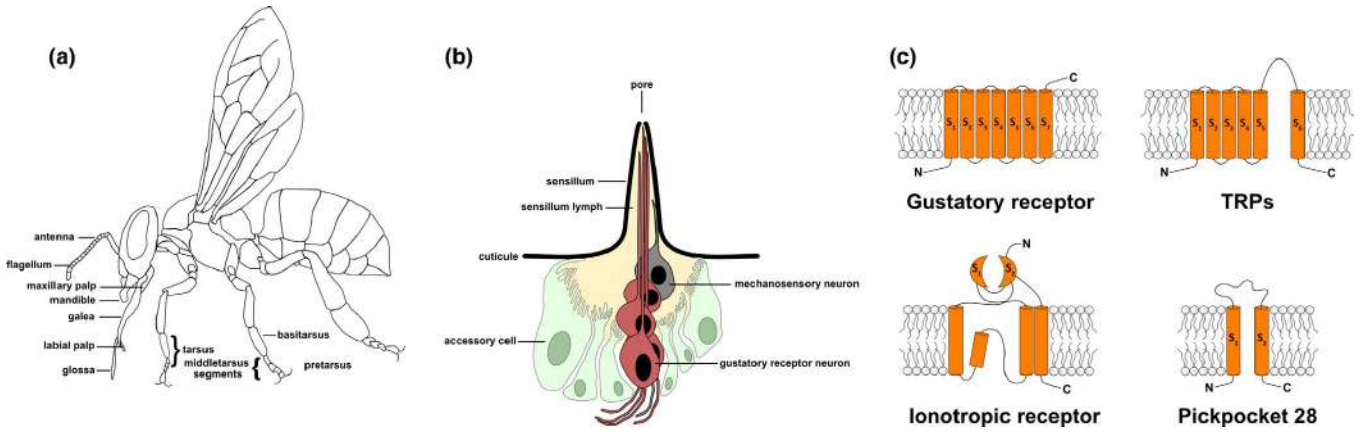

FIGURE 1 Honey bee taste, from body appendages to molecular taste receptors. (a) Anatomy of the honeybee. The main chemosensory organs involved in taste perception (antennae, mouthparts, and tarsal regions of the legs) are indicated. They bear gustatory *sensilla*, which are hair-like structures hosting gustatory receptor neurons. From de Brito Sanchez, 2011. (b) Schematic of a chaetic gustatory *sensillum*. Four gustatory receptor neurons (in purple) bathing in a cavity defined by auxiliary sensillar cells (in green) and filled with *sensillum* receptor hemolymph (in yellow) extend their dendrites toward the apex of the cuticular hair. A mechanoreceptor neuron (in gray) is attached to the basal wall of the hair. Tastants penetrate into the *sensillum* through a pore at the apex and stimulate molecular taste receptors located in the neuron membrane. (c) Four families of molecular taste receptors in insects. Schematics of gustatory receptors (GRs), ionotropic receptors (IRs), TRP transient receptor potential (TRP), and pickpocket (PPK) channels 28. In all schemes, the space above the lipid membrane represents the extracellular domain and that below the intracellular, cytosolic domain. The GR shown has seven (S1–S7) transmembrane domains, an extracellular C-terminal tail, and a cytosolic N-terminal region. The TRP channel shown corresponds to the TRPA1 protein in which four identical or similar subunits with six transmembrane domains (S_1–S_6), and cytosolic N- and C-terminal tails are combined to form a functional channel. The IR shown has an extracellular N-terminal tail, a bipartite ligand-binding domain whose two halves (S_1 and S_2) are separated by an ion channel domain, and a short cytoplasmic C-terminal region. PPK 28 belongs to the Degenerin/Epithelial sodium channel family (Deg/ENaC) in which each channel comprises three subunits (or multiples of three), and each subunit comprises two transmembrane domains (S_1 and S_2), two cytosolic N- and C- terminal tails and an unusually large and highly structured extracellular domain. Adapted from Scott, 2018

promote feeding, and to deterrent, bitter substances, which mediate food rejection (Schoonhoven & Loon, 2002). In the fruit fly *Drosophila melanogaster*, the insect whose taste has been most thoroughly studied in the last decades owing to the availability of neurogenetic tools and, which sets, therefore, a standard for comparisons, short, intermediate and long taste *sensilla* subtypes have been described. Each subtype contains one mechanosensory neuron and either two (intermediate *sensilla*) or four (short and long *sensilla*) GRNs (Hiroi et al., 2002). From the two GRNs located within intermediate *sensilla*, one responds to both sugars and low-salt concentrations, which are attractive for flies, while the other responds to bitter substances and high-salt concentrations, which are aversive (Hiroi et al., 2004). Short *sensilla* host a sugar-sensitive GRN (Hiroi et al., 2002, 2004), a water-sensitive GRN (Cameron et al., 2010; Chen et al., 2010; Dahanukar et al., 2007; Hiroi et al., 2002, 2004; Meunier et al., 2003; Weiss et al., 2011), a low salt-sensitive GRN (Meunier et al., 2003), and a bitter-sensitive GRN (Dahanukar et al., 2007; Hiroi et al., 2002; Weiss et al., 2011). Long *sensilla* host a sugar-sensitive GRN, a water-sensitive GRN, a low salt-sensitive GRN, and a high salt-sensitive GRN but no bitter-sensitive GRN (Hiroi et al., 2002; Weiss et al., 2011). GRNs convey, therefore, taste-specific information that is further processed in central regions of the insect brain such as the subesophageal zone (SEZ), which receives afferences from GRNs located at the level of the mouthpieces and antennae (Thorne et al., 2004; Wang et al., 2004).

The gustatory specificity of a GRN is conferred by various types of molecular gustatory receptors (GRs) located in the GRN membrane (Figure 1c). One family of GRs includes heptahelical transmembrane proteins termed GRs (Clyne et al., 2000; Dunipace et al., 2001; Scott et al., 2001). Tastant molecules that penetrate into a gustatory *sensillum* bind to these proteins triggering a transduction process resulting in neural activation (Figure 1c). GRs are encoded by gustatory receptor genes (*Grs*). The genes are given the name "*Gr*" (gustatory receptor) and are differentiated by a number added after the *Gr* prefix. This number is also extensive to the GRs they encode.

Most of the GRs tuned to bitter and sweet tastants in flies, and other insects are thought to form ligand-gated ion channels (Sato et al., 2011), differently from mammals where G protein-coupled receptors (GPCR) confer the molecular taste specificity to taste receptor cells. This difference indicates that mammals and insects detect the same classes of chemicals using taste receptors cells that are evolutionary distinct (Liman et al., 2014). However, GPCR signaling cascades are also involved in the fly gustatory system (Clyne et al., 2000), possibly acting in parallel to GR pathways, enhancing the response to low concentrations of ligands, and/or modulating the activity of GRs through phosphorylation (Liman et al., 2014).

Besides GRs, other families of molecular receptors allow detecting tastants in insects. Among them, ionotropic receptors (IRs), which differ from GRs in both their functional principle and gustatory tuning, are involved in chemosensation (olfaction and gustation) and have been characterized in the GRNs of insects (Benton et al., 2009; Koh et al., 2014; Zhang et al., 2013; Figure 1c). IRs are more ancient than GRs and have evolved from ionotropic glutamate receptors (iGluRs; Benton et al., 2009; Croset et al., 2010; Rytz et al., 2013). They function as ligand-gated ion channels (Benton et al., 2009; Croset et al., 2010) but do not belong to the well-described kainate, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) or *N*-methyl-D-aspartate (NMDA) receptor classes of iGluRs and have divergent ligand-binding domains that lack their characteristic glutamate-interacting residues. In the fruit fly, IRs form multimodal receptors mediating the perception of odors, tastes, or other sensory cues such as humidity or temperature. Their role in taste perception has been well documented in *D. melanogaster* in the case of salt, amino acid, and acetic acid detection (Ganguly et al., 2017; Jaeger et al., 2018; Rimal et al., 2019; Zhang et al., 2013). Some IRs, as for instance those belonging to the IR20a clade (35 IRs), are expressed in GRNs (Koh et al., 2014), and at least four other IRs are expressed in gustatory organs such as the labellum and the pharynx (Croset et al., 2010; Zhang et al., 2013). One of these IRs, IR76b, acts as a sensor of low salts (Zhang et al., 2013) and amino acids if it is co-expressed with IR20a (Croset et al., 2016; Ganguly et al., 2017). In addition, interactions between GRNS expressing IRs and GRNs expressing GRs have been observed in the fly for the case of salt sensing. It was shown, for instance, that fly attraction to low-salt concentrations depends primarily on sweet-sensing GRNs expressing GR64f, with additional input from neurons expressing IR94e (Jaeger et al., 2018). Overall, these studies indicate that in *D. melanogaster* IRs can function as molecular taste receptors (yet also as olfactory receptors) and mediate behavioral reactions to tastants in the environment (Rimal & Lee, 2018).

In addition to GRs and IRs, TRP (Transient Receptor Potential) channels have been identified as additional actors of peripheral taste detection (Figure 1c). These proteins belong to a superfamily of cation transmembrane proteins expressed in many sensory neurons and respond to a wide range of sensory stimuli. They play a role in sensory signaling in multiple behavioral contexts such as phototaxis, thermotaxis, and gravitaxis and also participate in taste detection. In *D. melanogaster* and in the moth *Manduca sexta*, for instance, the TRPA1 channel is required for the detection of aversive tastants such as aristolochic acid in a way that is independent of GR detection (Afroz et al., 2013; Kim et al., 2010).

Another receptor gene family with a gustatory role has been identified in the fruit fly. The amiloride-sensitive DEG/eNaC (degenerin/epithelial sodium channel) channels

(Kellenberger & Schild, 2002), which are known as pick-
1161 pocket (PPK) channels (Adams et al., 1998; Liu et al., 2003), participate in multiple sensory (Ben-Shahar, 2011), including water sensation and salt taste. Individual ENaC subunits associate as homomultimer or heteromultimer to form voltage insensitive, amiloride-sensitive sodium channels. Thirty-one members of the PPK family were identified in *Drosophila*, each representing a channel subunit (Ben-Shahar, 2011). One of them, the PPK28 channel, was shown to serve as the osmolarity sensor for gustatory water reception in the adult fruit fly (Chen et al., 2010; Figure 1c). In addition, PPK11 or PPK19 is expressed in gustatory organs and mediate responses to low-salt and high-salt concentrations in the larva of *Drosophila* (Alves et al., 2014; Liu et al., 2003) and to high-salt concentrations in adult flies (Liu et al., 2003). Other PPK channels could participate in modulating the detection of other tastes (e.g., salts) in the GRNs that express them.

To what extent the various molecular mechanisms are shared across insect species remains to be determined. The vast majority of studies on the molecular underpinnings of peripheral taste detection have been performed in the fruit fly, which provides an unmatched array of neurogenetic tools for addressing the specific roles of single neurons and receptors in perceptual phenomena. Yet uncovering these mechanisms in other insect species, in particular in those that bear economic importance or serve as models for other research areas, constitutes an important strategic goal. Such is the case of the honey bee (*Apis mellifera*), which has a fundamental environmental and socioeconomic importance given its crucial role as pollinator agent in agricultural landscapes and which has served as a traditional model for basic research on various sensory modalities (e.g., visual [Avarguès-Weber et al., 2011; Avarguès-Weber et al., 2012] olfactory [Paoli & Galizia, 2021; Sandoz, 2011], mechanosensory [Giurfa & Malun, 2004; Scheiner et al., 2005]). In the last decade, massive colony losses have been reported worldwide and described as the "colony collapse disorder" (Oldroyd, 2007; VanEngelsdorp et al., 2009). This dramatic decrease in honey bee populations may have multiple causes such as an uncontrolled use of pesticides (Goulson, 2013; Pisa et al., 2014; Sanchez-Bayo & Goka, 2014); the presence of parasites, predators, and diseases; and the reduction of natural habitats and biodiversity through intensive agricultural practices and monocultures (Brown & Paxton, 2009; Goulson et al., 2008, 2015). In addition, different forms of environmental pollution may also contribute to colony losses (Burden et al., 2019; Negri et al., 2015; Søvik et al., 2015). In this context, understanding the basic principles of honey bee taste is important to determine the capacity of this insect to detect and avoid potential noxious substances (e.g., pesticides and pollutants) present in the environment.

Here we review the current knowledge on honey bee GRs to provide an integrative view of honey bee peripheral taste detection. We describe behavioral and electrophysiological responses to several tastant categories and relate these responses, whenever possible, to known molecular receptor mechanisms. Overall, we adopted an evolutionary and comparative perspective to understand the neural principles of honey bee taste and define key questions that should be answered in gustatory research centered on this insect.

2 | THE GUSTATORY WORLD OF HONEY BEES

Despite their strategic importance, studies on the gustatory sense of honey bees remain scarce (de Brito Sanchez, 2011). Yet taste plays an important role throughout the different life stages of the honey bee. From the larval to the forager stage, detecting and responding appropriately to substances according to their nutritional values is crucial for individual and colony survival. This is particularly important for adult bees (typically 3-week-old bees), which after reaching the foraging stage are in charge of collecting nectar and pollen as sources of carbohydrates (e.g., sucrose, fructose, and glucose) and proteins, respectively. Foragers need, therefore, to be sensitive to these and other substances present in lower quantities in flowers such as amino acids, vitamins, or mineral salts (de Brito Sanchez, 2011; Harborne, 1994). Some bees may also collect water with saline content and resins (Drescher et al., 2019) to produce propolis, thus being exposed to different, additional tastes. These multiple foraging specializations suggest that the gustatory world of bees may include a relatively large spectrum of tastants (sugars, salts and amino acids) present in the natural products they choose and collect.

2.1 | Behavioral responses to sugars

Free-flying bees are responsive to sugars present in nectar and honeydew. The most impressive survey on honey bees' behavioral responses to tastants that are perceived as sweet by humans was performed by Nobel Prize winner Karl von Frisch, who quantified the choice of free-flying bees confronted with solutions of 34 different tastants offered in small dishes (von Frisch, 1934; Figure 2a).[1] Using 1-M sucrose solution as a reference (i.e., the solution to which foragers was trained), he determined that, besides sucrose, eight other substances are sweet for the bees: maltose, melezitose, glucose, fructose, trehalose, α-methyl glucoside, fructose, and inositol.

[1]Von Frisch's works on honey bee taste can be found in this impressive 156-page article, which is unfortunately mostly ignored by modern research on insect taste, probably because it is only available in German and because von Frisch himself included only a brief summary of these investigations at the end of the book that serves as reference for his life work (von Frisch, 1967). Yet his work on bee taste covered multiple taste modalities and proposed several hypotheses that were later verified or proposed again, ignoring his original statements.

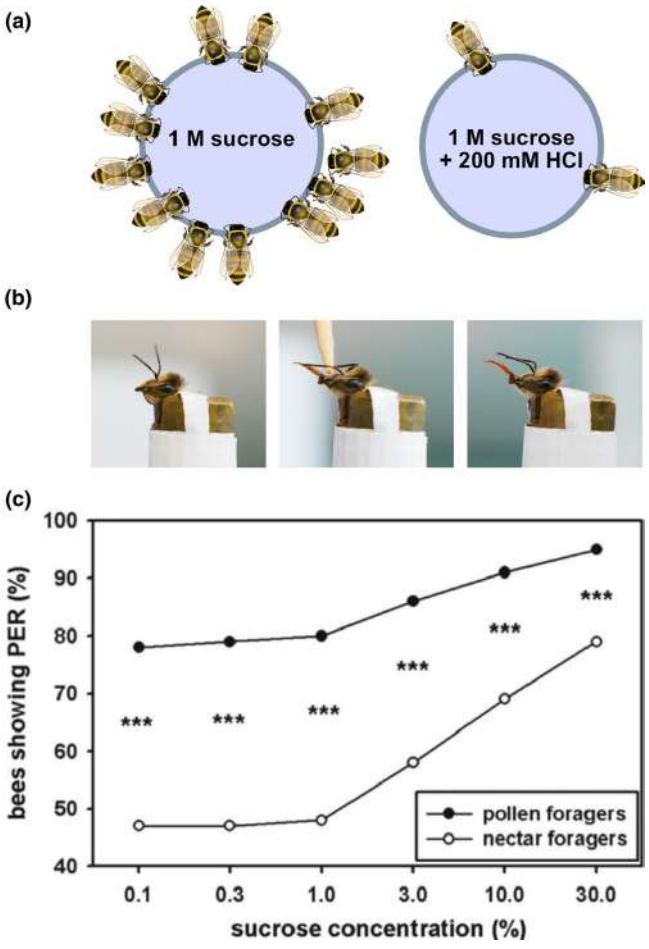

FIGURE 2 Behavioral methods for studying gustatory responses of honey bees in an appetitive context. (a) Choice of tastant solutions by free flying foragers. This procedure involves training free-flying bees to collect sucrose solution (e.g., 1 M) on a feeder or dish and then confronting them with the original appetitive solution versus the same solution to which a different tastant was added (e.g., 200-mM HCl). In this schema, foragers prefer the 1-M sucrose solution over a mixture of 1-M sucrose and 200-Mm HCl. Adapted from von Frisch, 1934. (b) A bee harnessed within a cylindrical tube before, during, and after antennal stimulation with a toothpick soaked in a sucrose solution. Contact of sucrose receptor neurons on the antennae with sucrose and other sweet tastants triggers the Proboscis Extension Reflex (PER), which is an appetitive response to food. (c) Sucrose-responsiveness curves of harnessed pollen and nectar foragers. Individual foragers prepared as in (b) were tested for their responsiveness (PER) to six increasing sucrose concentrations ranging from 0.1% to 30% (w/w). Upon each antennal stimulation with sucrose, the occurrence of PER was noted. A significantly higher percentage of pollen foragers responded with PER to each sucrose stimulation than nectar foragers, thus showing that nectar foragers are more selective and respond mainly to concentrated sucrose solutions (***: statistically significant difference). From Scheiner et al. (2004)

Twenty-five tastants (including sorbitol, mannitol, *l*- and *d*-arabinose, xylose, sorbose, and raffinose) that we perceive as sweet are unsweet for the bees. In other behavioral experiments performed with free-flying bees, sucrose was preferred over glucose, glucose over maltose, and maltose over fructose (Wykes, 1952) or sucrose over fructose and fructose over glucose (Waller, 1972). In other experiments with harnessed bees tested for their appetitive proboscis extension response (or PER), which is triggered by stimulation of the antennae with an appetitive stimulus (Figure 2b), fructose, and sucrose were ranked either equally (Miriyala et al., 2018) or, as in free-flying bees, sucrose was preferred to fructose (Bachman & Waller, 1977). A mixture of equal parts of sucrose, glucose, and fructose was reported to be less attractive than sucrose or a mixture in which sucrose was dominant (Bachman & Waller, 1977). Furthermore, sucrose solution was preferred over dry sugar, which requires the additional effort of dissolving the sugar for collection (Liao et al., 2020).

The responsiveness of bees toward sweet solutions may vary significantly between individuals of the same colony and is highly conditioned by internal (hunger and physiological state, genetics, age, task specialization, e.g., Figure 2c) as well as by external factors (season and weather, colony reserves, presence of brood, etc.; Scheiner et al., 2004). For instance, nectar foragers are more selective and respond mostly to the highest sucrose concentrations, while pollen foragers are less selective and respond to a broader range of sucrose solutions, including the more diluted ones (Page & Erber, 2002). Variability in sucrose responsiveness is considered a key element for division of labor and social organization within the colony: it reflects the existence of different thresholds of responsiveness for an appetitive stimulus and thus predisposes certain individuals to perform certain tasks (e.g., nectar collection) for the society (Bonabeau et al., 1996). Interindividual differences in sucrose responsiveness depend on genetic factors, among others (Junca et al., 2019; Scheiner & Arnold, 2010). These interindividual differences are important as they may explain contradictory results concerning sugar preferences or the acceptance of sucrose solutions contaminated with agrochemicals (Arce et al., 2018; Kessler et al., 2015; Muth et al., 2020).

2.2 | Behavioral responses to amino acids

Amino acids are common constituents of floral nectars and critical components in the diets of insect pollinators. Detecting amino acids is important as pollen consumption during early adulthood shapes amino acid levels in the bee brain, which may affect development (de Groot, 1952), neural circuitry, and behavior (Gage et al., 2020). In addition, dietary amino acids confer immunity and increased resistance to parasites such as *Nosema ceranae* (Glavinic et al., 2017).

Using his behavioral assay involving free-flying bees, von Frisch (1934) tested the response of bees to *d*- and *l*-valine, *d*-alanine, and glycine. He did not study if bees can detect these substances per se but rather focused on their effects on

sucrose solution acceptance. He reported a decrease in acceptance after adding these amino acids to sucrose solution, but he did not report the concentrations of amino acids used in his experiments. Later works showed that honey bees detect amino acids in pollen (Harborne, 1994) and prefer pollens enriched in essential amino acids that are required by bees (valine, leucine, and isoleucine; Cook et al., 2003). They also prefer and are more responsive to sucrose solutions that contain amino acids such as glycine (Kim & Smith, 2000) or proline and alanine (Bertazzini et al., 2010), which contradicts in part (at least for glycine) von Frisch's original report (1934). However, preference in these works depended on in the amino acid concentrations used, which may explain the contradictory results, besides differences in experimental methods.

In olfactory conditioning experiments, in which harnessed bees are trained to associate an odorant with a reward of sucrose solution delivered to the antennae and then to the proboscis (olfactory conditioning of the proboscis extension response or PER; Bitterman et al., 1983; Giurfa & Sandoz, 2012), the addition of glycine to the sucrose solution improved learning (Kim & Smith, 2000). This indicates that this amino acid enhances the appetitive value of the food reward. Preference for diets rich in amino acid contents may, however, vary with factors such as age and task specialization: when bees make the transition from within-hive duties to foraging, their nutritional needs shift toward a diet largely composed of carbohydrates at the expense of amino acid contents (Paoli et al., 2014).

2.3 | Behavioral responses to bitter substances

Honey bees may be exposed to bitter substances during foraging as some flowers produce nectars enriched in caffeine or nicotine (Liu et al., 2004; Singaravelan et al., 2005). The sensitivity of bees to nectars containing bitter substances is variable as some reports indicate that nectars containing them in higher concentrations can be deterrent (Johnson et al., 2006) while they can be attractive if the content of bitter substances is low (Singaravelan et al., 2005). Whether or not bees perceive bitter substances as distasteful is controversial as several works, including the pioneering experiments by von Frisch (1934), concluded to a remarkable lack of sensitivity of bees to these substances (see also Ayestaran et al., 2010), while other works reported that bees reject sucrose solution containing bitter substances based on the unpleasant nature of these substances (Mustard et al., 2012; Wright et al., 2010, 2013).

