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Neuropeptide Y-like signaling and nutritionally-mediated gene expression and behavior in the honey bee

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Abstract

Previous research has led to the idea that derived traits can arise through the evolution of novel roles for conserved genes. We explored whether Neuropeptide Y-like signaling, a conserved pathway that regulates food-related behavior, is involved in a derived, nutritionally-related trait, division of labor in worker honey bees. Transcripts encoding two NPY-like peptides were expressed in separate populations of brain neurosecretory cells, consistent with endocrine functions. NPY-related genes were upregulated in the brains of older foragers compared to younger bees performing brood care (“nurses”). A subset of these changes can be attributed to nutrition, but NPY peptide treatments did not influence sugar intake. These results contrast with recent reports of more robust associations between division of labor and the related insulin-signaling pathway and suggest that some elements of molecular pathways associated with feeding behavior may be more evolutionarily labile than others.

Introduction

It has been proposed that one route of behavioral evolution is through the adaptation of conserved genes to novel roles (Harris-Warrick, 2000; Toth & Robinson, 2007). In order to test this idea, it is necessary to identify sets of conserved genes that regulate behavioral traits across taxa, and to then elucidate their roles in the context of more derived behaviors. This approach is analogous to studies into the evolution of development that exploit the conserved yet adaptable ‘toolkit’ of genes involved in the regulation of morphology (Carroll *et al.*, 2005).

Recently, we and others have identified feeding- and nutritionally-related genes as one potential toolkit that has been exploited in the evolution of honey bee social behavior (Ament *et al.*, 2010). In honey bee colonies, food-related tasks are partitioned among workers of different ages (Winston, 1987). Foraging for food outside the hive is performed exclusively by older workers, whereas food storage and feeding of larvae (“nursing”) are performed by young bees that stay inside the hive. The transition from hive work to foraging is a regulated process and its timing depends on both social and nutritional factors (Toth *et al.*, 2005). Among the genes involved in this process of behavioral maturation are genes that regulate feeding in *Drosophila melanogaster* (Ben-Shahar *et al.*, 2002; Ben-Shahar *et al.*, 2004), the yolk protein gene *vitellogenin*, which has taken on roles as a general storage

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protein and is incorporated into brood food in sterile worker honey bees (Nelson *et al.*, 2007; Amdam *et al.*, 2003), and genes in the insulin/insulin-like growth factor signaling pathway (IIS) (Ament *et al.*, 2008). Together, the involvement of these diverse food-related genes suggests that the evolution of honey bee social behavior included the cooption of systems that regulate simpler food-related behaviors in solitary species (Toth & Robinson, 2007).

Despite the growing evidence that nutritionally-related pathways are involved in worker maturation, we know little about how these changes actually lead to differences in behavior. One approach to this difficult problem is to break the complex social system down to the underlying nutritional and behavioral components in order to understand the mechanisms by which these simpler phenotypes are related (Ben-Shahar *et al.*, 2004). Taking this approach, we found that in the context of worker honey bee maturation, unlike in flies and other well-studied models, the expression of insulin-related genes is negatively correlated with nutrient stores. We do not know why this is the case, but we have speculated that it relates to a novel role for IIS in regulating a nutritionally-related set point so that foragers are more sensitive to nutritional changes than are nurses (Ament *et al.*, 2008). More generally, this result suggests that complex social traits could arise through changes in gene regulation that influence underlying changes in more simple physiological and behavioral traits. If so, in order to explain social behavior it will be important to understand the signaling systems that link nutrition to physiology and simple feeding-related behaviors, and to determine whether these systems differ appreciably in the bee from those studied in solitary species.

Insulin-like peptides in both vertebrates and invertebrates act as nutrient sensors: their synthesis and release is coupled to the levels of circulating macronutrients, and they affect behavior through interactions with other neuropeptide systems in the brain (Wu *et al.*, 2005a; Morton *et al.*, 2006; Schwartz *et al.* 1992). The best characterized of these brain peptide signals is Neuropeptide Y (NPY), which regulates food searching and food intake in both mammals and invertebrates. NPY was the first feeding-stimulatory neuropeptide discovered in mammals; many experiments have shown that it is highly expressed in hypothalamic nuclei of rats and mice after a period of food withdrawal and that infusions of NPY peptide into the ventral hypothalamus increase food intake (Hahn *et al.*, 1998; Stanley and Leibowitz, 1985; Morton *et al.*, 2006). Sequenced insect genomes contain two homologs of NPY-like peptides, neuropeptide F (*npf*) and short neuropeptide F (*sNPF*) (Brown *et al.*, 1999; Lee *et al.*, 2004; Hummon *et al.*, 2006). In *Drosophila*, genetic manipulations of the NPF receptor, *npfr1*, suggest that this gene delays the naturally occurring transition from a larval feeding stage to the non-feeding wandering stage (Wu *et al.*, 2003). Moreover, upregulation of NPY-related genes mimic starvation-induced changes in larval foraging strategy: *Drosophila* larvae over-expressing *npf* or *npfr1* consume more of undesirable foods and under poor foraging conditions (Wu *et al.*, 2005a; Wu *et al.*, 2005b). However, NPF signaling apparently has no effect on the consumption of desirable sucrose diets in standard conditions in *Drosophila*, suggesting that this peptide regulates the motivation to feed rather than directly affecting consumption (Lingo *et al.*, 2007). An independent line of research has shown that the second Neuropeptide Y-like peptide in *Drosophila*, *snpf*, also regulates food intake in *Drosophila* larvae (Lee *et al.*, 2004; Lee *et al.*, 2008). Together, these results suggest a conserved role for NPY-like signaling as a regulator of the motivation to feed or, more colloquially, “hunger.”

