Article

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

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SUMMARY

The neuropeptide F (NPF) and its short version (sNPF) mediate food- and stress-related responses in solitary insects. In the honeybee, 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 honeybee 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.

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 honeybee (*Apis mellifera*), a species that 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 honeybees. 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 honeybee also offers unique

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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 honeybees. We compared honeybee 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; Ayestaran et al., 2010; 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 honeybees 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 plus 15 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 the 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 honeybee 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
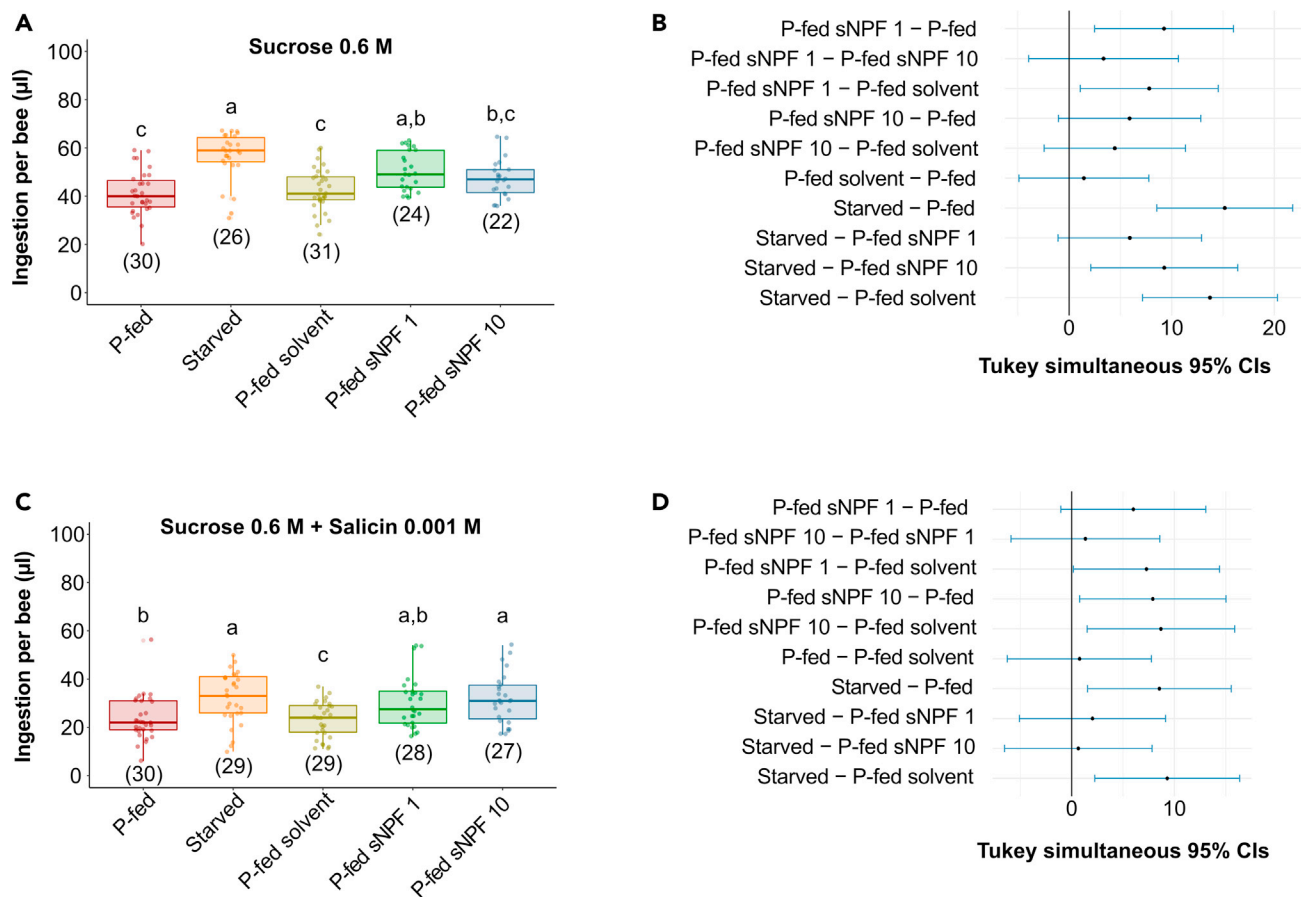
Figure 1. The effect of feeding status and sNPF on food consumption