Von Frisch indicated that the sensitivity of bees to tastants that are bitter to humans was surprisingly low: "*…bees are much less sensitive to bitter substances than we…it is possible to contaminate sugar with a bitter substance that does not interfere with its being taken up by bees but that renders it unacceptable to man*." (von Frisch, 1967). Similar statements can be found in von Frisch's original work (1934), where he verified the relative lack of sensitivity of bees for substances such as quinine, salicin, and arbutin. In behavioral experiments in which harnessed bees were offered pure bitter solutions, the insects readily consumed the solutions (Ayestaran et al., 2010), consistently with von Frisch's original observations (von Frisch, 1934), even if ingestion resulted in high mortality. Similarly, conditioning experiments in which bees had to associate a tastant stimulation presented on the antennae with an electric shock (gustatory conditioning of the sting extension response or SER; Figure 3a) showed that bees were unable to learn the difference between distilled water and quinine or salicin, thus suggesting a lack of specific bitter perception, at least at the antennal level (Guiraud et al., 2018).

The rejection of sucrose solution containing bitter substances reported in some works could either be due to the presence of bitter tuned receptors or to an inhibitory effect of bitter substances on sucrose receptors. Several bitter tastants inhibit the firing of sweet-responding GRNs in bees and other insects (de Brito Sanchez et al., 2005, 2014; French et al., 2015; Jørgensen et al., 2007; Liscia & Solari, 2000; Meunier et al., 2003). In the latter case, rather than inducing direct deterrence, bitter substances would degrade the perceived quality of the sucrose solution rendering it inacceptable because of the mismatch between a forager's expectation and the taste of an aqueous solution for which sweet taste has been degraded. Under this assumption, decreased responses to sucrose solutions supplemented with bitter tastants should be analyzed cautiously (Mustard et al., 2012; Wright et al., 2010, 2013) as such a decrease may not reflect aversion but a decrease in appetitive motivation upon stimulation with a denatured sucrose solution.

The same arguments could be applied to rejection responses of freely moving bees to pure bitter substances when these bees have been previously trained to fly to a feeding place to obtain an appetitive sucrose reward. The rejection of pure bitter substances delivered as punishment upon incorrect choices could again reflect the mismatch between the appetitive expectation of the trained bee and the sensing of a non-sugary solution. This could have been the case, for instance, in visual discrimination experiments in which bees were trained to distinguish similar colors using sucrose as reward and a 60-mM quinine solution as punishment upon incorrect choices (Avarguès-Weber et al., 2010); in this case, the presence of quinine improved the discrimination of similar but not of dissimilar colors but the reasons for this improvement remain unclear.

The experimental conditions under which the bees are studied, that is, the possibility for them to express or not food rejection (de Brito Sanchez et al., 2015; Desmedt

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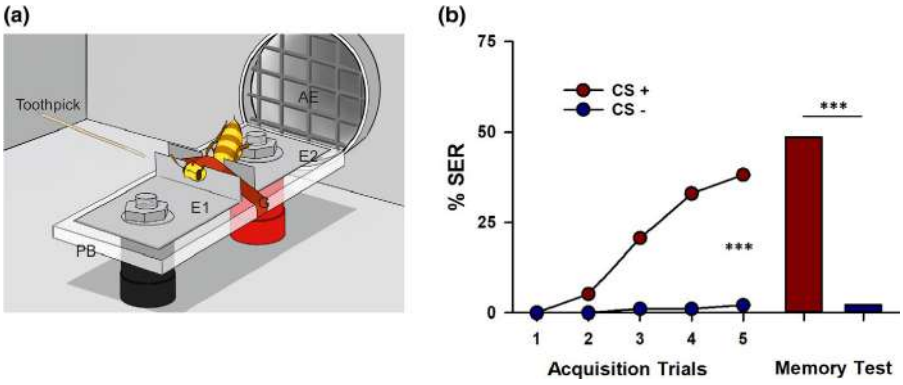

FIGURE 3 Behavioral methods for studying the gustatory responses of honey bees in an aversive context. (a) Associative gustatory conditioning of the sting extension reflex (SER) in honeybees. In this aversive conditioning, bees learn to associate a taste stimulus given on one antenna by means of a toothpick with a mild electric shock and a different taste applied on the opposite antenna by means of a different toothpick with the absence of shock. The bee is fixed between two brass plates (E1, E2) set on a Plexiglas basis (PB) by a girdle (G) that clamps the thorax to restrain mobility. The bee closes a circuit and receives a mild electric shock (7.5 V) which induces the sting extension reflex (SER). Odor contamination is avoided via an air extractor (AE) placed behind the bee. Aversive learning results in SER to the punished tastant but not to the unpunished tastant. From Guiraud et al. (2018). (b) Differential conditioning of 1 M sucrose versus 3 M NaCl. One group of bees had sucrose associated with shock and NaCl without shock while another group had the reversed contingencies. No differences were detected between both groups and performances were pooled and represented as a CS+ (tastant punished) versus a CS− (tastant non-punished) discrimination. The graph shows conditioned responses (% of bees exhibiting the sting extension reflex or SER to the punished tastant) along five CS+ and five CS— acquisition trials and in a memory test performed 1 hr after conditioning. Bees learned the gustatory discrimination and responded significantly more with a SER to the punished tastant than to the non-punished one at the end of training. One hour after conditioning, they remembered the learned associations. From Guiraud et al. (2018)

et al., 2016), seem determinant for measuring an enhanced or reduced aversive effect. For instance, when bees can choose between sucrose solution and sucrose solution spiked with a bitter substance, they prefer the pure sucrose solution and reject the mixture (Desmedt et al., 2016). Yet when bees are presented with a single feeding option and their escape possibilities are reduced, they consume the previously rejected mixture. This change in feeding behavior was interpreted as a case of feeding helplessness, in which bees behave as if they could not avoid the non-palatable food and consumed it (Desmedt et al., 2016). Similarly, in olfactory-learning experiments in which bees were trained to discriminate an odorant rewarded with sucrose from a different odorant punished with quinine, the aversive strength of quinine varied with the learning context (de Brito Sanchez et al., 2015). It was stronger when bees were trained in a Y-maze in which they could move freely and express rejection of the punished odorant, but it was milder when bees were harnessed and had to learn the odor discrimination in the absence of movement (olfactory PER conditioning; de Brito Sanchez et al., 2015).

2.4 | Behavioral responses to salts

Honey bees sense salts, in particular when they collect water, which is an important task for colony temperature regulation (Kühnholz & Seeley, 1997). Salts are also important as metabolites for the regulation of physiological state (Louw & Hadley, 1985) and for larval feeding (Nicolson, 2009). Bees, in particular water foragers, are known to prefer compound-rich "dirty" water sources with specific salt concentrations over clean water sources (Bonoan et al., 2017; Butler, 1940).

Low concentrations of salts are generally attractive to bees while high concentrations are aversive, which is consistent with observations in mammals (Chandrashekar et al., 2010; Oka et al., 2013). Von Frisch (1934) showed that increasing the concentration of a NaCl solution added to sucrose solution diminished progressively the attraction of bees trained to collect pure sucrose solution. In experiments with harnessed bees (Lau & Nieh, 2016), appetitive PER responses were observed when bees were stimulated with diluted saline solutions (1.5%–3% NaCl and 1.5% $MgCl_2$) but such responses decreased with increasing salt concentrations. A similar trend was found for KCl and Na_2PO_4, which were appetitive between 0.4% and 1.2% but not at higher concentrations (Lau & Nieh, 2016). These results account for the use of concentrated NaCl solution (e.g., 3 M) as an efficient negative reinforcement in olfactory PER conditioning with harnessed bees. When an odorant is paired with NaCl solution delivered to the antennae and proboscis, bees learn to inhibit their responses and reject the punished odorant (Aguiar et al., 2018; Bhagavan & Smith, 1997; de Brito Sanchez et al., 2015; Cook et al., 2005; Getz & Smith, 1987).

High sensitivity to different concentrations of saline solutions may be localized in the proboscis and associated appendages rather than at the level of the antennae. In the gustatory conditioning of the SER in which bees learn to differentiate tastants delivered to the antennae based on their association or not with an electric shock (Guiraud et al., 2018, Figure 3b), they learned to discriminate neither 3-M NaCl from distilled water nor from 100-mM NaCl solution, which in turn was not discriminated from KCl 100 mM (Guiraud et al., 2018). Therefore, salt detection appears mediocre at the antennal level. On the contrary, a high sensitivity to saline solutions was found at the level of the tarsomeres of the forelegs (de Brito Sanchez et al., 2014; see below).

As for sugars, honey bees exhibit interindividual differences in their sensitivity and preference for salts (Lau & Nieh, 2016), which may again reflect the existence of different thresholds of responsiveness underlying division of labor within the hive (see above). For instance, bees show variations in their salt preferences, with some individuals preferring lower salt concentrations and others slightly higher concentrations (Lau & Nieh, 2016). This variability suggests some level of specialization among water foragers (Lau & Nieh, 2016). This result is interesting in the light of the known specialization of some bees in the task of water collection (Robinson et al., 1984). In addition, foraging for salts is seasonally modulated (Bonoan et al., 2017), which may be due to seasonal variation in colony needs. For instance, $CaCl_2$, $MgCl_2$, and KCl, which are commonly found in pollen, are preferred in autumn when pollen is scarce but are avoided during summer when pollen is abundant (Bonoan et al., 2017).

2.5 | Behavioral responses to acids

Sour taste, the taste sensation evoked by acids, is less well understood than other taste modalities in bees and other insects. Acids are generally toxic to animals and may indicate that food is unripe or spoiled (DeSimone et al., 2001). Von Frisch reported that honey bee foragers trained to collect sucrose solution reject diluted acid solutions. Rejection was similar to that of pure water, which makes conclusions on the taste quality of these acid solutions difficult (von Frisch, 1934). In another set of experiments, von Frisch added different acids to sucrose solution so that the behavioral responses he recorded may have reflected either sensitivity to acids per se or the perception of a sucrose solution modified by the addition of acids (see discussion above on bitter taste; von Frisch, 1934). Von Frisch found that higher concentrations of acids induced a rejection of the sucrose solution in an acid- and concentration-dependent manner. Bees were particularly sensitive to formic acid, tartaric acid, and lactic acid, which induced higher rejection at lower concentrations. They were more tolerant to the addition of succinic acid and acetic acid and exhibited intermediate rejection for citric acid (von Frisch, 1934). For other acids, the concentration dependence effect may lead to a preference for the mixture of sucrose and acid solutions. This was the case in later experiments in which mixtures including caffeic and genistic acids were preferred to a pure sucrose solution (Hagler & Buchmann, 1993).

As for bitter substances, these results do not allow to conclude on the presence of a dedicated acid sensing channel in the gustatory system of honey bees. Increased rejection of sucrose spiked with different acids may reflect a decrease in appetitive motivation for a solution that has been denatured by the addition of acids rather than a direct rejection of the acids themselves.

3 | TASTANT DETECTION AT THE PERIPHERY – GRNS

In the honey bee, GRNs are housed within hair-like *sensilla chaetica* and *sensilla basiconica* (Esslen & Kaissling, 1976). Gustatory s*ensilla chaetica* can be found essentially on the antennae, while gustatory *chaetica* and *basiconica* are found on the mouthparts and forelegs (de Brito Sanchez et al., 2005, 2014; Whitehead, 1978; Whitehead & Larsen, 1976a, 1976b). *Sensilla basiconica* also exist on the antennae, but they mediate olfactory detection (Esslen & Kaissling, 1976). These *sensilla basiconica* contain olfactory receptor neurons that project to a specific region of the antennal lobe, the primary olfactory center of the insect brain, which participates in an olfactory subsystem responding to colony odors and pheromone odors (Carcaud et al., 2015; Kropf et al., 2014). As some of these odorants have low volatility, their detection may require direct contact chemoreception, thus establishing a diffuse separation between taste and olfaction. Here we will not further elaborate on this particular case but focus exclusively on electrophysiological responses of pure GRNs, recorded so far from *sensilla chaetica*.

The highest density of gustatory *sensilla chaetica* is found on the terminal antennomere, that is, on the tip of the antennae (Esslen & Kaissling, 1976). Each gustatory *sensillum* hosts three to five GRNs, each of which projects a dendritic branch to the *sensillum* apex (Mitchell et al., 1999; Whitehead & Larsen, 1976a; Figure 1b). The specificity of several GRNs located on different body appendages has been studied by means of single-*sensillum* recordings (Boeckh, 1962; Boeckh et al., 1965; Kaissling et al., 1989; Kaissling & Thorson, 1980; Olsson & Hansson, 2013; Schneider & Hecker, 1956). This electrophysiological technique consists in obtaining extracellular recordings of GRNs by means of an electrode establishing electrolytic continuity with the receptor hemolymph in which the dendrites of these neurons bathe. Thus,

stimulating the neurons via the same recording electrode loaded with the tastants to be tested provides information about GRN tuning and sensitivity. Individual GRNs can be distinguished based on their different temporal response patterns and amplitudes of their action potentials, which are mainly due to different dendrite diameters (Hansson et al., 1994; Kaissling & Colbow, 1987). Several studies have characterized the responses of GRNs located on different body appendages upon stimulation with the above-described tastant categories.

3.1 | Electrophysiological responses to sugars

The first recordings of GRNs responding to sugars in the honey bee were obtained from *sensilla chaetica* located on the galea (Whitehead & Larsen, 1976a; Figure 4a). GRNs responding to sugars exhibited a higher sensitivity and higher response rates upon stimulation with sucrose followed by glucose and then by fructose. Recordings of *sensilla chaetica* located on the labial palps showed also maximal responses to sucrose, but in this case, fructose induced higher responses than glucose (Whitehead, 1978). More recently, two GRNs were found in galeal *sensilla* of bumble bees (Miriyala et al., 2018), which exhibit bursts of spikes in response to stimulation with sucrose. Spike bursting is abolished when *sensilla* are exposed to the gap- junction blocker carbenoxolone. This suggests that bursting in response to a sugar ligand might arise from inhibitory interactions between GRNs connected by electrical synapses. The consequence of this lateral inhibition between GRNs would be a high resistance to sensory adaptation upon sucrose stimulation. A similar pattern of activity was observed in galeal *sensilla* of honey bees (Miriyala et al., 2018) so that the same mechanism was proposed for this insect.

Antennal GRNs were also recorded (de Brito Sanchez et al., 2005; Haupt, 2004) as the last antennal segment exhibits a high density of gustatory *sensilla* (Esslen & Kaissling, 1976). GRNs within these *sensilla* respond to sucrose concentrations down to at least 0.1%. A high degree of variability in the response of antennal *sensilla* to the same sucrose concentration was found, which was interpreted as a way to extend the dynamic range of sucrose perception over a large range of concentrations (Haupt, 2004). Responses to sucrose were inhibited by the addition of very low concentrations of bitter substances (e.g., 0.01-mM quinine added to 15-mM sucrose solution; see below), thus showing the suppressive effect of these substances on sugar receptor neurons (de Brito Sanchez et al., 2005; Figure 4b).

Tarsal GRNs responding to sugars were also recorded in *sensilla chaetica* located on the tarsomeres (third and fourth tarsomeres) and on the claws of the posterior pair of legs (de Brito Sanchez et al., 2014). Tarsomere *sensilla* responded to sucrose solution, but responses were rather due to the contact with the electrolyte (KCl) contained in the stimulating solution as these *sensilla* are particularly sensitive to salts (see below). These *sensilla* did not show a dose–response relationship when stimulated with different sucrose concentrations, thus suggesting that they do not host a sucrose receptor cell. On the contrary, claw *sensilla* exhibited high responsiveness to sucrose, indicating that the claws are essential for sensing sucrose via the forelegs (de Brito Sanchez et al., 2014).

3.2 | Electrophysiological responses to amino acids

From the five neurons hosted within galeal *sensilla*, one is a mechanoreceptive neuron, which ends at the base of the *sensilla* and senses deflections experienced by the cuticular hair upon contact with an object's surface. A second neuron responds to sugars and a third (and possibly a fourth) neuron responds to electrolytes (Whitehead & Larsen, 1976a). It was suggested that the fifth neuron could be responsive to amino acids, among other tastants (Whitehead & Larsen, 1976a). So far, only one study observed responses to amino acids delivered to the galea (Lim et al., 2019). Single-*sensillum* recordings from the 10 most distally located *sensilla* were performed upon stimulation with various concentrations of L-glutamate and L-aspartate (50, 100, and 200 mM), which are major components of pollen (Szczęsna, 2006). Responses increased linearly with the solute concentration of both amino acids (Figure 4c), thus showing the presence of a GRNs tuned to these tastants (Lim et al., 2019).

3.3 | Electrophysiological responses to bitter substances

Stimulation of *sensilla chaetica* located on the antennae with different concentrations of bitter substances such as quinine and salicin did not induce any action potential, consistently with an absence of sensitivity to these substances (de Brito Sanchez et al., 2005). A similar result was found when GRNs located on the tarsi were stimulated with bitter substances (de Brito Sanchez et al., 2014). Yet recordings of the galeal *sensilla chaetica* stimulated with quinine or amygdalin showed a delayed, specific pattern of action potentials (Wright et al., 2010). Thus, if bees can sense bitter substance per se, they might do so via these specific *sensilla*. Yet this would have the disadvantage of a "delayed" detection, and it would seem more adaptive for bees to react earlier to aversive, noxious substances (i.e., upon antennal or tarsal contact) before they reach the mouthpieces.

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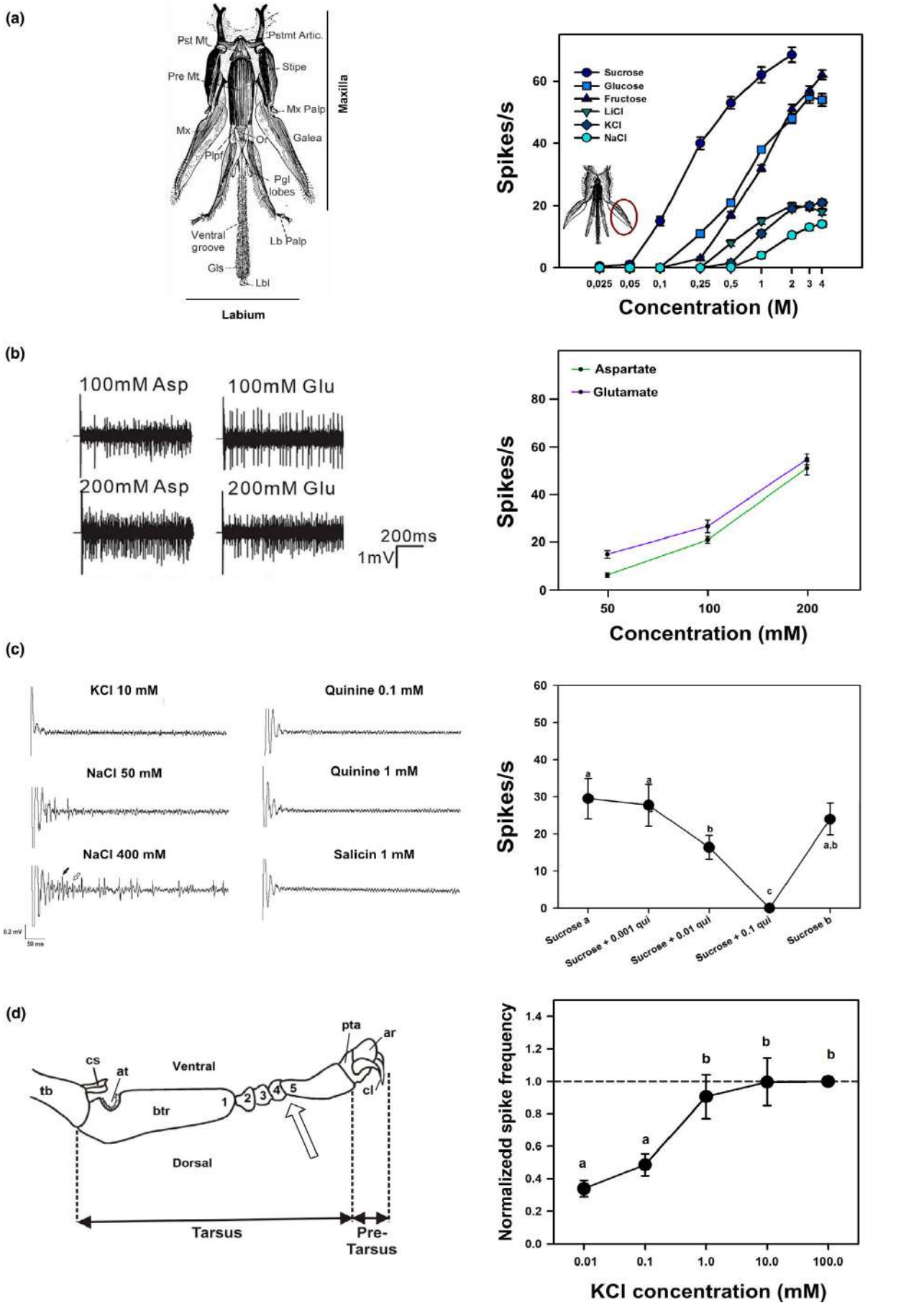
(a)
Pst Mt
Pstmt Artic.
Stipe
Pre Mt
Mx Palp
Maxilla
Mx
Galea
Or
Plpf
Pgl lobes
Ventral groove
Lb Palp
Gls
Lbl
Labium
Sucrose
Glucose
Fructose
LiCl
KCl
NaCl
Spikes/s
Concentration (M)
0,025 0,05 0,1 0,25 0,5 1 2 3 4
(b)
100mM Asp
100mM Glu
200mM Asp
200mM Glu
200ms
1mV
Aspartate
Glutamate
Spikes/s
Concentration (mM)
50 100 200
(c)
KCl 10 mM
Quinine 0.1 mM
NaCl 50 mM
Quinine 1 mM
NaCl 400 mM
Salicin 1 mM
0.2 mV
50 ms
Spikes/s
Sucrose a
Sucrose + 0.001 qui
Sucrose + 0.01 qui
Sucrose + 0.1 qui
Sucrose b
(d)
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at
Ventral
tb
btr
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2
3
4
5
cl
Dorsal
Tarsus
Pre-Tarsus
Normalizedd spike frequency
KCl concentration (mM)
0.01 0.1 1.0 10.0 100.0