Like *Drosophila* larvae, adult worker honey bees forage for food during only part of their maturation, so by analogy NPY-like signaling might regulate this transition in bees as in flies. However, maturational changes in food-related behaviors in bees differ from those in solitary species in that foragers collect food primarily for the colony rather than for themselves. Several aspects of bee biology suggest the hypothesis that foragers have increased NPY-like signaling relative to nurses. First, foragers consume a diet that is less

rich in protein and lipid than do nurses (Crailsheim *et al.*, 1992). Second, foragers have smaller protein and lipid nutrient stores than nurses (Snodgrass, 1956; Toth & Robinson, 2005). Third, foragers have a more vigorous lifestyle and faster metabolism than nurses associated with their energy-intensive flights outside the hive (Harrison, 1986). Finally, the response of foragers to changes in colony nutrition are more pronounced than those of nurses; when the colony requires food, only foraging-age bees leave the hive to seek pollen and nectar at distant floral sources. Therefore, foragers are nutritionally deprived compared to nurses with respect to nutrient consumption, nutrient storage, and nutrient utilization, and they appear to be more motivated to seek food than younger bees.

NPY-like signaling may influence behavior over several different timescales. Changes in diet and nutrient stores occur over the course of days to weeks as bees transition from hive work to foraging (Schulz *et al.*, 1998; Toth *et al.*, 2005). However, nutrition also affects foraging behavior itself on the timescale of hours to days. For instance, changes in the nutritional needs of the colony are thought to regulate the rate of foraging and the ratio of foragers collecting lipid- and protein-rich pollen vs. carbohydrate-rich nectar (Winston, 1987). Therefore, it is conceivable that hunger-related pathways such as NPY-like signaling could regulate processes both over the lifetime of a bee and over the course of a forager's daily activities. In support of a short-term role for NPY-like signaling in foraging behavior, sNPF peptide levels in the brains of foraging bees change during the period between when they arrive at a feeder and when they depart it a few minutes later (Brockmann *et al.*, 2009).

We explored relationships between NPY-like signaling and nutritionally-mediated traits of worker honey bees. We first characterized evolutionary changes in the sequences of NPY-related genes of hymenopteran insects. We further characterized the genes encoding NPY-like prepropeptides by localizing their expression to neurosecretory cells in the brain. We then measured the expression of NPY-related genes in the brains of nurses and foragers, and under a variety of different nutritional conditions. These experiments suggest that NPY-like signaling is more sensitive to nutritional status in the brains of foragers than of younger bees. However, these patterns were not as robust as for previously studied food-related genes such as insulin-signaling genes, and injection of synthetic NPF into the brains of foragers did not stimulate increased food intake.

Results

NPY-like peptides encoded in the genomes of hymenopteran insects

A previous study identified two NPY-like peptides, *AmNPF* and *AmsNPF*, in the sequenced honey bee genome (Hummon *et al.*, 2006). The predicted NPF peptide retains key attributes of NPY-family peptides, such as a proline-rich N-terminus that is likely to form the characteristic polyproline-like helix, but differs from most insect NPF peptides in that it has a –KARY rather than –RXRF C-terminus (Roller *et al.*, 2008). These C-terminal amino acids are critical for receptor binding, so the sequence changes may have functional consequences (Lindner, *et al.* 2008). Some lepidopteran genomes contain both a “standard” *npf* gene encoding a peptide with an –RXRF C-terminus, and a second gene encoding an NPF peptide with a tyrosine C-terminus (Huang *et al.*, 2010; Roller *et al.*, 2008). This does not appear to be the case in the bee; extensive searches of the assembled *A. mellifera* genome as well as of additional unassembled sequences revealed only the single *npf* gene. Moreover, the genome of a distantly-related hymenopteran, the jewel wasp *Nasonia vitripennis*, contains only a single *npf* gene, and this gene has a –KARY C-terminus (Hauser *et al.*, 2010). This suggests that hymenopteran genomes each contain a single *npf* gene, and this gene has an unusual tyrosine C-terminus. Similar analysis of the honey bee sNPF peptide showed that its sequence differs little from sNPF peptides found in other insects (Hummon *et al.*, 2006; Vanden Broeck, 2001).