(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_{P-fed} = 30; $n_{Starved}$ = 26; $n_{P-fed\ solvent}$ = 31; $n_{P-fed\ sNPF\ 1}$ = 24; $n_{P-fed\ sNPF\ 10}$ = 22. *Ingestion of 0.6 M sucrose solution spiked with 0.001 M salicin*: n_{P-fed} = 30; $n_{Starved}$ = 29; $n_{P-fed\ solvent}$ = 29; $n_{P-fed\ sNPF\ 1}$ = 28; $n_{P-fed\ sNPF\ 10}$ = 27.

a 0.6 M sucrose solution (Figures 1A and 1B) or with the same amount of 0.6 M sucrose solution spiked with 0.001 M salicin (Figures 1C and 1D) (Desmedt et al., 2016).

The feeding status and the treatments applied had a significant effect on food ingestion (Figures 1A and 1B, $F_{4,128}$ = 13.04, d.f. = 4, p = 6.12 × 10^{-9}; Figures 1C and 1D, $F_{4,138}$ = 6.1 d.f. = 4, p = 1.5 × 10^{-4}). As expected, starved bees ingested significantly more sucrose solution than bees of the control groups (Figures 1A and 1B; P-fed bees, p = 3.64 × 10^{-8}, [8.54; 21.79] CI 95%; P-fed solvent bees, p = 5.37 × 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 1C and 1D; P-fed bees, p = 8.44 × 10^{-3}, [1.54; 15.55] CI 95%; P-fed solvent bees, p = 3.42 × 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 (Figures 1A and 1B: p = 0.97, [-4.89; 7.77] CI 95%); Figures 1C and 1D: 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 1A and 1B, $p = 0.002$, [2.48; 16.02] CI 95%) and solvent-treated P-fed bees (Figures 1A and 1B, $p = 0.01$, [1.09; 14.53] CI 95%), reaching the ingestion level of starved bees (Figures 1A and 1B, $p = 0.14$, [-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 1A and 1B, $p = 0.71$, [-3.94; 10.65] CI 95%) and that of untreated P-fed bees (Figures 1A and 1B, $p = 0.38$, [-2.44; 11.34] CI 95%) and P-fed solvent bees (Figures 1A and 1B, $p = 0.38$, [-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 (Figures 1C and 1D, 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 (Figures 1C and 1D, 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 1C and 1D, $p = 0.09$, [-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.
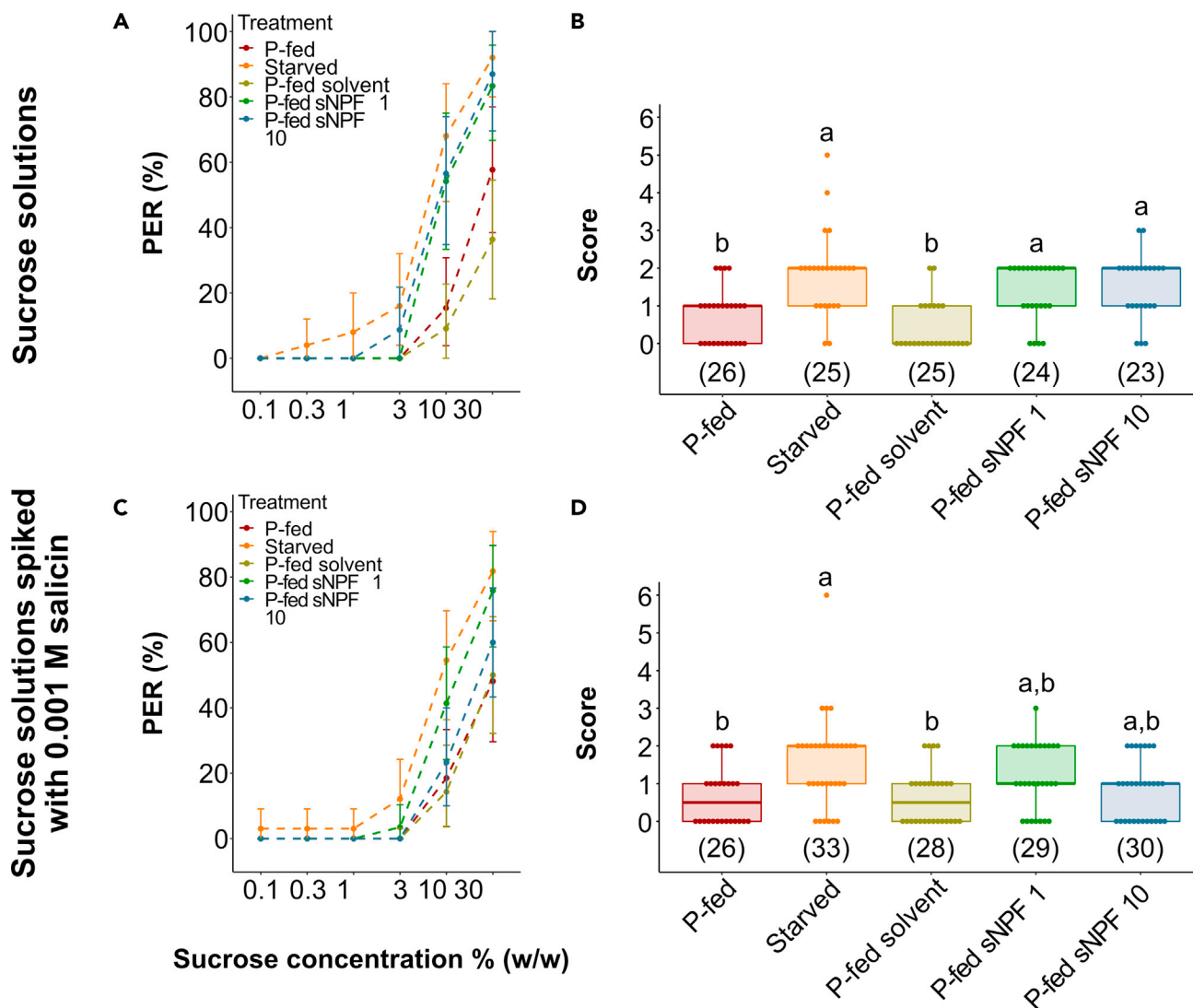
Experiment 2: sNPF increases gustatory responsiveness of P-fed honeybee foragers