FIGURE 4 Electrophysiological characterization of gustatory receptor neurons of the honey bee. (a) Left: The mouth pieces of the honey bee. Ventral view of the parts forming the proboscis, with the labium in the middle and the maxillae on the sides, flattened out (adapted from Snordgrass, 1956). Abbreviations: Gls, glossa; Lbl, labellum; Lb Palp, labial palp; Mx, maxilla; Or, salivarium opening; Pgl lobes, paraglossal lobes; Plpf, palpiger; Pre Mt, prementum; Pst Mt, postmentum; Pstmt Artic, postmental articulation. Right: Electrophysiological responses of chaetic *sensilla* on the galea. Single-*sensillum* recordings show that neurons within these sensilla respond linearly to the solute concentrations of sucrose, glucose, fructose, NaCl, KCl, and LiCl when these are expressed in a logarithmic scale. Each dot represents the mean response (±2 *SEM*) from an average of eight *sensilla* per 10 bees with two applications per *sensillum* (i.e., 160 responses recorded). The inset shows the proboscis, with a circle around the galea where these recordings were made (from Whitehead & Larsen, 1976b). (b) Electrophysiological responses of chaetic *sensilla* of the galea to amino acids. Left: Examples of recordings obtained upon stimulation with two concentrations of L-glutamate (Glu) and L-aspartate (Asp). Right: Responses to the solute concentrations of Glu and Asp increase significantly and linearly in these *sensilla*. Dots represent the mean response (±*SEM*) from an average of five *sensilla* per seven bees (i.e., 35 responses recorded). From Lim et al. (2019). (c) Electrophysiological responses of antennal chaetic *sensilla* to salt or bitter tastes. Left: Examples of recordings obtained upon stimulation with different stimulating solutions. KCl 10 mM; NaCl 50 mM; NaCl 400 mM; quinine 0.1 mM; quinine 1 mM; salicin 1 mM. Black and white arrows in (c) show different spike amplitudes. Right: Concentration threshold of quinine necessary to inhibit the response of sucrose receptor cells of the antenna to 15-mM sucrose (i.e., quinine concentration at which complete inhibition of sucrose receptor cells is reached). Sucrose-responding *sensilla* ($n = 8$ of 3 bees) were stimulated with 15-mM sucrose solution at the beginning (sucrose a) and at the end of the experiment (sucrose b) and with mixtures of sucrose solution 15 mM and quinine at three different increasing concentrations, 0.001, 0.01, and 0.1 mM. The quinine concentration threshold for inhibition of sucrose receptor cells lies between 0.01 and 0.1 mM. The abscissa displays the consecutive stimulations and the ordinate, the spike count. Error bars represent *SEM*. Different letters indicate significant differences between responses depending on quinine concentration. From de Brito Sanchez et al. (2005). (d) Electrophysiological KCl responses of chaetic *sensilla* located on the tarsi of the honey bee. Left: Scheme of the distal segments of a honey bee foreleg showing the tarsus and the pretarsus. The tarsus has five tarsomeres: a basitarsus (btr: 1), which is the largest tarsomere, and four smaller tarsomeres (2–5). The basitarsus presents a notch of antenna cleaner (at) and the tibia (tb) a closing spine (cs). The distally situated pretarsus (pta) bears a pair of lateral bifid claws (cl) and an arolium (ar), a small pad used to increase adhesion. Right: Normalized mean electrophysiological responses (to KCl 100 mM; ±*SEM*) of chaetic *sensilla* located on the small tarsomeres (six *sensilla* from five bees) stimulated with different concentrations of KCl (mM). These *sensilla* exhibit a high sensitivity to saline solutions. From de Brito Sanchez (2011)

In *sensilla chaetica* located on the antennae (de Brito Sanchez et al., 2005), galea (Wright et al., 2010), and tarsi (de Brito Sanchez et al., 2014), electrophysiological responses to sucrose were inhibited by the addition of quinine, consistently with the hypothesis that rejection of sucrose solutions containing quinine involves sucrose receptor inhibition (see Figure 4b and above). This inhibitory effect was also observed in adult blowflies (*Protophormia terraenovae*), fruit flies *D. melanogaster*, and moths (*Heliothis virescens*) stimulated with a mixture of sucrose and quinine, both at the behavioral and electrophysiological levels (French et al., 2015; Jørgensen et al., 2007; Liscia & Solari, 2000; Meunier et al., 2003).

The mechanism underlying sugar-sensing inhibition by bitter molecules such as quinine remains unknown. As "bitter" substances exhibit considerable variation in their chemical structures, a variety of modes of action may exist (French et al., 2015). Bitter molecules may interfere with the detection of sugar molecules at sugar receptors, as shown for *Drosophila* (Sellier et al., 2011), or they may block or interfere with transduction processes in sugar receptor neurons. In addition, some bitter molecules may suppress the activity of these neurons because of their toxicity (Tanimura & Shimada, 1981).

In antennal *sensilla* of honey bees, inhibition of neural activity was specific for quinine, as salicin, another bitter substance, did not inhibit cellular responses to sucrose. This difference may reflect differences in the structure of bitter substances (quinine is an alkaloid while salicin is a glucoside). Inhibition was reversible because stimulating with 15-mM sucrose solution after stimulating with a mixture of the same sucrose solution, and 0.1-mM quinine yielded a cellular response similar to that obtained for 15-mM sucrose solution alone before mixture stimulation (de Brito Sanchez et al., 2005). This reversibility indicates that quinine does not damage the sucrose GRNs.

3.4 | Electrophysiological responses to salts

Electrophysiological responses to saline solutions (e.g., NaCl, KCl, LiCl, $MgCl_2$, $CaCl_2$) have been recorded in various studies (de Brito Sanchez et al., 2005, 2014; Whitehead, 1978; Whitehead & Larsen, 1976a, 1976b) focusing on *sensilla* located on various gustatory appendages of the honey bee (e.g., palps, galea, and tarsi). In all cases, the presence of receptor cells responding to salts (in particular to NaCl, KCl and LiCl) was reported (see Figure 4a for galeal *sensilla*). Sensitivity to saline solutions was found in GRNs hosted by *sensilla chaetica* of the antennae (de Brito Sanchez et al., 2005), mouth parts (Whitehead, 1978; Whitehead & Larsen, 1976a; 1976b), and tarsi (de Brito Sanchez et al., 2014). Sensitivity to saline solutions was particularly enhanced in GRNs located on the third and fourth tarsomeres. Their spike frequency increased significantly with KCl concentration, especially

in the range of low concentrations (0.01, 0.1, and 1 mM), including concentrations that are normally undetectable by GRNs responding to salts in other body appendages (de Brito Sanchez et al., 2014). For instance, responses of tarsal GRNs were recorded at a 0.01-mM KCl concentration, which is normally used as contact electrolyte in electrophysiological recordings, while 10-mM KCl was needed to induce a response from antennal GRNs (de Brito Sanchez et al., 2005). This difference could be adaptive, as hovering bees in search of saline solutions available in water ponds would rather first contact water with their tarsi to assess salts content (de Brito Sanchez et al., 2014).

3.5 | Electrophysiological responses to acids

Data on honey bees'' electrophysiological responses to acids are lacking. The sensitivity of free-flying honey bees to certain acids added to sucrose solution shown in the behavioral experiments of von Frisch (1934, see above) could be due to the presence of an acid-tuned GRN or could reflect the effect of acids (e.g., inhibition) on the response of other specialized GRNs (e.g., sweet GRNs). Interestingly, von Frisch proposed a receptor-based theory to explain the variable rejection of sucrose solutions spiked with different acids. He argued that the degree of dissociation of an acid, which allows distinguishing between strong acids that dissociate completely to form ions in solution, and weak acids that ionize only partially and reversibly, is a key feature accounting for the bees' response. According to this dissociation theory, weak acids would be perceived as being more acid because only a small fraction would be dissociated in the hemolymph, leaving a large putative proton pool available in the non-dissociated form. If the acid reaches the GRN surface, more protons could be made available at the activation site, which would result in a higher perceived acidity (von Frisch, 1934, p. 112).

Eighty-three years later, the same idea was proposed to account for Ca^{2+} responses to acids of GRNs in the tarsi of *D. melanogaster* (Chen & Amrein, 2017), yet without mentioning von Frisch's original proposal. These GRNs are dedicated to sour taste and are more activated by weak than by strong acids. Coincidently with von Frisch's dissociation hypothesis, Chen and Amrein (2017) proposed that activation of these sour neurons might be mediated by proton translocation as protons were shown to be necessary and sufficient for activating these neurons, whereas the presence of the conjugate carboxylic base was not. They suggested that in the case of strong acids, translocation of free protons would induce activation of the neurons, but not all protons in the hemolymph may reach the pore channel of the receptor on the GRN surface. In the case of weak acids, only a small fraction would be dissociated in the hemolymph, leaving a large putative proton pool available in the non-dissociated form to reach the pore channel and activate the receptor site (Chen & Amrein, 2017).

4 | THE MOLECULAR BASIS OF PERIPHERAL TASTANT DETECTION

The sequencing of the honey bee genome (2006) was a critical step allowing to investigate the molecular basis of gustatory perception of this insect. Yet data on expression patterns of different GR types in different organs are still scarce or missing so that in many cases, conclusions on bee GR types are based on *Drosophila* homologs and their known functions.

Firstly, 10 GRs genes (*AmGr*) and 3 *AmGrs* pseudogenes (i.e., which do not code for functional proteins) were identified, thus indicating the presence of 10 functional GRs (Robertson & Wanner, 2006). Later, the sequencing of bumble bee genomes (*Bombus terrestris* and *Bombus impatiens*) led to a revision of the honey bee genome, and the number of GR genes and of functional GRs was extended to 11 and the number of pseudogenes to 4 (Sadd et al., 2015).

These numbers are low compared to those of other insects. For instance, the fruit fly *D. melanogaster* possesses 68 functional GRs encoded by 60 *Grs* (Dunipace et al., 2001; Robertson et al., 2003; Scott et al., 2001), the mosquito *Anopheles gambiae* 76 functional GRs encoded by 52 *Grs* (Hill et al., 2002), and the Argentine ant (*Linepithema humile*) 96 functional GRs encoded by 116 *Grs* (Smith et al., 2011). The reduced number of *Grs* found in the honey bee has been interpreted as the result of a feeding specialization on floral products (Robertson & Wanner, 2006), which would be associated with a reduction in tastant diversity. This interpretation has been questioned as a similar reduction in the number of *Grs* has been found in other Hymenoptera with different, omnivorous feeding regimes (e.g., 11 *Grs* and 6 *Grs* in the carpenter ant *Camponotus floridanus* and the jumping ant *Harpegnathos saltator*, respectively; Bonasio et al., 2010). Moreover, ecological analyses indicate that the gustatory world of bees does not seem as limited as it was long thought to be.

Besides GRs, the sequencing of the honey bee genome also revealed the presence of IRs. Twenty-one IR genes have been reported for the honey bee (Sadd et al., 2015), which is less than the 66 IR genes and the 9 putative pseudogenes of *D. melanogaster* (Benton et al., 2009; Croset et al., 2010). So far, no study has addressed a possible role of honey bee IRs in gustation or the possible interaction between IRs and GRs as a condition for detecting some tastants, as is the case in *Drosophila* (Jaeger et al., 2018). If such interactions exist in bees, they may greatly extend the number of functional combinations for detecting and discriminating tastants.

The same lack of knowledge applies to TRPA-based taste detection. Honey bees do not express the TRPA1 channel but a hymenoptera-specific channel, AmHsTRPA (Matsuura et al., 2009), which is involved in heat perception (Junca & Sandoz, 2015; Kohno et al., 2010). This is consistent with the fact that TRPA1 in *D. melanogaster* and *A. gambiae* is activated by changes in temperature (Kang et al., 2012; Kwon et al., 2008; Viswanath et al., 2003; Wang et al., 2009; Zhong et al., 2012). Whether AmHsTRPA also contributes to sense aversive chemical substances remains to be determined.

Three members of the pickpocket (Ppk) gene family (DEG/ENaC channels) are present in the honey bee genome: Ppk28, Ppk19, and a sodium channel protein Nach (The Honeybee Genome Sequencing Consortium, 2006). Their possible role in gustation has not been studied. Yet the Ppk28 found in the honey bee genome is not homolog of a Ppk expressed in the fruit fly genome as their structures are different (2,448 pb for the *Drosophila* Ppk28 and 5,968 pb for the honey bee Ppk28), thus asking for caution when elaborating on its possible function.

5 | EVOLUTION OF THE GR MULTIGENIC FAMILY

The sequencing of the honey bee genome allowed to identify the presence of GRs and IRs and enables, in addition, to use comparative analyses between species to address the evolution of taste mechanisms and search for orthologs guiding functional analyses of receptor function. To investigate the evolution of the GR multigenic family, we gathered all known 359 proteins of the GR family reported for 6 insect species, including 15 proteins for the honey bee, *A. mellifera* (AmGr); 25 proteins for the bumble bee, *B. terrestris* (BtGr); 76 proteins for the Malaria mosquito, *A. gambiae* (AgGr); 68 proteins for the fruit fly *D. melanogaster* (DmGr); 117 proteins for the Argentinean ant, *L. humile* (LhGr); and 58 proteins for the parasitoid wasp, *Nasonia vitripennis* (NvGr; Robertson & Wanner, 2006; Sadd et al., 2015; Smith et al., 2011). Some proteins are not functional and were labeled as pseudogenes (PSE) in our reconstruction. Multiple sequence alignments were carried out with ClustalW under default parameters (Thompson et al., 1994), resulting in a matrix of 359 terminals with 528 aligned amino acids. A Maximum Likelihood gene tree was reconstructed using a CAT model with RaxML v8.2.12 (Si Quang et al., 2008; Stamatakis, 2014); node support was estimated from 1,000 bootstraps. The RaxML reconstruction was performed on the CIPRES Science Gateway online server (Miller et al., 2010). In the absence of outgroups, the tree was rooted on the sugar receptor candidates to mirror the topology of Sadd et al. (2015).

Our phylogenetic reconstruction (Figure S1) yielded 121 out of 357 nodes exhibiting support values equal to or higher than 70. We identified nine sets of GR orthologs for Hymenoptera that are highly supported (bootstrap support value higher than 95%; or BS > 95). The Gr1, Gr2, Gr3, and Gr6 proteins are orthologs for all four hymenopteran species (honey bees, bumble bees, Argentine ants, and *Nasonia* wasps). The Gr4, Gr7, and Gr11 proteins are also orthologs but only for honey bees, bumble bees, and Argentine ants, suggesting that they might be specific to Aculeata or that an orthologue loss occurred in *N. vitripennis*. Finally, the Gr8, Gr9, and Gr12 are orthologs only for the Apidae (honey bees and bumble bees). This differentiation of Grs (at least for Gr4, Gr7-10, and 12) in Hymenoptera reveals specific Gr evolution within this group so that drawing straightforward conclusions on possible orthology between characterized DmGrs and unidentified AmGrs could lead to erroneous interpretations and should be avoided. For instance, our analysis highlights that no evident orthology relationships exist between DmGrs for bitter-taste detection and AmGrs despite previous suggestions in that sense (Simcock et al., 2017). This shows that the absence of bitter-sensing Grs may be Hymenoptera-specific (Figure S1). Interestingly, no Gr strictly tuned to amino acids has been identified in fruit flies until now whereas AmGr10 responds specifically to these tastants (see below). This suggests that this receptor is hymenoptera-specific (Figure 5c).

Although most species express a single protein in these orthologs, we identified six cases of duplication events. In the Argentinean ant *L. humile*, *Gr1* was duplicated two times (*LhGr1.1*, *LhGr1.2PJ*, *LhGr1.3PJ*) and *Gr2* a single time (*LhGr2.1PSE*, *LhGr2.2*); in *B. terrestris*, we identified four duplication events of *Gr8* (*BtGr8*, *BtGr14FIX*, *BtGr16*, *BtGr18*, *BtGr20*), of *Gr9* (*BtGr9FIX*, *BtGr15INT*, *BtGr17PSE*, *BtGr19*, *BtGr21*) and of *Gr12* (*BtGr12*, *BtGr22*, *BtGr23*, *BtGr24*, *BtGr25*). In *A. mellifera*, only *Gr4* was duplicated (*AmGr4*, *AmGr5*).

We detected four other important expansions by duplication in the Hymenopteran species considered. Most of the *L. humile* proteins result from a single intense expansion, with a highly supported clade that encompasses 94 out of 117 proteins (BS = 97). In *N. vitripennis*, 33 out of 58 proteins form a poorly supported clade that is strongly related to the Gr4 ortholog (BS = 54; and BS = 99, respectively), while 8 proteins form another clade that is strongly supported and related to the Gr7 ortholog (BS = 99 and BS = 100, respectively). Finally, 3 out of 15 proteins form a supported clade in *A. mellifera* (AmGrX, AmGrY, AmGrZ; BS = 100).

Although caution is needed when making functional conclusions on AmGRs based on GRs of *D. melanogaster*, matching orthologs between *AmGrs* and *DmGrs* proved to be useful in some cases and improved our understanding of the bees' gustatory sense. Using this approach, the AmGr1 and AmGr2 proteins were found to be most closely related to the DmGr5a and DmGr64f proteins (Figure 5b), which are sugar receptors among eight DmGr candidate sugar receptors

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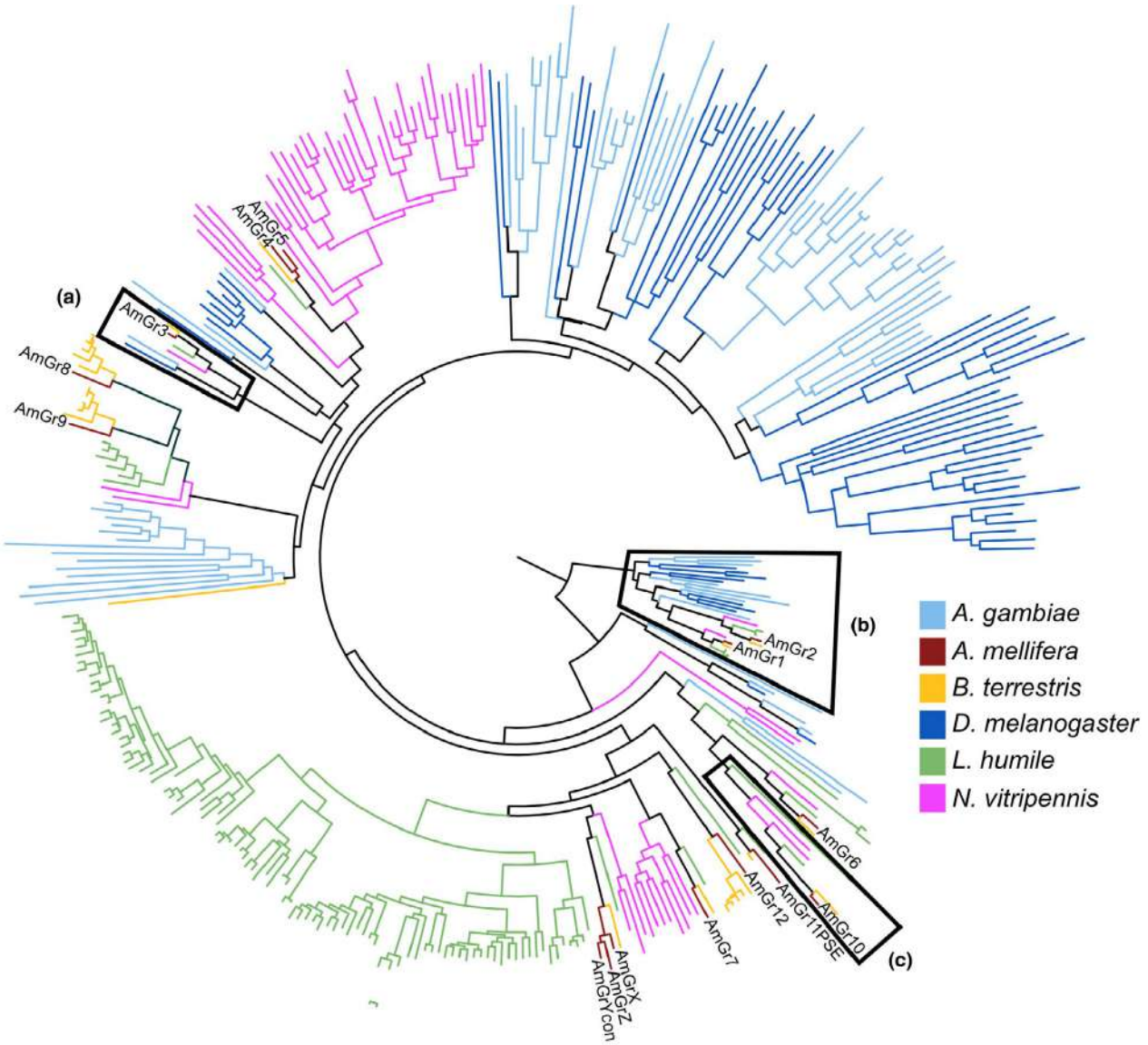
(a)
AmGr3
AmGr8
AmGr9
AmGr5
AmGr4
(b)
AmGr2
AmGr1
(c)
AmGr6
AmGr12
AmGr11PSE
AmGr10
AmGr7
AmGrX
AmGrZ
AmGrYcon
A. gambiae
A. mellifera
B. terrestris
D. melanogaster
L. humile
N. vitripennis

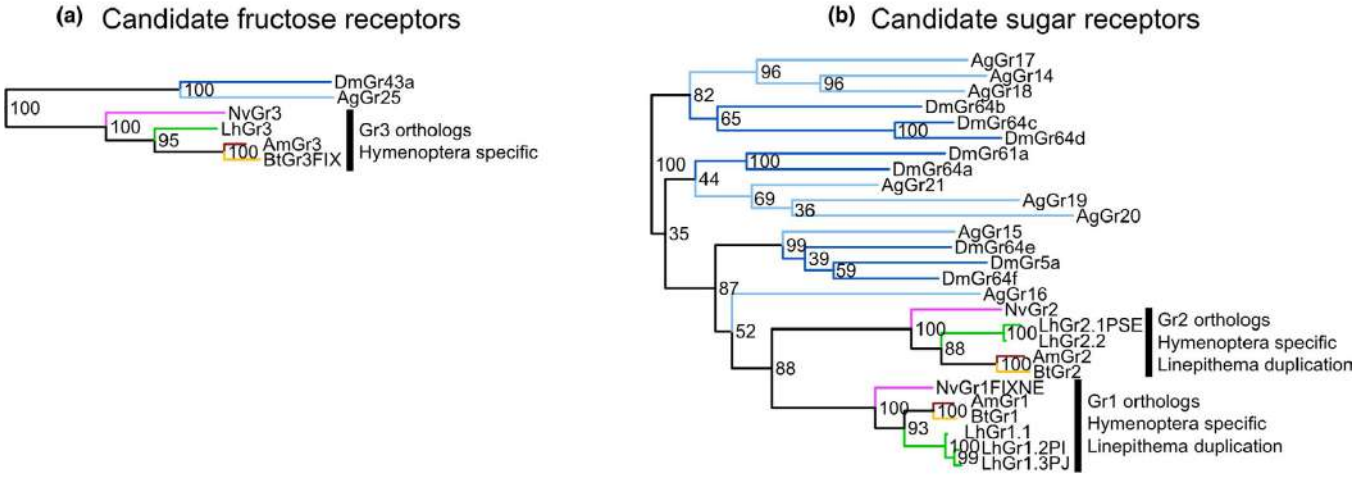
(a) Candidate fructose receptors
DmGr43a
AgGr25
NvGr3
LhGr3
AmGr3
BtGr3FIX
Gr3 orthologs
Hymenoptera specific
(b) Candidate sugar receptors
AgGr17
AgGr14
AgGr18
DmGr64b
DmGr64c
DmGr64d
DmGr61a
DmGr64a
AgGr21
AgGr19
AgGr20
AgGr15
DmGr64e
DmGr5a
DmGr64f
AgGr16
NvGr2
LhGr2.1PSE
LhGr2.2
AmGr2
BtGr2
Gr2 orthologs
Hymenoptera specific
Linepithema duplication
NvGr1FIXNE
AmGr1
BtGr1
LhGr1.1
LhGr1.2PI
LhGr1.3PJ
Gr1 orthologs
Hymenoptera specific
Linepithema duplication
(c) Candidate amino acid receptors
LhGr12
NvGr7
NvGr10
LhGr10
BtGr10PSE
AmGr10
Gr10 orthologs
Hymenoptera specific
Amino acid receptor and division of labor in Apis
0.6

FIGURE 5 Maximum likelihood reconstruction of the gustatory receptor gene family, with an emphasis on (a) candidate fructose receptors, (b) candidate sugar receptors, and (c) candidate amino acid receptors. The reconstruction was performed with a CAT model, using RaxML V8.2.12 on the CIPRES Science Gateway online server (Miller et al., 2010). Node support values were estimated from 1,000 bootstraps. In the absence of outgroups, the tree was rooted on the sugar receptor candidates to mirror the topology of Sadd et al. (2015). Protein sequences included in the reconstruction account for all known genes of four species of Hymenoptera (*Apis mellifera*, *Bombus terrestris*, *Linepithema humile*, and *Nasonia vitripennis*) and two species of Diptera (*Anopheles gambiae* and *Drosophila melanogaster*). Proteins and branches leading to them have been colored for each species to emphasize gene lineages, in red for *A. mellifera* (Am), orange for *B. terrestris* (Bt), green for *L. humile* (Lh), pink for *N. vitripennis* (Nv), light blue for *A. gambiae* (Ag), and dark blue for *D. melanogaster* (Dm). The complete phylogenetic tree is available in Figure S1

(Robertson & Wanner, 2006; Robertson et al., 2003; Scott et al., 2001, our work). Using the same approach, the AmGr3 protein was found to form a highly supported clade including the DmGr43a protein (BS = 100), indicating that they might all act as fructose receptors (Miyamoto et al., 2012; Robertson & Wanner, 2006), a hypothesis supported by recent experimental evidence (see below). Caution is nevertheless required when hypothesizing functions from orthologs because the effects of mutation, selection, and drift could alter a function or even lead to pseudogenization (Magadum et al., 2013). In addition, the possibility of elaborating such a comparative analysis was restricted to 3 out of 11 identified *AmGrs* (*AmGr1*, *AmGr2*, *AmGr3*), while 8 *AmGrs* could not be directly related to any *DmGr* gene.