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

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

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

Individual responsiveness scores of bees stimulated with pure sucrose solution (Figure 2B, $H = 38.97$, $d.f. = 4$, $p = 7.07 \times 10^{-8}$) or with the mixture of sucrose and salicin (Figure 2D, $H = 18.92$, $d.f. = 4$, $p = 8.13 \times 10^{-4}$) differed according to the treatment employed. In both cases, starved bees had significantly higher sucrose response scores than both P-fed controls, untreated P-fed (Figure 2B, pure sucrose solution; $W = 124$, $p = 7.46 \times 10^{-4}$; Figure 2D, sucrose solution with salicin; $W = 233$, $p = 0.02$) and P-fed solvent (Figure 2B, pure

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*; $n_{P\text{-fed}} = 26$; $n_{Starved} = 33$; $n_{P\text{-fed solvent}} = 28$; $n_{P\text{-fed sNPF 1}} = 29$; $n_{P\text{-fed sNPF 10}} = 30$.

sucrose solution; $W = 76$, $p = 1.48 \times 10^{-5}$; Figure 2D, 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 2B, $W = 227.5$, $p = 1$) and P-fed solvent bees (Figure 2B, $W = 114$, $p = 7.25 \times 10^{-4}$). Although the comparison between P-fed sNPF 1 bees and P-fed untreated bees was marginally non-significant (Figure 2B, $W = 174.5$, $p = 0.05$), a clear enhancement of appetitive responsiveness was detected in P-fed sNPF 10 bees. In this case, sucrose scores increased significantly to a level
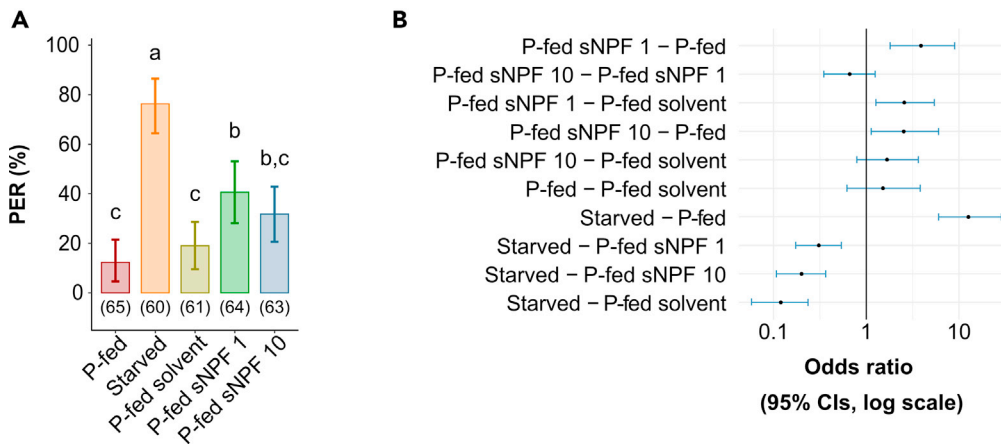
Figure 3. The effect of feeding status and sNPF on appetitive olfactory responsiveness

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 is significantly different.

comparable to that of starved bees (Figure 2B, W = 241.5, p = 1), so that they differed significantly from those of both P-fed controls, untreated P-fed (Figure 2B, W = 150, p = 0.01) and P-fed solvent (Figure 2B, W = 479.5, p = 2.48 × 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 2D), which were intermediate between those of starved bees and those of both P-fed controls (Figure 2D, 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 (Ayestaran et al., 2010; de Brito Sanchez et al., 2005; Desmedt et al., 2016).

Experiment 3: sNPF increases responsiveness of P-fed honeybee 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 Figure S1). Olfactory responsiveness varied according to the treatment employed (Figure 3A, Fisher's exact test, p = 1.69 × 10^{-7}). Starved bees were significantly more responsive to appetitive odorants than untreated P-fed bees (Figures 3A and 3B, p = 1.03 × 10^{-14}, OsR = 12.63 [6.04; 28.7] CI 95%), P-fed solvent bees (Figures 3A and 3B, p = 2.12 × 10^{-11}, OsR = 0.12, [0.06; 0.23] CI 95%) and bees treated with sNPF (Figures 3A and 3B, Starved *vs.* P-fed sNPF 1: p = 6.99 × 10^{-5}, OsR = 0.31, [0.17; 0.54] CI 95%; Starved *vs.* P-fed sNPF 10 μg/μL: p = 7 × 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 (Figures 3A and 3B, p = 8 × 10^{-4}, OsR = 3.89, [1.81; 8.99] CI 95%) and P-fed solvent (Figures 3A and 3B, 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 (Figures 3A and 3B, p = 0.07, OsR = 0.15, [1.13; 6.03] CI 95%) and P-fed solvent bees (Figures 3A and 3B, 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 honeybee 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 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 conveys 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 (Figures 4A–4C, top row). Yet, this decrease was rescued by the topical application of sNPF 10 μg/μL (Figures 4A–4C, 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 4D) (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-phenylethanol/solvent: $n = 55$ glomeruli, $p = 0.0004$; 2-phenylethanol/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 4E), 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 honeybee 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 5A, GLMM, $\chi^2 = 0.28$, *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
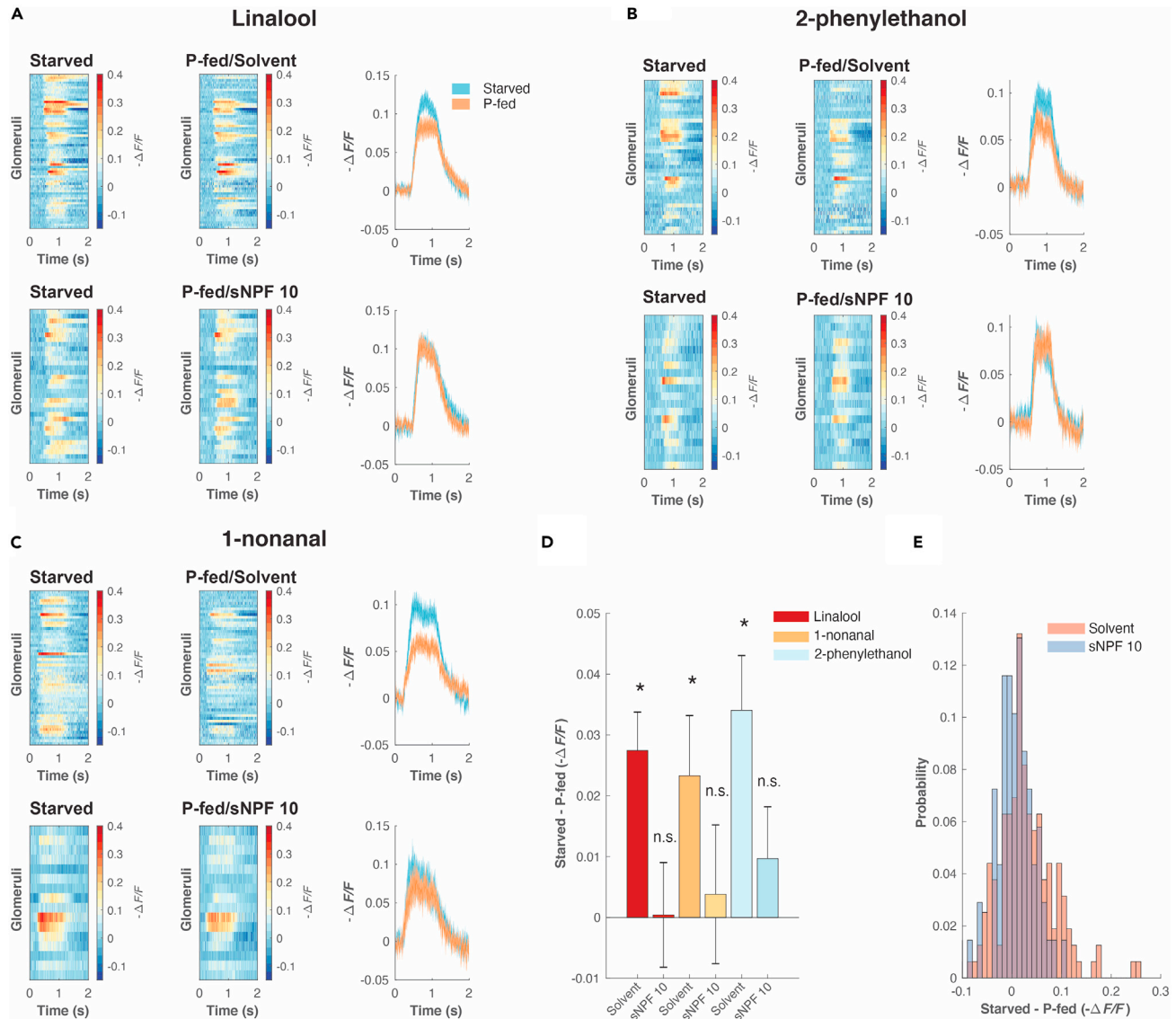
Figure 4. The effect of feeding status and sNPF on glomerular activity in the antennal lobe