Sadd et al. (2015) suggested that the *AmGr4/5* lineage, which was thought to be an ortholog of *DmGr28a/b* (Robertson & Wanner, 2006), may be a specific duplication in *Apis* as there is only one ortholog gene in *B. terrestris* (*BtGr4PSE*), which is a pseudogene that may have lost its function in bumble bees and cannot therefore duplicate. *AmGr6* to *AmGr9* have no apparent orthology to any of the *Grs* of the fruit fly Robertson and Wanner (2006), which may indicate that the GRs encoded by these genes potentially represent a Hymenoptera-specific lineage with unique functions (Sadd et al., 2015). The phylogenetic analyses of Sadd et al. (2015) suggest that *AmGr8*, *AmGr9*, and *AmGr12* belong each to one of the three set of duplicated genes in *B. terrestris*, meaning that their unknown functions could be related. In addition, *AmGr6*, *AmGr7*, and *AmGr10* are orthologs of *BtGr6*, *BtGr7*, and *BtGr10*, respectively. *AmGr6* is also an ortholog of *LhGr6* (Smith et al., 2011; Figure S1).

According to Robertson and Wanner (2006), the honey bee genome contains ~50-Gr pseudogenes. Only three of these pseudogenes, *AmGrX*, *Y*, and *Z*, were built in full-length versions. *AmGr11* is thought to be a pseudogene like *AmGr X*, *Y*, and *Z* but it is an ortholog of *BtGr11* and *LhGr11* (Sadd et al., 2015; Smith et al., 2011; Figure S1) which are functional genes. Although this suggests a loss of function in the case of *AmGr11*, the effect of such loss on honey bee's taste is unknown as the specificities of *BtGr11* and *LhGr11* are also unknown.

Coupling molecular approaches with functional neurophysiology provides a valuable strategy to overcome the deficits in genetic-tool availability in the honey bee. For instance, expressing GRs in *Xenopus* oocytes and coupling this expression with electrophysiological recordings (e.g., patch clamp recordings) enable the characterization of AmGr tuning (Değirmenci et al., 2018; Jung et al., 2015; Lim et al., 2019; Takada et al., 2018). Alternatively, the development of RNAi or CRISPR/Cas9 methods allows knocking out a GR gene and determining the consequences of its loss via electrophysiological and/or behavioral analyses (Değirmenci et al., 2020).

6 | THE MOLECULAR-RECEPTOR BASIS FOR TASTANT DETECTION

6.1 | Detection of sugars—AmGr1 and AmGr2

From the 11 functional GR genes identified in the bee genome, *AmGr1* and *AmGr2* are orthologs of eight candidate sugar receptor genes in *D. melanogaster* (Robertson & Wanner, 2006). Both are co-localized in antennal GRNs located within *sensilla chaetica* (Jung et al., 2015). *AmGr1* is highly expressed in the distal segment of the antenna, consistently with the highest density of *sensilla chaetica* found there (Esslen & Kaissling, 1976). When expressed in a *Xenopus* oocyte, *AmGr1*—which is closely related to the fruit fly sugar receptors *DmGr64a* and *DmGr5a* (Figure 5)—responds to sucrose, glucose, maltose, and trehalose in a dose-dependent manner but not to fructose. *AmGr2* does not respond to any of these sugars (Jung et al., 2015). However, a higher sensitivity to glucose and a lower sensitivity to sucrose, maltose and trehalose is observed when *AmGr1* and *AmGr2* are co-expressed in *Xenopus* oocytes compared with the sole expression of *AmGr1* (Jung et al., 2015). In addition, co-expression of *AmGr1* and *AmGr2* results in more stable responses of GRNs when compared to the responses of GRNs expressing only *AmGr1* (Jung et al., 2015).

These findings suggest that the sugar receptors AmGr1 and AmGr2 can form heterodimers, monomers, or mono-dimers and that AmGr2 may act as a co-receptor for AmGr1 conferring a wider detection range for sugars (Jung et al., 2015). Thus, AmGr1 may exhibit different ligand properties depending on the co-expression with *AmGr2* in the same gustatory neuron. In that sense, the role of *AmGr2* would be similar to

that of some *Grs* of *D. melanogaster* such as *DmGr64f* and
1173 *DmGr93a* (Jiao et al., 2008; Lee et al., 2009), which increase both the sensitivity and the range of detectable nutrients (Fujii et al., 2015; Miyamoto et al., 2013; Slone et al., 2007; Wisotsky et al., 2011) and stabilize GRN responses. A similar role has been found for olfactory co-receptor genes in the fruit fly olfactory system (Benton et al., 2006; Larsson et al., 2004).

6.2 | Detection of sugars—AmGr3

Interestingly, GRNs expressing *AmGr1* do not respond to fructose (Jung et al., 2015) although various behavioral experiments showed that honey bees distinguish this sugar from sucrose (Ayestaran et al., 2010; von Frisch, 1967). These findings suggested that fructose binds to another GR, possibly to that encoded by *AmGr3* (Robertson & Wanner, 2006). *AmGr3* is an ortholog of fructose receptor genes found in other insect species as suggested by our phylogenetic analysis (Figure 5a). Neurons expressing *DmGr43a* in the protocerebrum allow sensing fructose in the hemolymph, promote food intake in hungry flies, and suppress food intake in fed flies (Miyamoto & Amrein, 2014; Miyamoto et al., 2012). These results indicate that *DmGr43a* might act as a nutrient sensor. In the silk moth, *BmGr9* may have a similar role as the receptor it encodes specifically binds to fructose and is expressed in the larval gut (Sato et al., 2011). Recent studies in which *AmGr3* was transiently expressed in *Xenopus* oocytes have shown that in honey bees AmGr3 is specialized in fructose detection (Değirmenci et al., 2020; Takada et al., 2018). When double nonsense mutations were introduced into *AmGr3* using a CRISPR/Cas9 approach, the mutants exhibited a very low fructose responsiveness compared to control bees but responded normally to sucrose (Değirmenci et al., 2020; Figure 6a,b). Some mutant bees still responded to fructose in these experiments, thus leading to the suggestion that perception of fructose could occur, though in a reduced manner, via *AmGr1* and its co-receptor *AmGr2*, when co-expressed in the same gustatory neuron.

Analyses of *AmGr3* expression in tissues of adult bees revealed higher expression levels in the gut of foragers and in the antennae and legs of nurses (Takada et al., 2018). Further studies demonstrated *AmGr3* expression in the bee brain and showed high expression in starved bees and, conversely, lower levels in bees fed on a diet of fructose (Simcock et al., 2017). These results support the hypothesis that *AmGr3* acts as an internal sensor and regulator of sugar homeostasis and thus as a key element for sugar intake in the honey bee.

To sum up, from the 11 functional GR genes identified in the honey bee, three participate in different aspects of sugar sensing. While *AmGr1* confers sensitivity to various sugars, including sucrose, glucose, trehalose, and maltose, *AmGr2* seems to act as a co-receptor of *AmGr1*, increasing its sensitivity and the range of sugars detected. *AmGr3* is dedicated to fructose detection and besides its peripheral role, its brain and gut expression are consistent with an additional role as an internal nutrient sensor. Importantly, these receptor genes are not only expressed in peripheral taste organs (antennae, mouthparts, tarsi, etc.) and in the gut but also on the entire surface of the body and in the brain, where they may not act as conventional sugar receptors but may participate in signaling pathways of nutrient sensing.

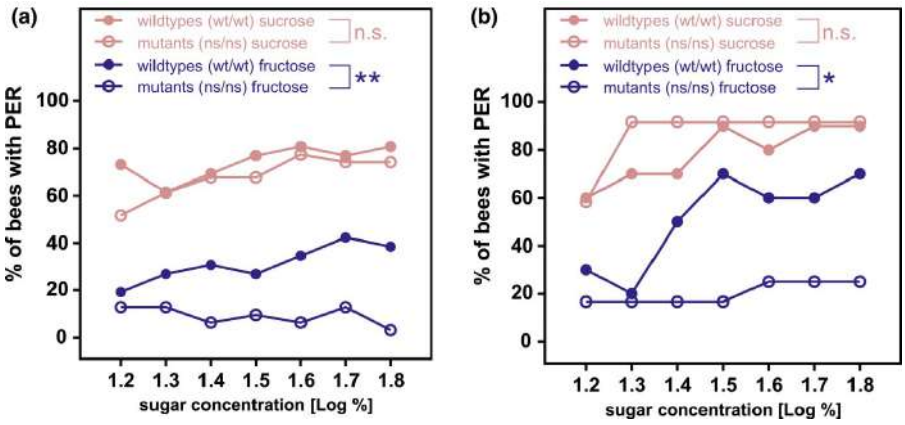

FIGURE 6 Molecular analyses of GRs in honey bees. Nonsense mutation introduced by CRISPR/Cas9 to *AmGr3* induced changes in behavioral sucrose responsiveness. The graph shows the percentage of bees responding to increasing sugar concentrations (16%, 20%, 25%, 32%, 40%, 50%, and 63%, corresponding to a log of 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, and 1.8, respectively). Responses were recorded upon stimulation with either fructose (blue) or sucrose (red). Panels (a) and (b) show two replicates of the same experiment. In both cases, *AmGr3* mutants (ns/ns—double mutants) displayed a reduced responsiveness to fructose (blue curve, white dots) but not to sucrose (red curve, white dots). ns, nonsense; w, wild type. n.s.: nonsignificant; **: significant. From Değirmenci et al. (2020). Courtesy of L. Değirmenci.

6.3 | Detection of amino acids—AmGr10

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The umami taste is related to the detection of amino acids. The basis for amino acid detection in honey bees is provided by *AmGr10* as shown by a study in which this receptor gene was cloned and expressed in *Xenopus* oocytes or transfected in HEK cells, to obtain two independent measures of receptor sensitivity using electrophysiological recordings (Lim et al., 2019). These recordings showed that *AmGr10* does not confer sensitivity to sweet and bitter tastants but confers sensitivity to a broad spectrum of amino acids such as aspartate, lysine, glutamate, glutamine, asparagine and arginine, and more particularly to L-glutamate and L-aspartate (Lim et al., 2019), which are major components of pollen (Szczęsna, 2006; Figure 3d). As in umami taste perception by humans, responses were enhanced by the addition of purine ribonucleotides such as IMP (inosine-5′-monophosphate) or GMP (Guanine 5′-monophosphate).

AmGr10 is expressed in *sensilla chaetica* of the galea. Single-*sensillum* recordings performed on these *sensilla* indeed showed responses to L-glutamate and L-aspartate, as well as to sucrose (Lim et al., 2019), thus suggesting that one of the four uncharacterized GRNs hosted by these *sensilla* is specifically tuned to amino acids. The specificity of the other three GRNs was already known: two respond to electrolytes and one to sugars (Whitehead & Larsen, 1976a). Alternatively, these results could be explained if different GRs were expressed in a single GRN like in mammalian sweet-umami cells (Grant, 2012).

AmGr10 was expressed not only in gustatory hairs located in the mouth parts of the bee but also in the fat body and other internal organs such as the brain and the hypopharyngeal glands (Lim et al., 2019). The distributed internal expression of *AmGr10* was confirmed by another study, which focused on the hypopharyngeal glands, brain, and ovaries of nurses (Paerhati et al., 2015). Expression levels were higher than those of foragers and *AmGr10* knockdown by dsRNA injection at the nurse stage caused earlier nurse-to-forager transition (Paerhati et al., 2015). Taken together, analyses of the internal expression of *AmGr10* at the adult stage suggest that, besides its role as a peripheral amino acid detector, the receptor encoded by this gene would monitor internal levels of amino acids for nutritional processes that may underlie division of labor.

In conclusion, the receptor encoded by *AmGr10* is clearly dedicated to amino acid sensing both at the periphery and internally. The existence of such a receptor in honey bees seems adaptive, given the biological importance of amino acids at multiple levels in this species (see above). What remains to be determined is the mechanistic basis of amino acid sensing by this receptor given its broad tuning. Identifying the structure and/or the molecular features of the amino acids that are recognized by the receptor would be an important goal per se.

6.4 | Detection of bitter substances

As discussed above, the detection of bitter taste by honey bees remains controversial. So far, no clear evidence for the existence of dedicated bitter-taste receptors has been provided. Some authors have remarked that *AmGr4* and *AmGr5* share similarities with the *Drosophila DmGr28a/b* complex (Robertson & Wanner, 2006), which was later related to bitter detection after its identification in bitter-taste neurons located in taste *sensilla* on the legs (Ling et al., 2014) and proboscis (French et al., 2015). Yet the basis for bitter detection in *Drosophila* is provided by six other GR genes (*DmGr32a*, *DmGr33a*, *DmGr39a*; *DmGr66a*, *DmGr89a* and *DmGr93a*), none of which is directly related to the *DmGr28a/b* complex (Dweck & Carlson, 2020). Thus, similarity between *AmGr4* and *AmGr5* and *DmGr28a/b* does not constitute a convincing argument to justify the involvement of the former in bitter perception.

A recent study by Leung et al. (2020) yielded a surprising result in *D. melanogaster*: three opsin receptor genes (*Rh1*, *Rh4* and *Rh7*) are expressed in the same GRNs and are required for the detection of a plant-derived bitter substance (aristolochic acid). In addition, the opsin receptor gene *Rh6* is expressed in bitter-taste GRNS of the fruit fly, where it is responsible for the rejection of cold sucrose solution (Li et al., 2020). This suggests that opsins may act as chemosensors or as thermosensors besides their well-known role in vision (Leung & Montell, 2017). Honey bees possess four opsin genes conferring sensitivity to UV-, blue and green light ranges (1 UV opsin, 1 blue opsin and two green opsins; Velarde et al., 2005; Wakakuwa et al., 2005), but no study has yet investigated their possible role as chemosensors or thermosensors.

Overall, there is no clear evidence supporting the existence of a receptor channel specialized in the detection of bitter tastants in honey bees. Some of the *Grs* that have not been functionally characterized until now might serve this function. Yet the lack of homology with fruit fly receptor genes that participate in bitter detection casts doubts about the presence of bitter-dedicated receptors. Detection of bitter taste might nevertheless be possible, via other receptor types (e.g., opsin-like) or via its suppressive effect on sugar GRNs (see above).

6.5 | Detection of salts

In *D. melanogaster*, peripheral detection of salts is mediated by a complex system including gustatory-receptor (*Grs*) and ionotropic-receptor (*Irs*) genes (Jaeger et al., 2018). Low salt attraction depends primarily on "sweet" neurons expressing Gr64f, with additional input from neurons expressing the ionotropic receptor IR94e, which has no identified ortholog in the

honey bee (Croset et al., 2010). High-salt avoidance is mediated by "bitter" neurons expressing DmGr66a and a population of glutamatergic neurons expressing the low osmolarity sensor Pickpocket23 (Ppk23). Moreover, when flies are deprived of salt, activation of Ppk23 is suppressed, thus showing a state dependency that is adaptive and conditioned by the insect's needs. In addition, responses of these Ppk23glut neurons require the presence of IR76b (Jaeger et al., 2018), while responses of Gr66a neurons, the other neuronal type mediating high-salt avoidance, do not require it (Jaeger et al., 2018). Responses of Gr64f neurons, which mediate attraction to low-salt concentrations, are completely dependent on IR76b, consistently with its proposed role in sensing low-salt taste (Jaeger et al., 2018). Interestingly, the IR76b-dependent salt responses of Gr64f neurons are sodium specific while those of Ppk23glut GRNs are not. It has also been suggested that IR76b and IR25a may act in a complex to mediate salt taste detection, which is consistent with evidence indicating that IR25a is a broadly expressed co-receptor (Ahn et al., 2017; Benton et al., 2009; Cameron et al., 2010; Chen & Amrein, 2017).

These results indicate a complex and state-dependent mode of salt detection, involving different salt transduction mechanisms and different classes of GRs and neurons (Jaeger et al., 2018). This may explain why prior reports differed on whether high-salt responses remain intact in IR76b mutants as its suppression and consequences may depend on the GRN type and transduction mechanism affected (Lee et al., 2017; Zhang et al., 2013).

In honey bees, GRNs responding to saline solutions definitely exist as electrophysiological and behavioral evidence indicates the presence of molecular-receptor mechanisms dedicated to salt detection (see above). Although it is conceivable that at least one of the GRNs hosted in gustatory *sensilla* is dedicated to saline solutions, so far no GR could be specifically ascribed to salt detection. Honey bees seem to have less receptors to sense salts than fruit flies, since no orthologs of Gr66a, IR94e, and ppk23 have been found in the bee genome. Honey bees express only three ppks (ppk19 and ppk28, sodium channel protein Nach) whose functions are still unknown. Yet they possess an ortholog to IR76b (Croset et al., 2010), which—as seen above—is required for both low-salt sensing and high-salt sensing and is expressed in all GRNs tested at the level of the fly labellum (Jaeger et al., 2018). Detection of saline solutions could be mediated either by specific GRs or by sweet-sensing neurons expressing this IR as in the fruit fly. If and how AmIR76b participates in salt taste detection in the honey bee remains to be determined.

6.6 | Detection of acids

As no electrophysiological evidence exists supporting the existence of GRNs responding to acids in the honey bee, the molecular basis of acid detection remains speculative in this insect. Bees might detect acids while foraging using IR25a and IR76a, which have corresponding orthologs in the fruit fly where they mediate acid detection for oviposition preference (Chen & Amrein, 2014). Yet the role of these IRs is unknown in honey bees.

7 | SENSING TASTANTS WITH IRS

IRs involved in gustation have been studied in the fruit fly but not in the honey bee. Gustatory IRs can be found in *sensilla* broadly distributed along the body of *D. melanogaster*, including the labellum, legs, pharynx, and wings (Koh et al., 2014). In the fruit fly, IR76b is necessary for low salt detection (Zhang et al., 2013), but this receptor also drives avoidance of high salt (Jaeger et al., 2018; Lee et al., 2017). Interestingly, both IR76b and IR25a are expressed in bitter and sweet GRNs (Benton et al., 2009; Croset et al., 2010) and more recently they have been also identified in acid-sensing GRNs located in tarsal *sensilla* (Chen & Amrein, 2017). IR76b and IR25a expressed in sweet GRNs are required for fatty-acid detection (Ahn et al., 2017) and also for detecting sour taste in acid-sensing GRNs (Chen & Amrein, 2017).

In the honey bee, genome, Sadd et al. (2015) identified 21 IRs. A similar number (22) was reported for the genome of the bumble bee *B. terrestris* (Sadd et al., 2015). So far, their functions, both in honey bees and in bumble bees, remain unknown. Expression analyses by means of RT-PCR have been conducted in the antennae and brain of the honey bee for only six *Ir* genes (*Ir8a*, *Ir25a*, *Ir68a*, *Ir75u*, *Ir76b* and *Ir93a*) because they are orthologs of *D. melanogaster* Ir genes (Croset et al., 2010). The mRNA of *Ir68a* and *Ir75u* was expressed in both the brain and the antennae of the honey bee. For instance, IR8a forms a functional subunit with IR64a that acts as an olfactory receptor mediating odor detection (Ai et al., 2013). IR25a, IR68a, and IR93a are required for humidity sensing (Enjin et al., 2016; Knecht et al., 2017). Also, IR25a and IR21a mediate thermosensation (Ni et al., 2016). Their localization in antennal *sensilla* of the honey bee is consistent with a role of these appendages for multimodal sensory detection. The functions ensured by these IRs in bees remain to be determined. Their participation in sensing salts as well as substances such as acids and fatty acids, for which not much is known in the taste biology of bees, could be highly relevant.