(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 and 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-phenylethanol solvent: n = 55 glomeruli from 9 bees; 2-phenylethanol 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 a 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).

untreated fed bees (Figure 5B, $W = 645$, $p = 0.009$). 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 (Figure 5B, $p > 0.05$ for all comparisons between sNPF fed bees and solvent-fed bees).
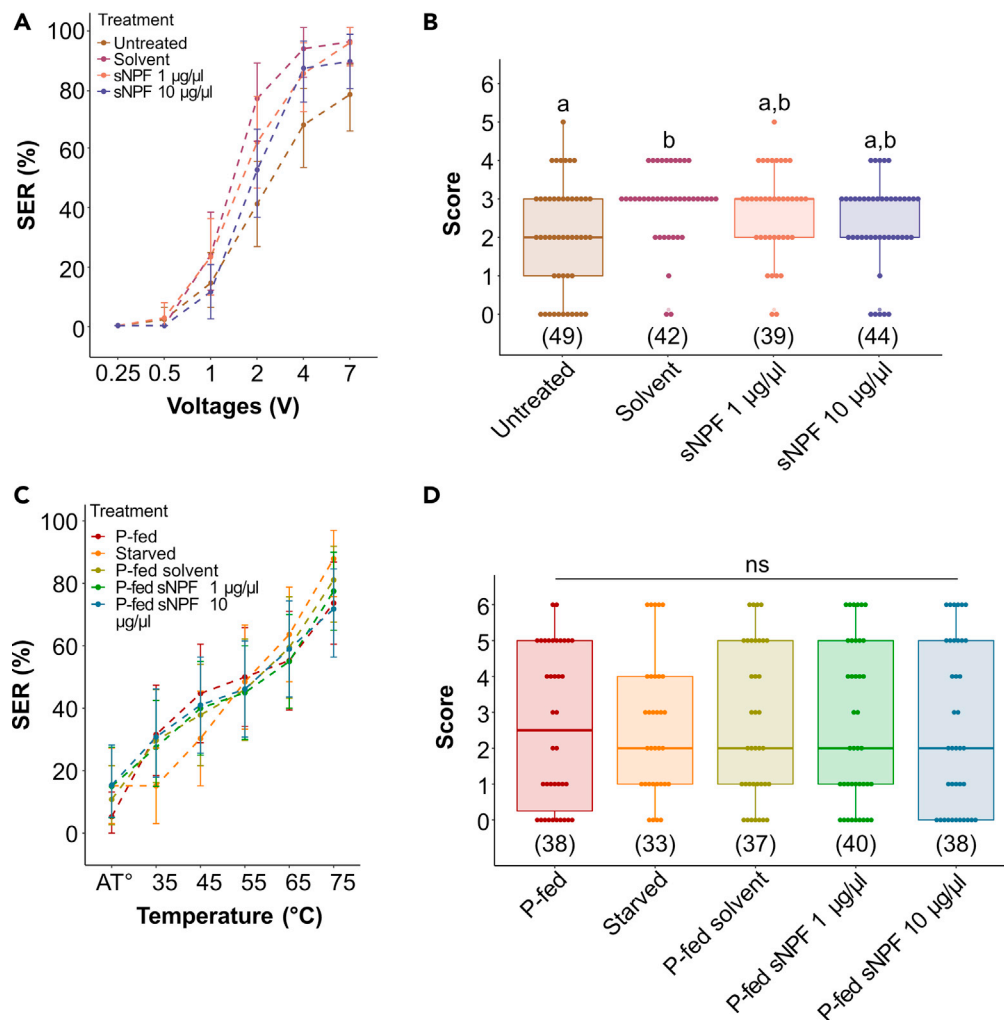
Figure 5. The effect of feeding status and sNPF on responsiveness to nociceptive stimuli