8 | CONCLUSION AND PERSPECTIVES

The multiple levels of analysis of bee gustatory perception presented here highlight the complexity of honey bees'

gustatory world and of the neural and molecular mechanisms
1176 mediating taste perception in these insects. Bees do not necessarily live in an impoverished gustatory world despite the specialization of some colony members on floral products. Task specialization within the hive, which is a fundamental feature of the social life style of bees, results in different bees collecting different products, which are not always derived from flowers. These include water with different mineral contents, resins, and even feces (Mattila et al., 2020). Moreover, we have focused on traditional tastants such as sugars, salts, acids, amino acids, and bitter substances, but the taste receptors of bees might also be used to sense long-chain fatty molecules such as cuticular hydrocarbons and low-volatile pheromones. These gustatory dimensions need to be further explored.

An important conclusion that can be drawn from our review is that "*not all the bees in the colony are equal*". Worker bees within a hive differ in terms of their genetic background as several patrilines can coexist within a hive. Accordingly, bees from different patrilines may differ in their behavioral and/or physiological responses to identical or similar events. Thus, in analyzing taste and taste-related behaviors, caution should be taken to specify which kind of bees are used and which are the reasons justifying this choice as gustatory and feeding processes and motivation may vary dramatically between bees. Taste processes and sensitivity may not only vary with age but also with season and task specialization. Thus, the common practice of capturing bees indiscriminately at a hive is not recommendable as it excludes any possible control of the kind of bee used in the experiments. Addressing questions on appetitive taste perception in winter bees, and extrapolating them to summer bees, or even worse, to all bees, could be misleading as winter and summer bees differ dramatically in their energy budgets, body reserves, and neurohormonal regulation (Behrends & Scheiner, 2010). Similarly, absence of control of the kind of bee used (guard, nurse, forager, etc.) may lead to erroneous conclusions as taste sensitivity may vary with task specialization. From this perspective, research agendas addressing if and how molecular taste receptors change their expression levels quantitatively but also qualitatively according to these factors would be extremely important to understand the link between task specialization, age, season and taste sensitivity, among others (Pankiw & Page, 1999; Pankiw et al., 2001; Scheiner et al., 2004; Tsuruda & Page, 2009). Another fundamental question that has been poorly addressed so far with respect to honey bee taste is the role of experience and the plasticity in shaping taste responses. Besides the existence of learning protocols in which taste is deprived of any reinforcement function to be presented as a stimulus to be learned and memorized (Guiraud et al., 2018), further questions on the effect of experience on molecular receptor expression need to be addressed. In the olfactory domain, it has been shown that odor learning changes the expression levels of olfactory receptors in honey bees (Claudianos et al., 2014). The olfactory receptor AmOr151, which is a broadly tuned receptor binding floral odorants such as linalool, and AmOr11, the specific receptor for the queen pheromone 9-oxo-decenoic acid, were both significantly downregulated after honeybees had learned these odorants in the olfactory PER conditioning assay. Long-term odor memory was essential for inducing these changes, suggesting that the molecular mechanisms involved in olfactory memory also regulate olfactory receptor expression at the periphery. Changes in taste receptor expression linked to repeated exposures to certain tastes may also occur, thus adding an additional source of variability that needs to be considered and evaluated.

Behavioral methods for analyzing taste responses in bees are diverse, but many of them have used the appetitive response of PER. This strategy is problematic as it makes dissociating taste from ingestive processes difficult. When a bee stops responding to a mixture of sucrose and a given tastant, straightforward interpretations that such response ceasing is due to the aversive nature of the tastant added to the sucrose solution are incautious. A variety of perceptual phenomena may underlie this phenomenon, from the inhibition of sucrose receptors by the added tastant to a change in taste that is not aversive but does not match a forager's expectation of high-quality sucrose solution. This problem renders difficult the analysis of mixtures of sucrose solution with other tastants (Ayestaran et al., 2010; Bertazzini et al., 2010; Desmedt et al., 2016; von Frisch, 1934; Hagler & Buchmann, 1993; Kim & Smith, 2000), which rely on the appetitive motivation of the bees to respond to food reward. From this perspective, the development of new protocols to study taste perception and discrimination, which try to reduce significantly the reinforcing function of a tastant is mandatory to advance our understanding of taste processes in bees.

The publication of the honey bee genome (Honeybee Genome Sequencing Consortium, 2006) expanded considerably our knowledge on the gustatory sense of bees, but further investigations are needed, guided by hypothesis derived with caution from this molecular data base. From the GR genes identified in the bee genome, only four have been characterized. Seven AmGrs remain to be characterized, which may allow solving pending questions and controversies such as the existence of bitter receptors or the process of fat taste detection. These receptors seem to build a unique lineage separating hymenopterans from other insect groups.

The development of new methods such as CRISPR/Cas9 or RNAi, which can be applied to the honey bee (Değirmenci et al., 2020; Guo et al., 2018; Wang et al., 2013), could provide valuable ways for addressing taste receptor function. Combining these strategies of receptor knock-down with behavioral and/or electrophysiological analyses requires time and considerable efforts but appears at the present time as

a privileged choice for studying GR genes in the honey bee. These molecular techniques could also serve for characterizing other mechanisms and receptors involved in taste perception in the bee. If information about Grs is still scarce, our current knowledge on the IRs of the bee is even more limited. The same applies to other types of receptors that might be used by bees to sense tastes (opsins, TRPs, ppks, etc.). Which functions they mediate and what is their gustatory tuning remains to be determined.

Another dimension of taste processing that requires thoughtful investigation is the central neuromodulation of taste processing pathways. Another reason for variability in gustatory responses may be the top-down modulation of the activity of GRNs and higher-order gustatory pathways. Bees, like most animals, are subjected to central neuromodulatory processes, which are crucial to define motivational states and which set the occasion for performing specific behaviors. Neurotransmitters such as biogenic amines (e.g., dopamine, octopamine, serotonin, among others) released in the central nervous system can act as facilitators or depressors of behavior or as instructive signals during learning and play a crucial role for an animal responsiveness toward specific sensory stimuli (Mercer & Menzel, 1982; Tedjakumala et al., 2014). For instance, appetitive responsiveness of bees, evaluated through PER to increasing concentrations of sucrose solution, is enhanced by octopamine (Scheiner et al., 2002, 2006) and is also influenced by tyramine (Scheiner et al., 2017). Other factors have been shown to modulate appetitive responsiveness via biogenic amines (e.g., pheromones; Baracchi et al., 2017, 2020), thus showing their importance for appetitive motivation.

These molecules may also act at the peripheral level, changing the sensitivity of receptors. For instance, in the male of the silk moth *B. mori*, octopamine increases the amplitude of receptor and action potentials elicited by the pheromone components Bombykol and Bombykal (Pophof, 2002). This shows that central neurotransmitters can have a modulatory action at the peripheral level, changing the responses of sensory receptors. Top-down modulation of gustatory processes has been shown in vertebrates; in the mouse, labeled-line circuits transmitting sweet and bitter signals from the tongue to the cortex are modulated by top-down processes (Jin et al., 2021). In this case, the gustatory cortex and the amygdala exert positive and negative feedback onto incoming bitter and sweet signals from the periphery in the brainstem. Top-down modulation of peripheral responses to taste has been shown in fruit flies where orthogonal neuromodulatory cascades control oppositely sweet and bitter peripheral taste sensitivity (Inagaki et al., 2014). Starved animals exhibit enhanced sugar sensitivity and decreased bitter sensitivity, allowing them to accept food resources that would be otherwise rejected. Bitter sensitivity is independently modulated during food deprivation, in the opposite direction as sugar sensitivity. While sugar sensitivity is increased via the neuropeptide F (dNPF) and dopaminergic signaling acting on sweet taste receptors, bitter sensitivity is reduced via the action of the adipokinetic hormone (AKH), the short neuropeptide F (sNPF) and GABA-ergic neurons inhibiting bitter-taste receptors (Inagaki et al., 2014). In this way, state-intensity-dependent, reciprocal regulation of appetitive and aversive peripheral gustatory sensitivity permits flexible, adaptive feeding decisions (Inagaki et al., 2014). Thus, motivational factors change the levels of neurotransmitters in the insect brain and affect thereby both the activity of taste receptors and eventually their expression levels. In consequence, the analysis of taste processes would benefit from considering factors that may alter the ratio of neurotransmitters in the brain such as the genetic background, hunger state, nutrient needs, age, social cast, or season (Harris & Woodring, 1992; Schulz et al., 2002; Taylor et al., 1992). We suggest here that neurotransmitters and neuropeptides can exert a top-down control on GRs and expression levels of Grs, which can be an additional explanation for the variability observed in behavioral and electrophysiological experiments addressing gustatory responses.

Besides the neuromodulatory action exerted by neurotransmitters and neuropeptides, gustatory sensitivity can also vary with the diet consumed by the insects. In the locust, for instance, a diet rich in some nutrients (e.g., amino acids) results in an increase of their levels in the hemolymph and in a concomitant decrease in the sensitivity and gustatory responses to the abundant substances (Abisgold & Simpson, 1988; Simpson & Simpson, 1992). The mechanism by which this nutrient increase changes the sensitivity of GRNs is still unclear but it was suggested that the abundant nutrients in the hemolymph could enter into receptor hemolymph of the *sensilla* and bind to GRs, inducing thereby an adaptation of GRNs and a decrease of sensitivity (Abisgold & Simpson, 1988; Simpson & Simpson, 1992). Exploring this possibility in honey bees should take into account both nutrient reserves in the fat body, which may decrease the impact of artificial diets, and the foragers' social life as ingested nutrients may be transiently stored in the crop for delivery in the hive, without being fully consumed and metabolized.

Finally, in the light of vivid debates on current agricultural practices employed by humans and a resulting massive, worldwide mortality of honey bee colonies (the so-called “colony collapse disorder”) induced by multiple factors including the indiscriminate use of agrochemicals, it is now crucial to better understand the gustatory world of honey bees. Some studies already assessed the preference of free-flying bees when given the choice between pure sugar solutions and solutions contaminated with different types of agrochemicals (Arce et al., 2018; Kessler et al., 2015; Liao et al., 2017). Yet more work is required to answer the crucial socioeconomic question of if and how

pesticides and other molecules released in the environment (e.g., weed-killers or even sanitary products for veterinary use) affect per se gustatory responses, the activity of gustatory neurons and their molecular receptors (Kessler et al., 2015). Addressing this question with the tools and approaches described in this article would add a fundamental dimension to these debates, uncovering unknown and possibly unsuspected effects of these molecules on the behavior and neurophysiology of bees.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

L. Bestea, conceptualization, writing-original draft, writing-review and editing; A. Réjaud, phylogenetic tree reconstruction, writing-original draft, writing-review and editing; J.-C. Sandoz, funding acquisition, writing-review and editing; J. Carcaud, writing-review and editing; M. Giurfa, conceptualization, funding acquisition, writing-review and editing; M.G. de Brito Sanchez, conceptualization, funding acquisition, writing-review and editing.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Discussion

My thesis studied the role of sNPF in various sensory domains (olfactory, visual, gustatory), hedonic contexts (appetitive, aversive) and organization levels (individual behaviour and neural processing) in a social insect, the honey bee. My goal was to determine if sNPF exerts a modulatory action in these domains, contexts and organization levels, comparable to the one it exerts in other insect species in which it has been explored [49, 35]. I explicitly asked if sNPF 1) modulates both appetitive and aversive responsiveness in honey bees, 2) promotes the acquisition and formation of visual memories and 3) promotes the acquisition and formation of both appetitive olfactory and aversive gustatory learning and memory. Below we discuss the answers provided to these questions, which have been presented in Chapters 1, 2 and 3, respectively.

sNPF modulates appetitive but not aversive responsiveness in honey bees (Chapter 1)

In this chapter we demonstrated a functional link between sNPF and behavioural expression of food-related sensitivity in honey bee foragers. Increasing artificially sNPF levels in partially fed bees with decreased appetitive motivation turned them into the equivalent of starved animals as they increased significantly food ingestion and gustatory responsiveness to pure sucrose solution, matching the behaviour of starved bees. sNPF topical application also increased olfactory responsiveness to odorants with intrinsic appetitive value. These odorants have been shown to elicit spontaneous PER in naïve bees irrespective of their colony origin and to detract bees from defensive responses upon alarm pheromone release [165]. This effect was also observed at the neural level, as shown by calcium imaging recordings of projection neuron activity at the level of the antennal lobe (AL), the primary olfactory neuropile in the bee brain. Our results show that sNPF increases neural activity of projection neurons conveying the olfactory message from the AL to higher-order centres, and that in doing so, it can rescue the decrease in neural activity induced by satiety in these same neurons. Remarkably, the enhancing effect of sNPF on behavioural responsiveness was

totally absent when aversive stimuli (electric and thermal shocks) were used to stimulate the bees. In this case, sNPF treatment did not induce higher tolerance to aversive stimuli, contrary to what has been shown in vertebrates and flies [103, 166]. In flies, for instance, overexpression of NPFR1 in PAIN neurons, which mediate nociception [104], delayed the aversive response of *Drosophila* larvae [103]. These results suggest that PAIN-mediated thermal nociception is modulated by NPF1 expressed in PAIN neurons. No equivalent result was observed in our experiments. Our results thus show how sNPF affects multiple behavioural modules (ingestion, gustation, olfaction) and central odour processing related to appetitive behaviour in the honey bee while being dispensable for aversive responses.

In the ingestion and gustatory responsiveness experiments, we modulated the nutritional state of bees and tested both palatable and unpalatable compounds as hunger mediates a direct influence on food perception, hence leading an animal to accept food that is normally rejected [167]. In the common starling (*Sturnus vulgaris*) consumption of toxic insect's larvae increases when the bird's body weight and lipid reserves diminished [168]. *Calliphoridae* flies reduce their sucrose acceptance threshold while their hunger state increased, leading them to tolerate more bitter compounds that are usually avoided [169]. This trade-off has been investigated in *Drosophila* focusing on NPF-sNPF signalling given the enhancing effect exerted by these peptides on both palatable and toxic food consumption [72, 71, 80, 82]. For instance, overexpression of *npf* or *npfr1* in *Drosophila* larvae increase intake of unpalatable food under starvation via the modulation of the reward circuitry which inhibits food avoidance normally elicited [71, 72]. The study of Inagaki et al. [249] demonstrated that NPF/sNPF signalling is involved in orthogonal neuromodulatory pathway cascades controlling oppositely sweet and bitter peripheral taste sensitivity under starved condition. While sugar sensitivity is increased via the neuropeptide F (dNPF) and dopaminergic signalling acting on sweet taste receptors, bitter sensitivity is reduced via the action of the adipokinetic hormone (AKH), sNPF and GABA-ergic neurons inhibiting bitter-taste receptors [249].

Consistently with findings on *Drosophila*, treating partially fed bees with sNPF increased food ingestion of pure sucrose solution and of a less palatable mixture of sucrose and salicin to levels comparable to those observed under starvation. However, gustatory responsiveness results presented an asymmetry compared to ingestion as sNPF treatment increased PER to pure sucrose concentrations but not to sucrose solutions spiked with salicin. Electrophysiological recordings of sweet-sensing gustatory receptor neurons (GRNs) should be conducted upon sNPF treatment to unravel whether this peptide exerts a top-down modulation of peripheral responses to taste, and/or modulates central taste processing. The preliminary results of electrophysiological recordings in foragers show that hunger increases the spike activity of sweet-sensing GRNs towards pure sucrose solutions compared to partially fed bees (Appendice B). In this case, we could hypothesize that sNPF topical application would increase spike activity of sweet-sensing GRNs. In honey bees,

there is no clear evidence of a dedicated bitter-sensing GRN, but bitter compounds mixed with sucrose were shown to inhibit the response of sweet-sensing GRNs to the sucrose [170, 252]. However, the absence of effect in gustatory responsiveness towards sucrose solutions spiked with salicin may indicate that rather than acting peripherally on sweet-sensing GRNs, sNPF could modulate central gustatory processes. The role of NPF still remains elusive in honey bees.

Our results of olfactory responsiveness suggest that sNPF does not act alone in the modulation of appetitive behaviours in honey bees. In this experiment partially fed bees significantly increased their olfactory responsiveness but not to the extend reached by starved bees (Chapter 1). Studies in *Drosophila* revealed that sNPF is part of cascades of reactions [249, 84] where it interacts upstream or downstream other neurotransmitters signalling to modulate behavioural expressions. Several works have demonstrated enhancing or inhibitory effects induced by biogenic amines on sucrose and olfactory responsiveness in honey bee, as well as in associative appetitive and aversive learning [171, 172]. For instance, dopamine is said to decrease sucrose sensitivity in honey bee foragers [173] whereas tyramine and octopamine have the opposite effect [173]. The inhibitory effect of dopamine (DA) on sucrose signalling is now questioned by several lines of evidence, including the demonstration in *Drosophila* that a subset of dopaminergic neurons convey sucrose signalling to the mushroom bodies of fruit flies [174, 175, 176, 177]. In bees, also, experiments using a visual version of PER conditioning [177], DA-receptor blockade impaired appetitive visual learning and memory while DA administration improved them, thus calling for a revision of the role attributed to DA in honeybees appetitive responsiveness and learning. In flies, the pathway activated by sNPF upon starvation leads to increased acceptance of bitter, aversive compounds and at the same time to an inhibition of dopaminergic signalling associated with enhanced sucrose sensitivity [249]. Whether the same effect exists in honey bees remains to be determined. Alternatively, the traditional view of sucrose signalling in the bee brain posits that octopamine (OA) accomplishes this function as depolarization of the octopaminergic neuron VUMmx1 can replace sucrose stimulation in olfactory PER conditioning and can lead to olfactory learning [178]. In addition, octopamine increases peripheral olfactory sensitivity in honey bees [179]. Thus, a link that needs to be determined may exist between sNPF and OA signalling in the bee brain. The same applies to serotonin, which reduced food intake when injected into the brain of foragers [180].

Overall, future studies should assess the interaction between these biogenic amines and sNPF in behavioural responsiveness experiments combining topical application of sNPF as used in our work, with injection of agonists/antagonists of biogenic amines into the bees' nervous system. This would help to understand whether this peptide functions downstream or upstream the tested neurotransmitters to further establish a neuronal pathway model. Other peptides such as insulin may interact with sNPF in honey bees as injection of insulin in the brain of naïve bees increases spontaneous odour responsiveness [181]. Surprisingly, ilp1 (insulin like peptides) and *npf* are both

upregulated in forager's brain compared to those of nurses [182, 234] whereas in *Drosophila* both signalling pathways are opposed. Ament et al [234] suggested that this singularity was relevant in a foraging context to make bees highly sensitive to both hunger and satiety cues.

In *Drosophila* adults and larvae, a few works have studies how NPF-sNPF signalling interact with biogenic amines and insulin to modulate food-seeking behaviours and olfactory sensitivity towards appetitive odorants. Feeding usually begins with search of the appropriate food source for which the sense of smell is essential [183]. During food-seeking behaviour, animals encounter many odorants of different attractiveness. Generalist species need then to select the best possible from many options [184]. Studies have shown that the modulation of this behaviour is evoked by NPF and sNPF in the adult fruit fly, at the central and peripheral level respectively [84, 185, 186]. On the contrary, NPF neurons are not involved in olfactory information processing in Drosophila larvae [95].

In *Drosophila* adults, there is a close link between olfactory processing in the antennal lobe (AL) and sNPF. Specifically, analyses of neuropeptide expression in the AL using a matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry and immunocytochemistry showed that sNPF (together with other neuropeptides) is expressed in subsets of olfactory sensory cells and different populations of local interneurons and extrinsic neuron. Thus, the olfactory network possesses the capacity to either produce or respond (via sNPF receptors) to sNPF, which explains the results obtained in our work.

A similar neuropeptidergic expression in the AL of honey bees could underlie our findings showing an increase of projection-neuron activity in the AL upon sNPF topical application in partially fed bees. The olfactory system in honey bees, briefly presented in fig. 4.2*a*, is well-described and has been intensively studied over the past decades [187, 188]. Further neuronal analyses are required to unravel the neuronal pathway of sNPF in olfactory coding and whether it affects the activity of higher order brain centres such as the lateral horn (LH) and the mushroom bodies (MB). Determining the expression of sNPFR in local neuronal populations of the honey bee AL is necessary in this context. In the case of odorants with intrinsic appetitive values, we would expect an additional effect of sNPF on the LH and its associated neuronal activity as this structure is thought to mediate responses to odorants with innate biological valence [189] whereas MBs are thought to be mainly involved in the responses to conditioned odours [60, 61].

In fruit flies, NPF signalling modulates the peripheral response of a specific class of olfactory sensory neurons (ab3A neurons) that detect a range of esters associated with fruits that signifies food and a place to lay eggs [190]. NPFR activation increases the number of odour-evoked action potentials in this subset of neurons and the ab3A neuron-specific knockdown of NPFR reduces attraction of flies to apple juice baits [190]. Starvation influences olfactory processing that mediates

food-search behaviours at the first olfactory synapse in glomeruli receiving inputs from ab3A neurons [84]. This change is mediated by sNPF expressed in ab3A neurons [59, 62] whose action facilitates synaptic transmission from selected olfactory sensory neurons [84]. In addition, insulin functions as a global satiety signal and is involved in a negative feedback loop controlling sNPF expression to inhibit food intake [84, 82, 186, 81] (fig. 4.1). Starvation modulation of the odour map increases the saliency of glomerular activity through the action of sNPF to match the changing internal needs of an organism. It is still unclear how the two pathways (NPF and sNPF) relate to modulate ab3A neurons' response. Even less clear is the potential role of NPF in honey bees, which will require further studies, not only at the level of the olfactory network, but more generally at multiple levels including the behavioural and the neurobiological one.

Olfactory information is further processed downstream the ALs in higher-order brain centres such as the mushroom bodies (MBs) and the lateral horn (LH). In *Drosophila* adults, NPF neurons respond to a variety of attractive odorants in both fed and starved individuals [185]. This activity level increases with hunger and food preference: the higher the odour-evoked NPF response, the greater the attraction to that odour [185]. Besides, strongly driving NPF neurons is sufficient to flip the valence of an odorant from aversive to attractive [185]. In *Drosophila* larvae, appetitive olfactory inputs are modulated by dopamine and NPF activities [57, 191]. Deficiencies in NPF signal disrupts dopamine-mediated higher-order olfactory processing, thus inhibiting appetitive odour-induced feeding [57]. The authors identified a small number of dopaminergic neurons expressing NPFR that are likely postsynaptic to the second-order olfactory neurons and project to the lateral horn region which mediate innate odour response [192]. NPF neurons also project to the lateral horn. Appetitive odour excitation of these dopaminergic olfactory neurons is gated by NPF via its receptor NPFR [57]. Taken together, these results show that NPF neurons are necessary for food odour-induced foraging [185] and also for food-odour stimulated feeding [57].

The absence of effect of sNPF on aversive responsiveness was intriguing as a clear modulation was found in the fruit fly [103]. This may reflect the peculiarity of the responsiveness to aversive, nociceptive stimuli in the honey bee, which are under the control of social cues such as alarm pheromones, which are absent in fruit flies. In the fruit fly larva [193], serotonergic signaling is necessary to inhibit the olfactory sub-circuits of the larval AL that mediate food attraction upon starvation. In the bee, the main defensive signal, the sting alarm pheromone, enhances serotonergic signaling, which in turn depresses responsiveness to aversive stimuli such as electric shocks [194]. One interpretation to this downregulation of aversive responsiveness is based on the colony need that bees defending the hive do not flee potential dangers and nociceptive stimuli but engage in defensive activities [195]. This hypothesis was supported by findings showing that responsiveness to electric shocks is downregulated by alarm pheromones, based also on an activation of the equivalent of an opioid system inducing analgesia. How sNPF may interact with serotonergic and opioid-like

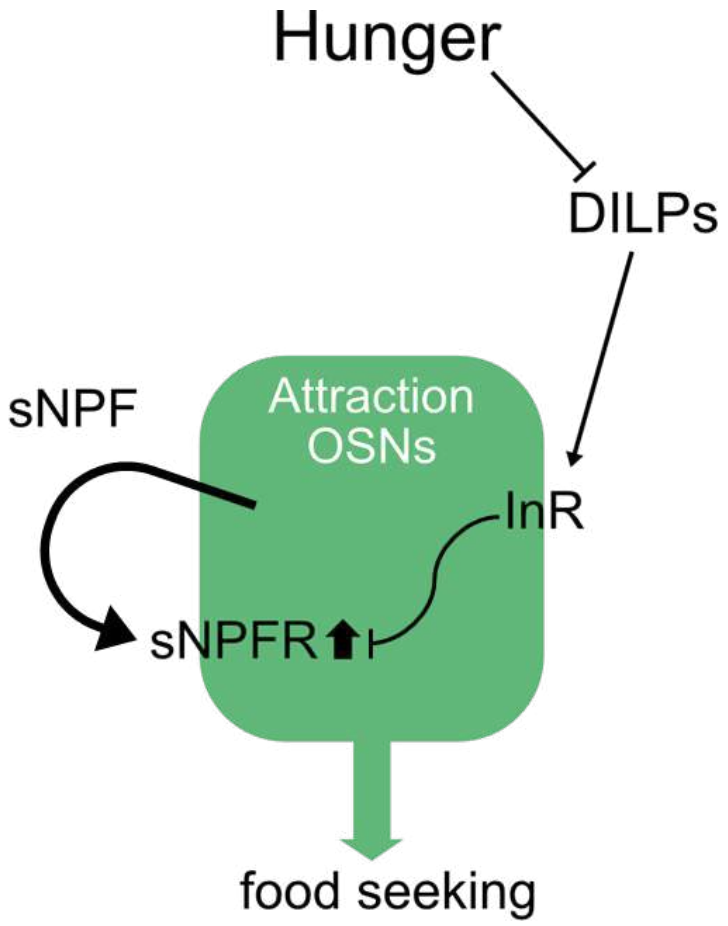

Figure 4.1: **Olfactory responsiveness to appetitive odorant in the adult *D. melanogaster*.** Starvation inhibits the release of DILPs (*Drosophila* Insulin-Like Peptides) binding to insulin receptor (InR), reducing insulin signalling in both attraction OSNs. The reduced insulin signalling leads to increased expression of sNPFR in the attraction OSNs. sNPFR receives sNPF secreted from the same OSNs, which in turn enhances their attraction to food odours. Adapted from Lin et al. 2019 [74].

signalling remains to be determined. A possibility to be explored is that the enhancing effect of sNPF is counteracted by the serotonergic and opioid-like systems to result in an absence of change in behavioural responses.

sNPF promotes the acquisition and formation of visual memories in honey bees (Chapter 2)

In this chapter we studied whether sNPF topical application enhances visual learning and memory of partially fed foragers. Bees were thus trained to discriminate colours within a miniature maze and we manipulated both their feedings state (i.e. comparing starved and partially fed bees with

a reduced appetitive motivation) and the levels of sNPF. We show that sNPF counteracted the negative effect of satiety on learning and memory formation and determined cognitive performances that were in some cases even better than those of starved animals. Our results thus identify sNPF as a critical component of motivational processes underlying learning and memory formation in bees and thus, of their foraging activities, which rely on these capacities [196].

As in the previous chapter we showed that sNPF enhanced both sucrose and olfactory responsiveness, it could be postulated that the enhancing effect of sNPF in these visual-learning experiments occurred at the level of sucrose and colour processing circuits. Visual processing engages a series of visual neuropils such as the lamina, medulla and lobula, and higher-order structures such as the central complex and the mushroom bodies. Studies determining whether the different neuronal populations of these brain structures express sNPFR are necessary to further elaborate on the effect of sNPF on visual circuits. In addition, behavioural tests using light sensitivity protocols could be done to determine if sNPF changes the perception of visual stimuli. For instance, it could be possible to combine topical application of sNPF with experiments on phototactic sensitivity in which bees exhibit phototactic attraction to lights of variable intensities. sNPF could enhance the sensitivity to lower light intensities in such an experiment.

In our experiments, the effect of sNPF was clearly visible mostly at the level of a memory test performed one hour after acquisition, thus showing that it required time to act on experience-dependent plasticity circuits. This need of time contrasts with the faster effect of sNPF on innate responsiveness (Chapter 1), for which changes in responsiveness were already visible ca. 30 min after topical application. This suggests that sNPF acts on the circuits engaged in memory formation and eventually interact with molecular pathways leading to memory formation. In the first case, Kenyon cells, the constitutive neurons of the mushroom bodies could be important targets of sNPF signalling. In the second case, the interaction between sNPF signalling and molecular actors such as phosphokinase A (PKA) could be of particular relevance. PKA has been shown to be elevated in foragers [92] and higher levels of PKA are necessary to induce long-term memory formation in honey bees [197]. If and how sNPF and PKA interact needs to be determined. In this work, we only studied the role of sNPF in honey bees at the individual level. Our results raise other questions related to the modulation exerted by sNPF on appetitive behaviours at the colony level. Ament et al. [234] showed that mRNA expression level of *snpfR* was upregulated in the brain of workers of food-deprived colonies compared to that of workers belonging to well-fed colonies. In addition, food-deprived colonies initiate foraging earlier than bees from well-fed colonies [234]. The authors postulate that *snpfR* expression may relate to nutrition. Here, we demonstrated that sNPF modulates visual learning and memory in honey bees, suggesting that this peptide also influences foraging activity which relies on cognitive abilities as bees are flower constant and such constancy depends on the capacity of bee foragers to learn and memorize the

sensory traits that characterize the flowers exploited [198, 199, 200].

Núñez and Giurfa [201] postulated that foraging must be considered as a motivational system. Motivation was defined in their view as 'reversible changes in the internal state of the animal that are related to changes in responsiveness to external stimuli' [202]. In this framework, the sensitive variable of this motivational state is the crop load of honey bee foragers. Bees do not fill their crop in conditions in which reward is particularly profitable (e.g. food sources that are close to the hive or food sources with a high sugar production rate); in this case, crop load is partial as bees prioritize a fast return to the hive to recruit other bees of the colony via their communication system to exploit collectively the profitable food sources. This social control of individual appetitive motivation could also be affected by sNPF. In particular, it would be interesting to determine if and how sNPF affects the levels of crop load attained by foragers and thus their decision to return faster or not to the colony. Our results showing that sNPF increases ingestion of partially fed bees to a level comparable to that of starved bees in lab conditions (Chapter 1) do not considered an important factor influencing crop load which is the flow rate delivering the nectar [201, 116]. A low flow is associated with low crop load and extended foraging time and *vice versa* [201]. In other social insects such as ants *Camponotus mus*, starvation increases crop filling and fluid-intake (flow) rate of foragers [203]. We could use artificial flowers measuring the flow rate of sucrose solutions in ingestion experiment to better assess the effect of sNPF on crop load and motivation.

Effect of sNPF on appetitive and aversive learning in honey bees (Chapter 3)

In this chapter, we extended our study of the effect of sNPF on learning and memory by focusing on appetitive olfactory learning and memory, evaluated via the olfactory conditioning of PER, and on aversive gustatory learning and memory, evaluated via the gustatory conditioning of SER. In this way, we aimed at determining whether the facilitatory effect on appetitive visual learning and memory found in Chapter 2 could be observed in another form of appetitive learning. The interest was promoted by the enhancing effect on gustatory and olfactory responsiveness and neuronal activity of the antennal lobe detected in Chapter 1. Using the well-established protocol of the absolute olfactory conditioning of the Proboscis Extension Response [148], a Pavlovian protocol in which bees learn the association between an odorant and sucrose reward, we studied the impact of sNPF and feeding state on acquisition and 24 h retention performances. Contrarily to previous results reported in fruit flies and *C. elegans* [106, 56], we were not able to demonstrate a clear facilitatory effect of sNPF on olfactory memory formation in an appetitive context. We found some partial tendencies supporting the presence of this effect but they failed to reach significance

and they were not present in all odor combinations assayed. These tendencies should be further explored by repeating these experiments and adjusting the protocol of drug administration to render it more efficient.

In the case of the aversive associative learning, we used a differential aversive conditioning protocol of the Sting Extension Reflex, in which antennal taste stimulation is paired with an electric shock [129]. This protocol thus allows studying the capacity of bees to learn and memorize the aversive association between taste and shock, and to discriminate tastes based on differential punishment (one taste is punished and the other not). It thus provides a valuable tool to study gustation, a sense that remains largely unexplored despite its relevance for foraging activities and social communications [252, 251]. Our prediction in this case was that no effect of sNPF should be visible in this case due to the negative results obtained when testing the effect of sNPF on electric-shock responsiveness (see Chapter 1). Indeed, no effect of sNPF was detected in the context of the gustatory SER conditioning but further replications would be needed as our experiments revealed some inconsistent trends. One of them refers to the fact that taste discrimination was better in one case than in the other. Indeed, bees elicited significantly more SER towards the punished 1 M sucrose compared to 3 M NaCl in the reverse contingency, although previous results with the same tastants 3 M NaCl and 1 M sucrose revealed that discrimination learning was symmetric [129].

The subesophagic zone (SEZ) (represented in fig. 4.2*b*) is the first central area of gustatory processing in the insect brain. In *D. melanogaster* central representations of tastants are segregated according their hedonic value. For instance, afferences of gustatory receptor neurons (GRNs) expressing a gustatory receptor tuned to sweet tastants, Gr5a, are lateral and anterior to afferences of GRNs expressing Gr66a tuned to bitter tastants [205]. No equivalent data exist for honey bees. Thus, caution is required when extrapolating findings in D. melanogaster taste encoding to honey bees. In addition, the life history traits of these two insect species differ considerably. Despite the lack of effect of sNPF on our gustatory conditioning protocol, it could be important to study if this peptide affects gustatory encoding at level of the SEZ, in a way similar to what it induces at the level of the AL. Experiments combining imaging o the SEZ (to determine how tastes are encoded therein) with local application of sNPF in this region may allow to determine in the future if taste processing at this level is modified by this neuropeptide.

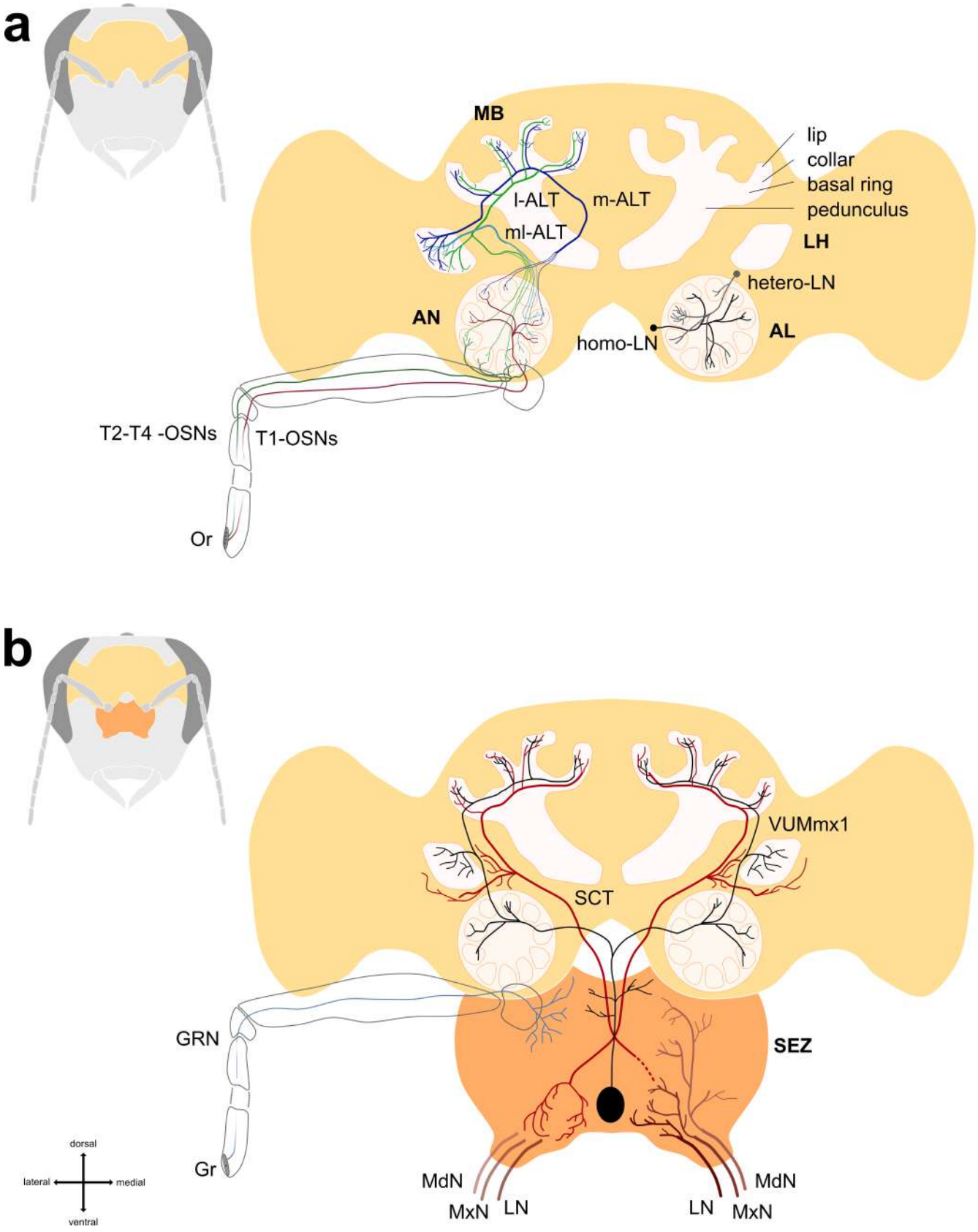
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Figure 4.2

Figure 4.2: **Olfactory and gustatory circuitry in the honey bee brain.** For clarity, different neuron types and neuronal tracts are labelled with different colours and presented in different hemispheres. Mechanosensory and motor neurons are not represented. Only one OSN and GRN have been represented. **(a)** Olfactory circuitry. The axons of the olfactory sensory neurons (OSNs) form the four antennal nerve (AN) tracts (T1 to T4) that innervate the first olfactory primary centre of the bee brain, the antennal lobe (AL). Each OSN innervates a single glomerulus and all OSNs bearing the same olfactory receptor converge onto one of the 163 glomeruli constituting the AL. Within the AL, the olfactory input is processed by homogenous and heterogeneous local interneurons (homo- and hetero-LNs), before being relayed to higher order processing centres by the AL output neurons, the projection neurons (PNs). A first group of PNs receive uniglomerular input (uniglomerular PNs) and leave the AL via the medial and lateral antennal lobe tracts (m- and l-ALT) projecting to the mushroom body (MB) and lateral horn (LH). A second group of PNs collects sensory information from multiple glomeruli (multiglomerular PNs), converges into three mediolateral ALTs (here compressed into one for clarity, ml-ALT), and conveys olfactory information to the LH only. Adapted from Paoli & Galizia, 2021 [188]. **(b)** Gustatory circuitry. The subeosophagial zone (SEZ) has been enlarged for clarity. The axons of the gustatory receptor neurons (GRNs) innervate the rostral part of the SEZ, the primary centre of gustatory processing in the bee brain. The SEZ is innervated by the mandibular, maxillary and labial nerves (MdN, MxN and LN, respectively) and results of the fusion of the mandibular, maxillary and labial neuromeres. The representation of the dendrites of the MdN, MxN and LN have been simplified for clarity. The VUMmx1 neuron body cells lie in the ventral part of the SEZ and its primary neurite innervates the ALs, the LHs and the MBs. Sensory projections from the proboscis are confined to the ventral portions of the maxillary and labial neuromeres of the SEG, overlapping with the arborizations of neurons of the subesophageal calycal tract (SCT). The SCT links the ventral SEG to the calyces of the mushroom bodies. Personal communication with Julie Carcaud.

Perspectives

Overall, this work highlights the singularity of the honey bee as a model in neuroscience research for which exploring the underlying mechanisms of food-decision making and motivation is challenging. The functional link identified between feeding behaviours and sNPF levels opens new perspectives to study ingestion processes and appetitive behaviours in bees. Many questions arose regarding the underlying mechanisms of sNPF effect on honey bees. Although genetic tools are not available in honey bees, molecular tools and pharmacological treatments have been developed and enable the study of mechanisms underlying behavioural expression. The following paragraphs suggest some ideas for a future research agenda.

RNAi knockdown of *snpfR*

In this work, we chose topical application to increase artificially sNPF levels and assess the impact of this manipulation on honey bees' behaviours. A missing approach in our work was performing the opposite manipulation, i.e. reducing sNPF signalling via RNAi knockdown of *snpfR*. This approach is in principle feasible, but our attempts in this direction were not successful. Further work is required to elaborate an efficient RNAi strategy against *snpfR*. For instance, this technique significantly reduced food intake of adults pea aphid (*Acyrthosiphon pisum*) [66] and desert locust (*Schistocerca gregaria*) [78]. In this way, by enhancing and suppressing artificially sNPF signalling, the necessity and sufficiency of this neuropeptide for the behaviours of interest could be assessed. We expect starved bees to behave as partially fed foragers with a decrease of food intake, gustatory and olfactory responsiveness.

Quantitative analysis of *npf*, *snpf* and *snpfR* gene expression

To understand the role of sNPF, we should also investigate how sNPF-related genes are regulated in the honey bee following topical application. Although we performed qRT-PCR on *npf*, *snpf* and *snpfR* in forager's abdomen after topication, comparison of mRNA expression levels between treatments and over time did not provide convincing results (see Appendix A). Therefore, I suggest to revise some aspects of the protocol described in Appendix A to proceed new quantitative analyses in the abdomen and the head of foragers. For instance, we should capture bees coming from hives having the same quantity of brood as it might impact sNPFR signalling. This relationship has already been demonstrated in a social insect, the fire ants (*Solenopsis invicta*), where a decrease in sNPFR immunoreactivity in worker's brain was observed in colony without brood compared to those with brood [244]. These qRT-PCR analyses could be conducted after injection of RNAi knocking down sNPF or sNPFR. In addition, we could analyse whether sNPF is expressed in the crop. If this is the case, comparing mRNA expression level of sNPF between partially fed and starved foragers would provide new insight about a potential role as nutrient sensor for this peptide. These results could thus be correlated with the work of Pankiw et al. [117] which shows that crop volume affects sucrose response thresholds directly and independently of sucrose concentration.

Other neuropeptides modulating the feeding behaviour of honey bees

Most studies investigating the neuronal mechanisms regulating the feeding behaviours of insects have mainly focused on NPF/sNPF signalling. However, food intake involves the coordinated action of numerous neuropeptides and hormones that are expressed in the central nervous system as well as in the endocrine cells of the gut [8]. In the case of honey bees, novel findings have been recently reported that indicate the important and diversified role of other neuropeptides in the modulation of food-related behaviours. The following paragraphs focus on three neuropeptides recently studied in this context in bees, even if their action had been studied earlier in other insects.

Leucokinin

This neuropeptide has been thought to be a potential homolog of the mammalian tachykinin due to initial phylogenetic analyses with a low resolution [207, 8]. However, more recent analyses suggest that leucokinin and tachykinin have an independent origin [50]. In *Drosophila*, leucokinin receptor neurons are expressed in the brain, the ventral ganglia and innervate the foregut [208]. Leucokinin may signal the crop volume in order to terminate meal ingestion [208]. Accordingly, mutation in the peptide and receptor genes of adult flies provokes an increase in meal size and a compensatory reduction of meal frequency. The authors suggested that this effect may arise from impaired communication of gut distension signals to the brain [208]. Another study showed that silencing leucokinin receptors in insulin-producing-cells leads mutant flies to display an altered expression of insulin-like peptides and increased resistance to starvation [209], suggesting that leucokinin signalling occurs via insulin like peptides [210, 209]. In addition, serotonin receptors expressed in leucokinin neurons diminish the activity of the latter and modulate thereby functions regulated by leucokinin [211]. Leucokinin also modulates taste detection in fruit flies as shown by experiments in which leucokinin neurons are inhibited through targeted expression of inward rectifier K(+) channels. In this case, the preference for trehalose decreased aversive responses to bitter tastants was enhanced for some but not all bitter substances (López-Arias et al., 2011).

In females *A. aegypti*, a leucokinin receptors are expressed within taste sensilla on the legs and mouthparts [212]. Kwon et al showed that a kinin analog engineered to be peptidase resistant binds to leucokinin receptors and inhibits thereby sucrose taste detection directly at the level of the taste organs, eliciting a fast and highly aversive response in females upon labellar or tarsal contacts with a sucrose source [212]. Alternately, when the leucokinin receptors were silenced via RNAi, the feeding-aversion behavior resulting from contact with the kinin analog disappeared,

thus showing the direct involvement of leucokinin signalling in food aversion.

In Hymenoptera, the leucokinin gene structure varies among species. It is weakly expressed in honey bee workers [213] and is totally absent in ants [214] and in *N. vitripennis* [215]. In the Asian honey bee *Apis cerana*, the relative expression of the leucokinin receptor gene was higher in both antennae and brain compared to the thorax and gut [216]; in addition, antennal and brain levels were higher in foragers than in nurses, suggesting a relationship with division of labor [216]. RNAi knockdown of leucokinin receptor induced a significant increase in the sucrose response threshold when sucrose solution stimulated the bees' antennae, thus showing that signalling through the leucokinin receptor regulates sucrose sensitivity [216]. Consistently, nectar foragers showed significantly lower leucokinin receptor expression than pollen foragers, which supports the role of the leucokinin receptor in foraging division [216]. These results suggest that leucokinin and sNPF are antagonist in honey bees.