(A and 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 and 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 and 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.

Experiment 6: sNPF does not affect aversive thermal responsiveness of fed honeybee 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 with dimethylformamide (DMF). A control experiment (see 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 5C, GLMM, χ^2 = 0.033, $d.f.$ = 4, p = 0.99). Furthermore, focusing on individual thermal-responsiveness scores did not reveal significant differences between groups (Figure 5D, 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 honeybee, sNPF facilitates responsiveness to appetitive, food-related stimuli, but seems to be dispensable for responding to aversive stimuli.

sNPF enhances food intake in honeybee 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 (see Itskov and Ribeiro, 2013 for review) 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., 2012, 2015). 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 honeybees, 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 honeybees, 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 (Figure 1B) found upon sNPF treatment contrasts with the lack of a clear effect of this treatment on responsiveness to the same mixture (Figure 2D). 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 midgut (Christie, 2020), suggesting a possible role as an internal energy sensor, similar 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 honeybees, 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 the 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 honeybees 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 the aversive responsiveness of honeybees 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 honeybees, the TRP channel AmHsTRPA modulates thermal responsiveness (Junca and San-doz, 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 lifestyle of honeybees, in which nociception and resistance to stressors like electric shocks are under the 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 honeybee 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 honeybees

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 honeybee, 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 neurotransmitters 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 speeds (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 the 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 San-chez 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 an RNAi approach targeting the sNPF receptor identified in the honeybee. 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 honeybees are not straightforward (i.e. difficulties for generating mutants given the complexity of the life cycle and lifestyle requiring the hive environment and exposure to the environment). We are currently working on the development of this RNAi approach.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.103619.

AUTHOR 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 M.G. de B.S. The article was written by L.B., M.G., and M.G. de B.S. All experiments were supervised by M.G. and M.G. de B.S. Funding was obtained by M.G., M.G. de B.S., and J.-C.S All authors reviewed and approved the final version of the article.

DECLARATION OF INTERESTS

The authors declare that they have no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Short neuropeptide NPF (sNPF)	NovoPro Bioscience Inc. (Shanghai, China)	317818-20MG
Salicin	Sigma-Aldrich	138-52-3
Eicosane	Sigma-Aldrich	112-95-8
Fura-2 Dextran 10 kDa	ThermoFisher Scientific	108964-32-5
Deposited data		
Repository data	Figshare	10.6084/m9.figshare.15134859
Experimental models: Organisms/strains		
Honey Bee workers *Apis mellifera*		N/A
Software and algorithms		
R version 3.6.0	R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/	N/A
MatLab R2018a (9.4.0.813654)	The MathWorks, Inc.	N/A
Affinity Designer	Serif (Europe) Ltd. https://affinity.serif.com/fr/	N/A
Custom MatLab scripts	This paper	https://doi.org/10.6084/m9.figshare.16864408
Custom R scripts	This paper	https://doi.org/10.6084/m9.figshare.16864408

RESOURCE 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 supplemental information.