Tachykinins

As NPF/sNPF, tachykinin is also a member of the structurally related RF-amide peptide family referred as tachykinin-related peptides (TRPs) [217]. They have been studied in several invertebrate species such as the crab *Cancer borealis* [218], *C. elegans* [219], *B. mori* [220], *L. migratoria* or the cockroach *Leucophaea maderae* [221] and seem to act as a nutrient sensor. TRPs have been identified in the brain and the endocrine cells in the midgut of the fruit fly [222]; they are released upon starvation to regulate contractions of the foregut via myoactivity, food intake and processing in several insect species [223]. For instance, injection of TRPs in *Bombyx* larvae shorten the latency to the first bite following the treatment, suggesting a stimulatory effect of food intake [224]. A different effect was found in *Drosophila* where a neural circuit responsible for controlling fructose sensing and involving tachykinin signalling has been recently characterized [225]. The circuit is active when hemolymph glucose is high; suppression of activity in this circuit, either by starvation or by genetic silencing of its neural components, promotes fructose ingestion when glycemia is high. This effect is achieved via tachykinin signalling, which acts on Gr43a brain neurons, which are central sensors of internal fructose. Under satiated conditions, tachykinin inhibits Gr43a brain neurons, preventing them from responding to internal fructose, and thereby preventing feeding promotion by fructose ingestion [225].

In honey bees, TRPs are predominantly expressed in the mushroom bodies and in some neurons of the antennal and optic lobes [226] and some expression has been found in the antennae [227]. A functional analysis of the consequences of tachykinin on honey bee behaviour was performed recently in two honey bee species (*Apis mellifera* and *Apis cerana*) in which appetitive responses (PER) of nurses, pollen foragers and nectar foragers to brood, pollen and sucrose contacting

their antennae were quantified upon TRPs injection and RNAi-mediated knockdown of the *TRP* and its receptor (*TRPR*) [228]. TRPs signalling regulated response thresholds to these three types of stimuli in a task-specific manner: while injection decreased task-specific response, RNAi knockdown increased the same specific response [228]. For instance, injection of the tachykinin related peptide TRP2 decreased sucrose responsiveness in nectar and pollen foragers but not in nurse bees. On the contrary, the same injection decreased PER to antennal larval stimulation in nurse bees but neither in pollen nor in nectar foragers. RNAi-mediated knockdown of tachykinin-related peptide (TRP) and its receptor (TRPR) had the opposite effect: it increased sucrose responsiveness in nectar and pollen foragers but not in nurse bees, and responsiveness to larval stimulation in nurse bees but neither in pollen nor in nectar foragers [228]. Thus, compared to sNPF signalling, TRP signalling seems to act in an opposite way, inhibiting rather than promoting appetitive responses. Compared to the knowledge gathered recently on TRP signalling [228], we require further experiments to assess the effect of sNPF on pollen and brood responsiveness. Conversely, studies on TRP signalling should determine the effect of this neuropeptide on food intake and aversive responsiveness in honey bees.

Crustacean cardioactive peptide (CCAP)

The CCAP gene is expressed in endocrine cells of the midgut and in the central nervous system of the cockroach *P. americana* and has been identified as a gut factor [229, 230]. It is secreted by identical cells to that of sNPF and the two peptides affect each other in an antagonist way, creating autocrine negative feedback loop to regulate response to food accessibility [229]. Thus, starvation increases the number of sNPF-immunoreactive cells and decreases the CCAP-immunoreactive cells in the subesophagic zone (SEZ), whereas refeeding reverses these effects. In addition to their role in the digestive mechanisms, these peptides also regulate locomotion behaviour in the cockroach; while CCAP injection suppresses locomotor activity in starved cockroaches, sNPF activated in satiated ones [229].

In *Drosophila* larvae, the CCAP has been well studied in the regulation of ecdysis [231, 232], which corresponds to a stereotyped sequence of behaviours used to shed the remains of the old cuticle. A recent study has addressed the role of CCAP in food intake in the adult fruit fly [233] and identified 2 CCAP-expressing neurons that regulate feeding behavior and metabolism. This regulation is exerted via a tight association with NPF signalling. Indeed, loss of CCAP or knocking down the CCAP receptor (CCAP-R) in two dorsal median neurons, inhibited the release of NPF [233]. As a consequence, starved flies, which have normally increased sugar sensitivity, measurable in a PER assay, exhibited a reduced sugar responsiveness upon loss of CCAP, or CCAP-R in two dorsal median NPF neurons. This inhibitory effect was also observed in fed flies,

which also decreased their sucrose responsiveness [233]. CCAP signalling was shown to trigger PER whereas NPF neurons were necessary but not sufficient [233]. In addition, mutant flies lacking CCAP or CCAP-R in NPF neurons could not differentiate between nutritional and non-nutritional sugars. Taken together, these results show that CCAP is a key peptide in the regulation of feeding behaviour via NPF signalling and also regulate metabolism [233].

CCAP is also expressed in the honey bee genome but no study has yet investigated its function neither in bees nor in any social insect. It would be interesting to determine whether there is a positive relationship between CCAP and sNPF signalling, similarly to the one existing between CCA and NPF in fruit flies, which could be determined via a sucrose responsiveness assay.

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Appendices

Appendix A

qRT-PCR analysis of *npf*, *snpf* and *snpfR* in honey bee's abdomen

Alice Rouzes, Louise Bestea, Rodrigo Velarde, Martin Giurfa and Maria Gabriela de Brito Sanchez

This appendix presents preliminary results

An integrative approach was required to further explore the relationship observed between sNPF and appetitive behaviours in forager honey bees (see Chapters 1 and 2). A single topical application of sNPF was sufficient to induce an increase of sucrose and olfactory responsiveness in fed individuals. In addition, the same treatment enhanced the neuronal activity of antennal lobes, the primary olfactory centre in the bee's brain and promoted visual memory formation. Yet, mechanisms underlying NPY-signalling in honey bees have been solely addressed [234], thus are still poorly understood. We measured the expression of NPY-related genes (*npf*, *snpf* and *snpfR*) in forager's abdomens using qRT-PCR to investigate the effect of sNPF topical application through different times at the molecular scale. We do not expect an increase of mRNA expression level of *snpf* but we wonder whether mRNA expression level of the receptor *snpfR* is influenced by sNPF topical application. The preliminary obtained results do not allow any conclusion and require a second experiment with additional controls.

Material and methods

Insects

Honey bee foragers from colonies located in the apiary of the Research Centre on Animal Cognition (Toulouse, France) were collected in the morning in September 2020 at an artificial feeder they were previously trained to visit. Since sNPF brain levels can vary depending on crop filling, as other neuropeptides [235], foragers were caught upon landing on the feeder, just before they started feeding, and placed individually into syringes with an open hub to allow for respiration. To overcome a potential daytime effect, the capture phase was repeated over four days.

Behaviour experiment and pharmacological treatments

Bees were then divided into five groups. One group was kept deprived of food ('starved'). The other four groups were fed by fitting within the open hub of the syringe an Eppendorf tip so that the enclosed bee could feed from it. Bees were presented with a first tip containing 5 µl of a mixture of honey, pollen, sucrose and water, and then with a second tip containing 15 µl of a 1.5 M sucrose solution in which case the feeding state was labelled as partially fed (henceforth P-fed). Feeding lasted between 15 and 30 min, depending on the number of bees assigned to an experiment. After feeding, the syringes with all the bees were placed in ice during 5 min. This allowed to take out the cold-narcotized bees and proceed to the topical-application phase. One of the fed groups was left untreated ('P-fed'). Two other fed groups received a topical application [236] of 1 µl of sNPF

solution on the thorax. One group received sNPF at a concentration of 1 µg/µl ('P-fed sNPF1') and the other at a concentration of 10 µg/µl ('P-fed sNPF10'). The fourth P-fed group received a topical application of the solvent used to dissolve sNPF ('P-fed Solvent'). The solvent was a mixture of 20 % dimethyl sulfoxide (DMSO) and 80 % acetone (DMSO/Acetone). Starved bees were also chilled on ice as control. As sNPF is supposed to enhance appetitive responsiveness, it was not delivered to starved bees, which were already at a ceiling level regarding appetitive responsiveness. Starved bees constituted therefore a control for the physiological effects of sNPF and allowed establishing whether sNPF treatment turned fed bees into starved-like animals. Each bee was replaced within its individual syringe after topical treatment where it recovered from the cold treatment. Experiments started between 20 and 30 min after the first topical application. Sucrose and solvents were purchased from Sigma-Aldrich (Steinheim, Germany) while honey bee sNPF was purchased from NovoPro (Shanghai, China; sequence: SDPHLSILSKPMSAIPSYKFDD [237]).

Bees of each treatment were then flash-frozen in a nitrogen solution 30 min, 1 h or 2 h (Sacrifice schedule) after recovering from being chilled on ice. Each abdomen was dissected on dry ice and samples were stored at -80 °C until RNA extraction.

Gene expression

RNA extraction

Each abdomen was homogenized in 900 l of Qiazol® Lysis Reagent (Qiagen®, Courtaboeuf, France) containing a 5 mm stainless steel bead using TissueLyser® (Qiagen®) (3*30 s at 30 Hz). The phenol and guanidine lysis reagent is optimized for the lysis of fatty tissues found in the brain and abdomen. Then, homogenates were incubated for 5 min at room temperature in order to dissociate the nucleoprotein complexes. To allow effective reduction of contamination by genomic DNA, 100 µl of gDNA Eliminator Solution and 180 µl of Chloroform (Qiagen®) were added to the solution before incubation for 3 min at room temperature and centrifugation (14 000 g for 15 min at 4°C). 550 l of the aqueous phase containing the total RNA was transferred to a new tube and used for RNA extraction. RNA extraction was carried out as indicated in the RNeasy® Plus Universal Mini kit (Qiagen®). Total RNA was eluted in 100 µl of nuclease free water. The RNA concentration and purity were measured with a Nanodrop One spectrophotometer (ThermoScientific®, Illkirch, France). All samples had very low contamination (OD 260/280 $>$ 2.0). The extracted RNA was stored at 80°C until use.

Reverse Transcriptase

Reverse transcription (RT) allows the obtain of a complementary DNA strand (cDNA) from a messenger RNA (mRNA) strand within a single amplification cycle. cDNA synthesis was performed using the High capacity RNA to cDNATM kit (Applied Biosystems™, Courtaboeuf, France) following the manufacturer's instructions in order to obtain cDNA. A quantity of 1 g of RNA in a final volume of 20 l is used for each RT reaction: the mix, consisting of an incompressible volume of 10 l of 2X Buffer Mix (consisting of dNTPs, random octamers and oligo (dT) 16) and 1 l of 20X RT Enzyme Mix (MultiScribeTM Reverse Transcriptase, 50 U/l), supplemented with Nuclease-free H2O. The RT reaction proceeded according to the following schedule: an incubation step at 37°C for 60 min, followed by 5 min at 95°C. In order to avoid inhibition of the qPCR reaction by the constituents of the RT, these cDNAs are diluted ten-fold in Nuclease-free water. The resulting cDNA was stored at -80 °C until use.

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

The expression of *npf*, *snpf* and *snpfR* mRNA level was determined using the SYBR Green Method including ROX passive reference dye, according to the protocol described in [238]. Quantitative real-time PCR were carried out in Hard-Shell® 384-Well PCR Plates cover with Microseal® 'B' PCR plate sealing Film (BIORAD®, Marnes la Coquette, France). Each reaction contained 3 µl of ten-fold diluted cDNA, 5 µl of Power SYBR® Green PCR Master Mix (Applied Biosystems™) and 1 µl of forward and reverse primers (10 µmol) (Sigma Aldrich, "Saint Quentin Fallavier, France) of selected genes. Amplification was performed using the following program: 10 min à 95°C followed by 40 cycles of 15 s at 95°C, 30 s at 60°C and 20 s at 72°C followed by a melt curve from 60 to 95°C with 0.5°C/5 s. The reaction was performed in the Biorad CFX thermocycler (BIORAD®) and analysed with the software Bio-Rad CFX Manager. Three technical replicates were performed for each biological replicate. If the triplicates showed too much variability (SD $>$ 0.3), the furthest triplicate was discarded.

The reference and target genes were selected, based on previous studies with some modifications [239, 240, 241] (fig. A.2). Four reference genes: *RP49*, *RPS18*, *GAPDH* and *actin* were selected in this experiment because they show a great stability in abdomens of forager bees [240, 241]. To ensure similar properties for each primer and amplicon of reference and target genes, the primers were accurately designed to amplify between 103-174 bp regions of each gene with primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) based on the sequence information of selected genes obtained from the NCBI database (https://www.ncbi.nlm.nih.gov/).

The efficiencies of all the primers used were between 90-120 % (fig. A.2). The selectivity was

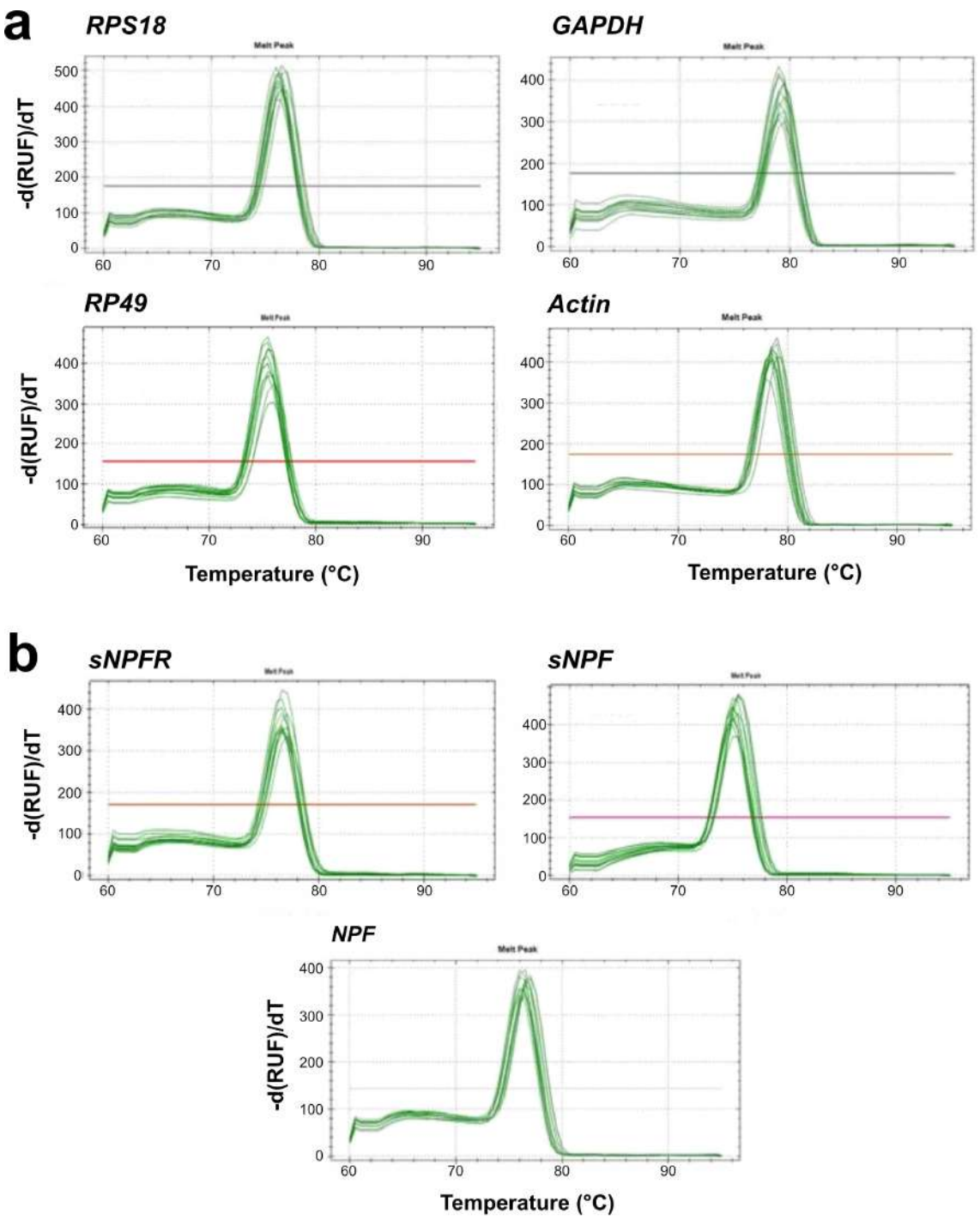

Figure A.1: **Validation selectivity of gene-specific primers.** Melting peaks of qRT-PCR. **(a)** Reference genes. **(b)** Target genes.

Target	Primer sequence 5' ➢3'	mplicon length (bp)	Efficiency (%)	
snpfR	ACG ACA GCC AGT AAC GTG TC (forward) CCT CCA GGG AAA GTA CAG CTA (reverse)	107	103.0	0.996
snpf	ATC GAC GGA TAC CCT TGA CTT (forward) TTC AGT ACC AAC AAC GAA GCC (reverse)	174	120.3	0.993
npf	ACG ATG AAA ACA GTG GTG GAG (forward) CAT AGG TTT CGT GCT CAC CC (reverse)	103	112.1	0.995
RPS18	GCA GTT TTG CAA GAT GTC TCT C (forward) CAT AAC GAC GAC CAA CAC CT (reverse)	134	103.7	0.996
RP49	AAA GAG AAA CTG GCG TAA ACC(forward) CAG TTG GCA ACA TAT GAC GAG (reverse)	125	94.7	0.993
GAPDH	CCC TTC AAA TGA CAC ACT GCT(forward) ATT AAT GGC AAC AAC CTG AGC (reverse)	149	111.1	0.992
actin	TGC CAA CAC TGT CCT TTC TG(forward) AGA ATT GAC CCA CCA ATC CA (reverse)	156	98.9	0.998

Figure A.2: **Description, sequence information, amplicon length, efficiency and correlation coefficient (R^2) of the genes.**

verified by analysing melting curves of the qRT-PCR products (fig. A.1). The stability of reference gene was verified by using the qPCR analysis software who calculated two quality parameters: Coefficient of Variation (CV) of the normalized relative amounts of the reference genes and a M value. A CV < 0.25 and a M value < 0.5 must be respected for the use of two genes as reference gene. In our analysis, normalization of the target genes with the combination of RPS18 and RP49, yields a CV $= 0.0165$ and an M $= 0.0477$. Thus, for the following results, the combination of RPS18 and RP49 is used for the normalization of the target genes. The relative mRNA expression levels were calculated after normalization with the geometric mean of RP49 and RPS18 by using the CT method.

Data analysis

All statistical analyses were done either with R software [242]. Statistical differences in gene expression of *snpfR* and *snpf* were analysed with a two-factor (Treatment $\times$ Sacrifice schedule) Anova (package R *car*) followed by a Tukey HSD *post-hoc* test. The *npf* gene expression was analysed with a Kruskal-Wallis rank test followed by multiple pairwise Wilcoxon comparisons (Bonferroni p-value adjustment method).

Results

Treatments and sacrifice schedules had a significant effect on *npf* mRNA expression level (fig. A.3*a*: W = 31.37, d.f. = 14, $p = 4.92 \times 10^{-3}$) but the only relevant and significant difference was between P-fed sNPF 10 and P-fed solvent bees sacrificed at 2 h after the topical application (fig. A.3*a*, $p = 0.02$). *snpf* mRNA expression level varied significantly (fig. A.3*b*: ANOVA, $F_{14,164} = 2.55$, $p = 2.49 \times 10^{-3}$) within P-fed sNPF 10 bees where a downregulation was observed between bees sacrificed at 30 min and 2 h after topical application ($p = 0.01$). *snpfR* mRNA expression level were affected by treatments and sacrifice schedule (fig. A.3*c*: ANOVA, $F_{14,164} = 2.43$, $p = 3.98 \times 10^{-3}$). This result was driven by starved bees which had higher *snpfR* mRNA expression level at 2 h than P-fed solvent bees (fig. A.3*c*, $p = 0.03$), P-fed sNPF 1 bees (fig. A.3*c*, $p = 0.04$) and P-fed sNPF 10 bees (fig. A.3*c*, $p < 0.01$). The remaining comparisons did not show any significant difference.

Discussion

Overall, we obtained miwed results on genes expression in forager's abdomens which not provide relevant data to understand underlying mechanisms of NPY-signalling of honey bees in our experiments. We would have expected consistency with the differences between appetitive behavioural experiments already observed and RT-qPCR, that is to say, at least no difference between P-fed and P-fed solvent groups and potentially differences between starved bees and P-fed/P-fed solvent groups. Consistently with previous works [234, 243], *npf* was expressed in the abdomen of bees. We did not separate the different tissues of the abdomen during RNA extraction but evidence shows that npf and snpf are expressed in the midgut of honey bees [243].

To further investigate the mechanisms underlying sNPF role in appetitive behaviours of honey bees, this experiment should be conducted again with foragers from a few selected hives to reduce variability that may be more visible in molecular biology analysis than in behavioural experiments. In our case, bees were not tagged, thus identification and affiliation to colony were impossible to determine. In addition, we should capture bees coming from hives having the same quantity of brood as it might impact sNPFR signalling. This relationship has already been demonstrated in a social insect, the fire ant (*Solenopsis invicta*), where a decrease in sNPFR immunoreactivity in worker's brain was observed in colony without brood compared to those with brood [244].

In honey bees, queens start to produce winter bees before the end august [245]. In september we might have caught forager bees in transition to winter physiology [246, 247]. Winter bees exhibit

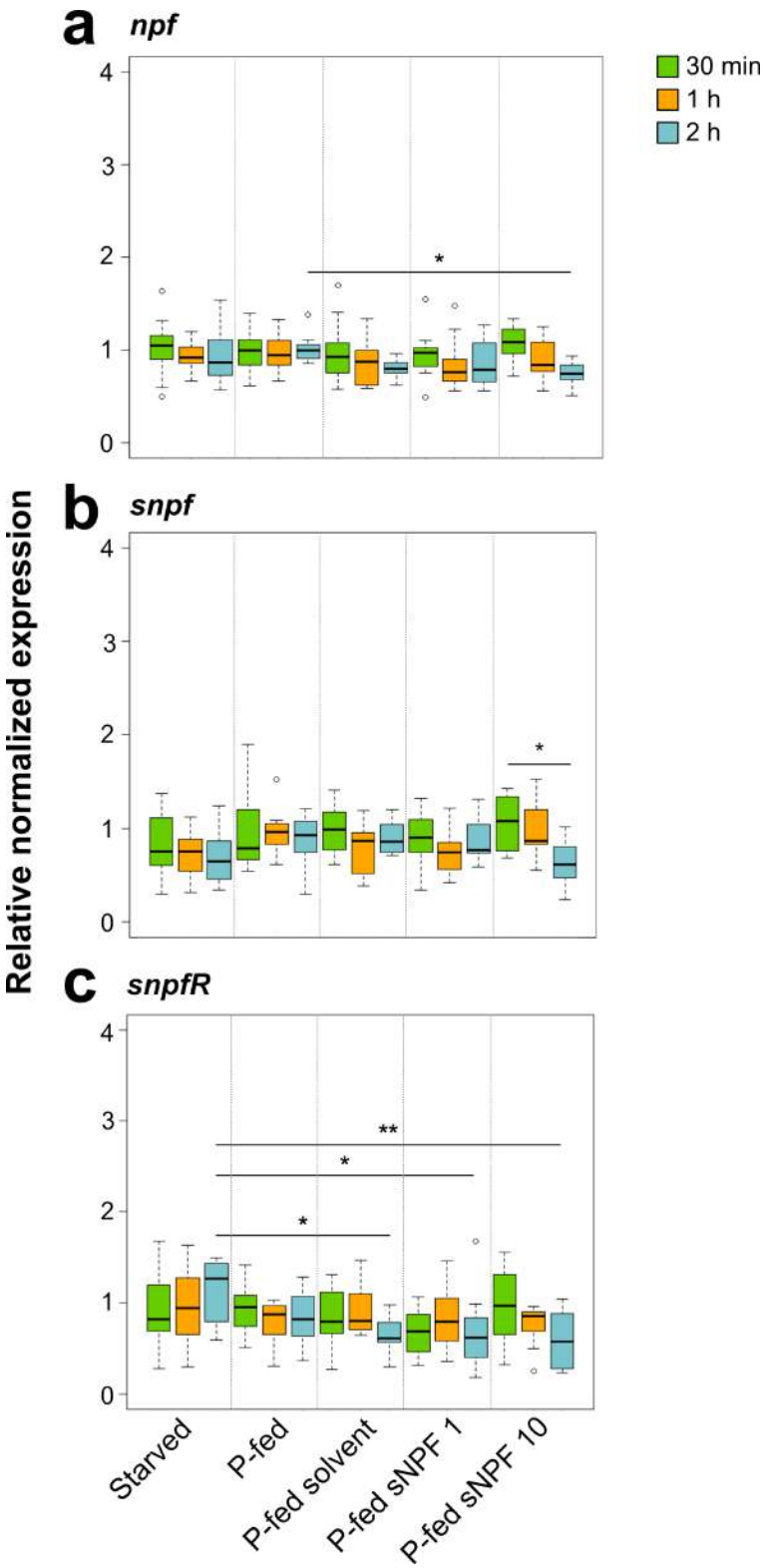

Figure A.3: **mRNA level expression of *npf*, *snpf* and *snpfR* in forager's abdomens at different times.**

fat storage comparable to that of nurses in summer [245, 247], which renders them less sensitive to starvation [248]. Therefore, to avoid such confusing effect we should conduct this experiment in spring instead of late summer.

Our results are yet too preliminary to draw any conclusion. As previously reported [234], the observed patterns are not as robust as for already studied such neuromodulatory pathways. By conducted a second experiment in spring with better control of the provenance of foragers we might avoid mixed results. In addition, we should perform qTR-PCR of forager's brain following the same procedure to assess the effect of sNPF topical application at the central level.

Appendix B

Nutritional state influences the activity of sweet-sensing gustatory receptor neurons

Louise Bestea and Maria Gabriela de Brito Sanchez

This appendix presents preliminary electrophysiological recordings.

Sweet taste perception is dynamically regulated according to the internal nutritional state in honey bees (see Chapter 1) but underlying mechanisms are still unclear, although studies in *D. melanogaster* have been investigated what molecular systems control taste acuity within the taste machinery itself [249, 250]. The modulation of sweet taste sensitivity in honey bees is poorly understood at the level of tastant detection in sweet-responsive GRNs.

Electrophysiological recordings were performed to investigate whether nutritional state modulate the gustatory receptor neurons (GRNs) activity in antennal taste *sensilla* when stimulated with sucrose solutions. These taste *sensilla* are mostly tunes to detect sugars and salts [251, 252].

Material and methods

Insects

Forager bees were caught at the experimental apiary and individually enclosed in syringe. They were partially fed (5 µl of honey + 15 µl of sucrose 50 %) ('P-fed') or were kept deprived of food ('starved'). After being chilled on ice until they stopped moving, bees were mounted individually in Eppendorf tubes (Dutscher, France). The antennal flagellum was immobilized with a metal thread stuck with wax and a glass electrode with an external diameter of 10–20 µm was placed over a single taste *sensillum*. A silver wire inserted into the contralateral eye was used as a grounded reference electrode. Electrodes were pulled from borosilicate glass capillaries, filled with different solutions.

Recording sites

Electrophysiological recordings were performed on taste *sensilla* (chaetica *sensilla*; see [253]) which can be easily identified by their external morphology [254]. Taste *sensilla* were localized in the ventral zone of the antennal tip [255], which is devoid of olfactory *sensilla* [253]). Only sensilla tuned to sucrose (Type II *sensilla*) were selected for recordings.

Stimuli and single sensillum recordings

The gustatory stimuli employed were KCl and sucrose (purchased from Sigma–Aldrich). All sucrose solutions were prepared as dilutions in 1 mM KCl, which ensures the necessary conductivity for

recordings, and were kept at 4°C. Taste *sensilla* were stimulated in the following order: 1 mM KCl (used as the reference); 1, 10, 30 and 100 mM sucrose. Taste *sensilla* were stimulated three times with each solution for 2 s with an interstimulus interval of 1 minute. The recording and reference electrodes were connected to a preamplifier (TasteProbe - Synthech, Kirchzarten, Germany). The electric signals were amplified (× 10) using a signal connection interface box (Syntech, Kirchzarten, Germany) in conjunction with a 100-3000Hz band passfilter. Experiments started when the recording electrode contacted the *sensillum* under study, which triggered data acquisition and storage on a hard disk (sampling rate: 10kHz). Data were analysed using Autospike (Syntech) and quantified by sorting and counting the number of spikes after stimulus onset.

Data analysis

Data analysis were performed using R software [242]. Electrophysiological data were analysed by comparing frequencies of recorded spikes using negative binomial general linear mixed model (GLMM) (*glmer.nb* function of the *lme4* package [256]). Treatment was entered as fixed effect while bee identity was entered as random effect to take into account the repeated measurements per individual. ANOVA (*car* package) was performed on GLMM and followed by post hoc multiple comparisons (Tukey p-value adjustment method, *emmeans* package).

Results and discussion

Nutritional state had a significant effect on the activity of GRNs (fig. B.1*b*, GLMM: $\chi^2 = 10.91$, d.f. $= 1$, $p = 9.55 \times 10^{-4}$). The number of spikes recorded was higher in GRNs of starved bees than of P-fed bees only when stimulated with 30 and 100 mM sucrose solutions (fig. B.1*b*, 30 mM sucrose: $p = 3.33 \times 10^{-5}$; 100 mM sucrose: $p = 0.03$), which correspond to the lowest sucrose concentrations used for sucrose responsiveness assay (0.01 % and 0.03 % respectively) (see Chapter 1, **??**).

These results are consistent with the increase of sucrose responsiveness observed in starved bees compared to their P-fed counterpaces (see Chapter 1) although the behavioral differences were observed for higher pure sucrose concentrations (10 and 30 %). These results suggest that the modulation of sucrose responsiveness may come from a top-down modulation of the activity of GRNs. In other words, the nutritional state could influence the sensitivity of GRNs tuned to sweet tastants to cause a compensatory selective feeding. In the migratory locust (*Locusta migratoria*), the nutritional states modulates the responsiveness of GRs on the mouthparts [257] and of GRNs that may act as nutrient sensors [258] which in turn quickly adjust sensory perceptions to meet

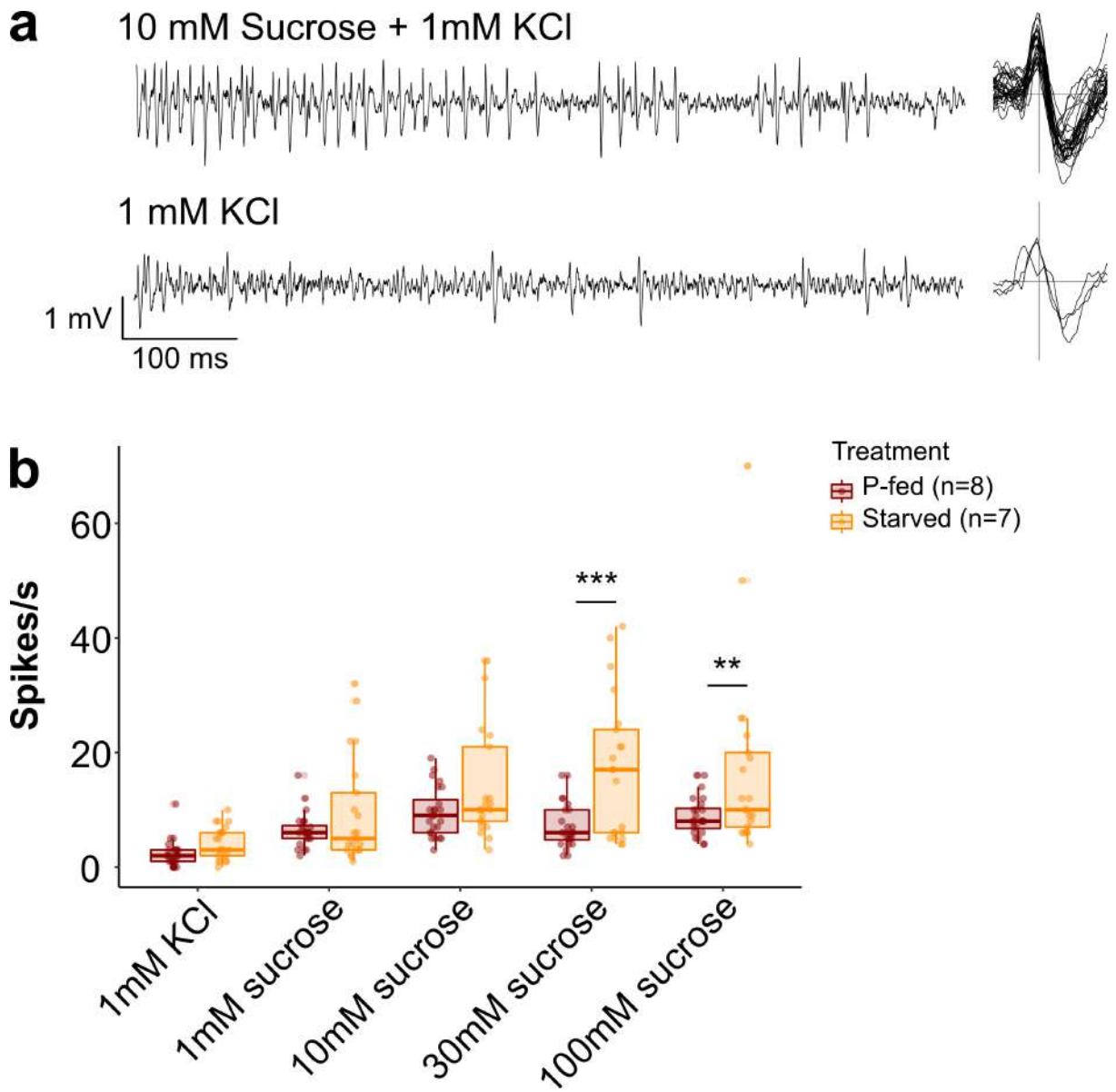

Figure B.1: **Electrophysiological recording of the gustatory receptor neurons from the antennae. (a)** *Left* - Examples of spike trains recorded from a taste *sensilla* (Type II) in response to 10 mM sucrose + 1 mM KCl and 1 mM KCl solutions. The smallest spikes to 10 mM sucrose + 1 mM KCl corresponds to the responses to 1 mM KCl. Note the decrease of spike frequency without the sucrose in the tested solution. *Right* – Action potentials of the recording. **(b)** Boxplots of the spiking responses to sucrose and KCl of P-fed (red) and starved (orange) bees for a type II sensilla. Each boxplot extends from the 25^{th} to the 75^{th} percentiles; the line in the middle of the box shows the median. $^{**}p<0.01$,$^{***}p<0.001$.

hunger needs. In *D. melanogaster* independent neuromodulatory cascades, in which NPF and sNPF are involved, control oppositely sweet and bitter peripheral taste sensitivity [249]. sNPF enhances sucrose responsiveness of P-fed foragers (see Chapter 1) but the underlying mechanisms remain unknown. In honey bees, there is no clear evidence of a dedicated bitter-sensing GRN, but bitter compounds mixed with sucrose were shown to inhibit the response of sweet-sensing GRNs to the sucrose [252, 251]. However, the absence of effect in gustatory responsiveness towards sucrose solutions spiked with salicin may indicate that rather than acting peripherally on sweet-sensing GRNs, sNPF could modulate central gustatory processing.

Electrophysiological recordings of sweet-sensing (GRNs) should be conducted upon sNPF treatment to unravel whether this peptide exerts a top-down modulation of taste peripheral responses or modulates central taste processing.

Appendix C

F1000Prime Recommendation of Wada-Katsumata A et al., Changes in taste neurons support the emergence of an adaptive behavior in cockroaches, 2013 Science, 340(6135):972-975

Martin Giurfa and Louise Bestea

DOI: 10.3410/f.718012945.793558385

In insects, tastes are detected by gustatory receptor neurons (GRNs) located in cuticular hairs distributed all along the body surface or located in specific gustatory appendages such as the mouth parts, the antennae, or the tarsi (last segments) of the legs. In this work, the authors used extensive electrophysiological recordings performed at the level of these cuticular hairs (called *sensilla*) to characterize the gustatory sensitivity of the GRNs located therein. The technique used is the so-called single-*sensillum* recording, which allows the extracellular recording of the neurons contained in the cavity of a gustatory *sensillum*. By means of meticulous analyses, the authors characterized the GRNs of cockroaches (*Blattela germanica*) and addressed the question of why some cockroach populations became resistant to bait traps containing pesticides. A key point of the analysis is the fact that these traps use glucose as attractant and that some populations of cockroaches seem to have developed an aversion to this tastant.

The authors showed that each glucose-sensitive *sensillum* expresses four distinct gustatory receptor neurons (GRN1-4) with different taste specialization (sweet, bitter, salt and water). Using a panel of 11 tastants, they showed that phagostimulants (e.g. sweet substances such as glucose) stimulate GNR1 but not GNR2 while deterrents (e.g. bitter substances such as caffeine) stimulate GNR2 but not GNR1 in the wild-type (WT) population of cockroaches. However, in cockroaches, which developed aversion to glucose (GA cockroaches), glucose and other related sweet compounds did not stimulate GRN1 but GNR2, the "deterrent" gustatory neuron, and in a way similar to caffeine.

The authors also established dose-response behavioral curves with (wild-type) WT and (glucose-averse) GA cockroaches that were hungry, or hungry and thirsty. They found — as expected — that hungry WT cockroaches increased their acceptance of the attractant glucose when its concentration increased. Yet, GA cockroaches showed the opposite behavior and rejected it when the glucose concentration increased, thus mimicking the response obtained when confronting both types of cockroaches to increasing concentrations of caffeine. Interestingly, one related compound - the methyl--D-glucoside - was rejected by WT cockroaches when they were only hungry, but it was accepted when they were hungry and thirsty without stimulating the GNR2 as in their GA counterparts. This suggests that the methyl--D-glucoside receptor is expressed by the GNR2 and might have a conformation close enough to recognize glucose and its relative compounds.

An additional behavioral experiment demonstrated that when the attractive fructose was mixed with glucose, it enhanced its attraction to WT due to the combined sweetener nature of the food, but the opposite effect was found on GA cockroaches where fructose loss its attractive nature via the combination with aversive glucose. In WT cockroaches the same effect was observed when fructose was combined with aversive caffeine.

Taken together, these results uncover the cellular basis of an acquired gustatory aversion in

cockroaches; they demonstrate that the aversion developed for glucose, which renders ineffective bait traps using this attractant, relies on a peripheral change in the perception of glucose by the GA cockroaches. The fact that conformational changes in the GRN2 were selected to bind glucose resulted in an acquired aversion to this attractant, as GRN2 is probably the gateway to a central circuit mediating aversive responses. The missing point in this impressive work is the structural analysis of the conformational changes experienced by membrane receptors in GRN2, which would have confirmed the hypothesis deduced from the electrophysiological recordings. Notwithstanding, the paper constitutes a fascinating analysis of gustatory receptor responses and of their associated changes due to natural selection.

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Résumé (french)

Chez les vertébrés, le neuropeptide Y (NPY) joue un rôle crucial dans la survie individuelle en modulant à la fois les comportements liés à la nourriture et au stress. Des niveaux élevés de NPY corrèlent avec une augmentation de la faim provoquant une ingestion plus importante et réduisent la sensibilité aux stimuli stressants. Chez les invertébrés, deux homologues indépendants de NPY ont été identifiés : le neuropeptide F (NPF) et le neuropeptide F court (sNPF). Chez l'abeille domestique (*Apis mellifera*), *npf* et *snpf* ainsi que leur peptides respectifs NPF et sNPF ont été identifiés or seul sNPF possède un récepteur, suggérant un rôle fonctionnel de ce neuropeptide chez cet insecte. Nous avons étudié l'impact de sNPF sur une multitude de comportements comprenant l'ingestion de nourriture de bonne et mauvaise qualité, les réponses appétitives et aversives, les apprentissages et la mémoire appétitifs et aversifs.

Nos résultats révèlent qu'une élévation artificielle des niveaux de sNPF via une application topique chez les butineuses augmente la prise alimentaire de nourriture bonne et mauvaise qualité. De plus, en utilisant une variété de tests pour étudier les réponses sensorielles, nous avons montré que sNPF a un rôle clé dans la modulation des réponses appétitives, mais cet effet est absent pour les réponses aversives. Les abeilles nourries et traitées avec du sNPF augmentent leur réponse au saccharose et aux stimuli olfactifs appétitifs, de façon similaire aux abeilles affamées. En adéquation avec les derniers résultats, des enregistrements *in vivo* multi photoniques de l'activité neuronal du lobe antennaire, le premier centre olfactif dans le cerveau de l'abeille, montrent une baisse des réponses aux odeurs appétitives chez les abeilles nourries qui est rétablie par le traitement avec le sNPF au même niveau que les abeilles affamées. Par ailleurs, l'effet modulatoire du sNPF était totalement absent sur les réponses aversives contrairement à ce qui a été observé chez la drosophile et les vertébrés, indiquant que chez les abeilles, sNPF n'augmente pas la tolérance aux stimuli stressants.

Etant donné l'amplification causée par le traitement sNPF sur la réponse au saccharose, nous avons étudié si cet effet se retrouvait dans des protocoles d'apprentissage pour lesquels les abeilles étaient entraînées à discriminer un stimulus récompensé par du saccharose d'un autre qui ne l'est pas. Nous avons étudié l'effet du sNPF sur les apprentissages et mémoires appétitifs visuels et

olfactifs. Dans le premier cas, des abeilles en semi libre vol ont été entraînées à discriminer deux couleurs dans un labyrinthe en Y après une application topique de sNPF. Dans le second cas, des abeilles en contention ont été entraînées à discriminer deux odeurs après une application topique de sNPF via le conditionnement du réflexe d'extension du proboscis. En parallèle, nous avons étudié les effets du sNPF sur l'apprentissage aversif gustatif pour lequel les abeilles en contention apprennent l'association entre une stimulation gustative de l'antenne avec un choc électrique après une application topique de sNPF. Nos résultats montrent une nette amélioration de l'apprentissage et mémoire appétitifs visuels et des tendances allant dans le même sens dans le cas de l'apprentissage appétitif olfactifs. A l'inverse, aucun effet n'a été observé quant à l'apprentissage et la mémoire aversifs gustatifs, ce qui est cohérent avec l'absence d'effet de sNPF sur les réponses sensorielles aversives.

Ce travail de thèse a montré que le sNPF affecte plusieurs modalités de comportements (ingestion, gustation, olfaction, vision, apprentissage, mémoire) et les processus neuronaux (lobe antennaire) liés aux comportements appétitifs, mais non aversifs, chez l'abeille. Par conséquent, ce travail fournit de nouvelles perspectives pour étudier les processus d'ingestion et le comportement alimentaire des abeilles.

Abstract (english)

Neuropeptide Y (NPY) signalling plays a crucial role for individual survival in vertebrates as it mediates both food- and stress-related behaviours. High NPY level correlates with increased hunger and leads to a larger food intake while it also reduces sensitivity to stressful stimuli. In invertebrates, two independent homologs of NPY have been identified: the neuropeptide F (NPF) and the short neuropeptide F (sNPF). In honey bees (*Apis mellifera*), both NPF and sNPF have been reported but only sNPF was found to have a dedicated receptor sNPFR, thus indicating that sNPF/sNPFR provides a functional signalling pathway in this insect. We thus studied the impact of sNPF on multiple behavioural components, including food-related behaviours such as ingestion of palatable and unpalatable food, appetitive and aversive responsiveness, and appetitive and aversive associative learning and memory retention.

Our results show that increasing artificially sNPF levels in honey bee foragers via topical exposure, increases significantly their consumption of both palatable and unpalatable food. In addition, using various responsiveness tests, we showed that sNPF is a key player in the modulation of appetitive but not aversive responsiveness. Fed foragers treated with sNPF exhibited a significant increase in their responsiveness to sucrose solutions and to appetitive olfactory stimuli, matching the levels of starved bees. In agreement with this last finding, *in vivo* multiphoton recordings of neural activity in the antennal lobe, the primary olfactory centre of the bee brain, showed a decreased responsiveness to appetitive odours in fed bees, which was rescued by treatment with sNPF to the level exhibited by starved bees. Interestingly, the modulatory effect of sNPF was totally absent in responsiveness to aversive stimuli contrarily to what has been observed in vertebrates and flies, thus indicating that in bees, sNPF dos not increase tolerance to stressors.

Given the enhancing effect of sNPF on appetitive responsiveness, we next studied if this effect translates to different appetitive learning protocols in which bees are trained to discriminate a stimulus that is rewarded with a sucrose solution from another that is not. We studied the effect of sNPF on both appetitive visual and olfactory learning and memory retention. In the first case, free-flying bees were trained to discriminate two colors in a Y-maze following topical increase of sNPF. In the second case, harnessed bees were trained to discriminate two odorants following

topical application of sNPF, using the conditioning of the proboscis extension reflex. In parallel, we studied the effect of sNPF for aversive gustatory learning in which harnessed bees learning the association of antennal taste with electric shock, following topical application of sNPF. Our results revealed a clear improvement of appetitive color learning and retention and a mitigated tendency in the same direction in the case of appetitive olfactory learning. On the contrary, no effect was observed in the case of the aversive gustatory learning and retention, consistently with the lack of effect of sNPF on aversive responsiveness.

To sum up, this work showed that sNPF affects multiple appetitive behavioural modules (ingestion, gustation, olfaction, vision, learning, memory) and central processing (antennal lobe activity) in the honey bee while being dispensable for aversive ones. It provides therefore a rich and multi-faceted view of the effects of this neuropeptide on the behaviour of a social insect and opens new research perspective to study ingestion processes and appetitive behaviour in bees.