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

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.

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: $n_{Starved/P-fed\ solvent}$ = 9; $n_{Starved/P-fed\ sNPF10}$ = 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 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: $n_{P-fed} = 38$; $n_{Starved} = 33$; $n_{P-fed\ solvent} = 37$; $n_{P-fed\ sNPF\ 1} = 40$; $n_{P-fed\ sNPF\ 10} = 38$.

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 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).

Article

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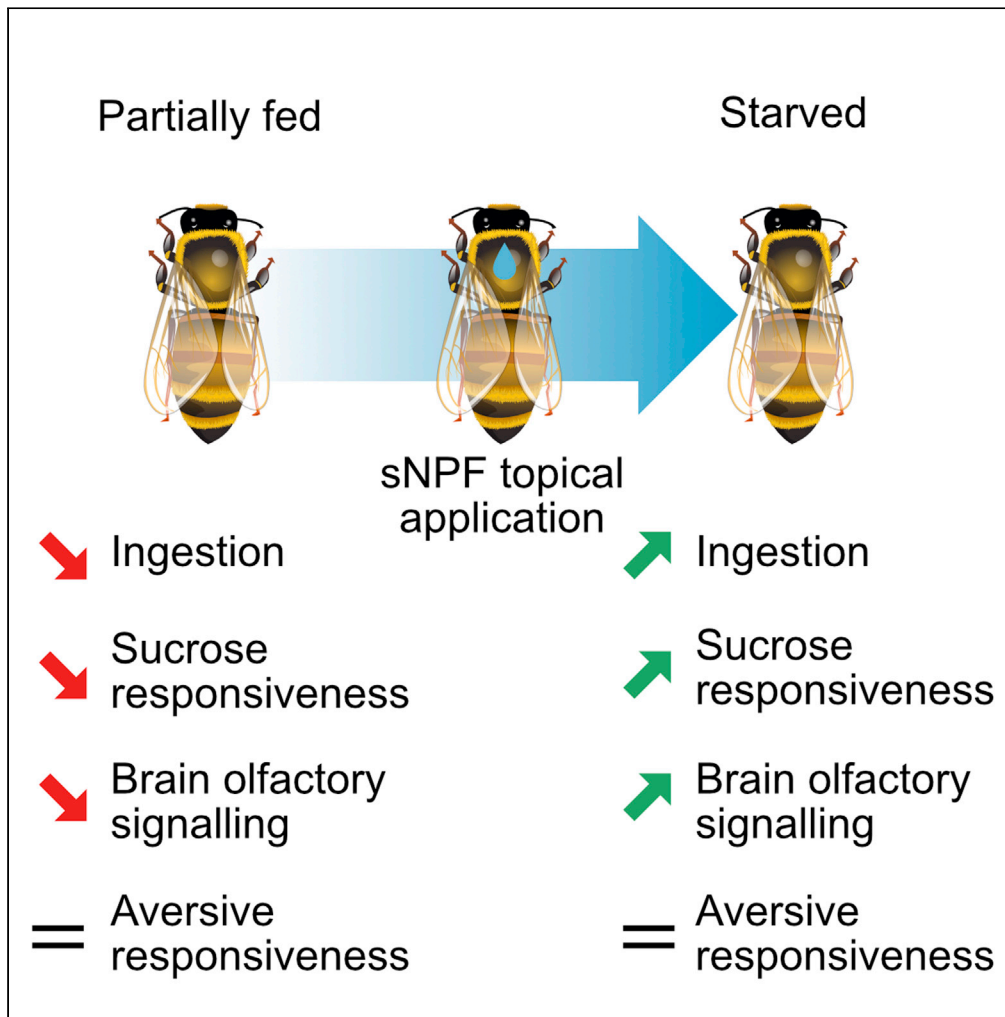
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Highlights

sNPF increases food
consumption and
appetitive responsiveness
of honeybees

Feeding reduces neural
activity in odor circuits;
sNPF restores it to the
starved level

sNPF has no effect on
responsiveness to
nociceptive stimuli in
honeybees

sNPF is a key modulator of
hunger and food-related
responses in bees