NPY-family receptors encoded in the genomes of hymenopteran insects

Unlike the genomes of most insects, which contain two NPY-family receptors, *npfr1* and *snprR*, which have been shown to activate NPF and sNPF, respectively (Garczynski *et al.*, 2002; Mertens *et al.*, 2002; Hill *et al.*, 2002; Hauser *et al.*, 2006), only an ortholog to *snprR* was identified previously in the honey bee genome (Chen & Pietrantonio, 2006; Hauser *et al.*, 2006). Extensive searches both in the assembled *A. mellifera* genome sequence as well as in trace archives failed to reveal an *A. mellifera* homolog for the *npfr1* gene, suggesting that this may be a real gene loss.

To further validate this finding, we searched the *N. vitripennis* genome and brain Expressed Sequence Tag databases from 11 additional bee species for NPY-family receptors (GER). These searches identified orthologs of *snprR* but not of *npfr1* (data not shown), suggesting that hymenopteran genomes lack orthologs of *npfr1*. However, we identified orthologs of both *snprR* (XP_001952283.1) and *npfr1* (XP_001943708.1) in the genome of the pea aphid, *Acyrtosiphon pisum* (International Aphid Genomics Consortium 2010), which is basal to all holometabolous insects. These results suggest that the *npfr1* gene was present in the ancestors of bees and wasps but was lost early in hymenopteran evolution.

All the insect genomes examined also contained homologs for a third gene with similarities to NPY-family receptors, *NepYr*, including the *A. mellifera* gene GB13527. However, this gene is generally discounted as a potential receptor for NPY-like peptides because it has been shown in *Drosophila* to have low affinity for NPF and to instead bind –PQGRF-amide peptides (St-Onge, *et al.*, 2000; Taghert and Veenstra, 2003), so we did not examine it further. Overall, these analyses indicate that a number of changes in NPY-like signaling genes occurred early in hymenopteran evolution -- prior to the divergence of *A. mellifera* and *N. vitripennis* ca. 150-200 million years ago (Dowton *et al.*, 2009) -- with few recent changes within the Hymenoptera.

npf and *snpr* are localized to separate populations of neurosecretory cells in the honey bee brain

NPY-related genes in the bee had not previously been characterized experimentally. We used *in situ* hybridization to determine where *npf* and *snpr* are expressed in the brains of foragers. Transcripts were localized to separate clusters of neurosecretory cells. *npf* was expressed bilaterally in 8-10 medial neurosecretory cells (Fig. 1A). *snpr* was localized bilaterally in 4-6 pairs of lateral neurosecretory cells (Fig. 1B). These results are consistent with the predicted endocrine functions for these genes, and they suggest that *npf* and *snpr* function in independent circuits.

npf is upregulated in the brains of foragers

We next examined whether any of the NPY-related genes in the bee were differentially expressed between nurses and foragers. We focused primarily on the expression of these genes in the brain because we were interested in understanding their effects on behavior. *npf* was upregulated in the brains of foragers compared to nurses (Fig. 2, significantly different in 3 of 4 individual trials and in a combined analysis). *snpr* was expressed at similar levels in nurse and forager brains in all most trials (Fig. 2). *snprR* was upregulated in foragers in two trials, but was downregulated in a third trial, and not different in the last trial and was not significantly different between groups in a combined analysis (Fig. 2). These results suggest that only *npf* expression is consistently associated with behavioral state in the brains of honey bee workers.

Previous studies have shown that NPF peptides are expressed in the midgut as well as the brain (Huang *et al.*, 2010). qPCR assays confirmed that *npf* was expressed in the abdomen,

but its expression did not differ between nurses and foragers (Fig. 2B). This suggests that maturational changes in *npf* expression are specific to the population of *npf*-expressing cells in the brain.

***snpfR* is upregulated by food deprivation**

We next examined whether diet quality influences the expression of NPY-related genes. We fed young, caged bees either a nutrient-poor diet (sugar syrup) or a nutrient-rich diet (ground pollen, honey, and sugar syrup). Diet quality did not affect the expression of *npf*, *snpf*, or *snpfR* in brain (Fig. 3A). This negative result was not due to a treatment failure because bees in the same cages differed in their lipid stores and in the expression of *insulin-like peptide-1* (these were some of the same bees used in Ament *et al.*, 2008, and qPCR assays for the two studies were performed in parallel). This suggests that although *npf* and *ilp1* are both upregulated in the brains of foragers, only the expression of *ilp1* is directly influenced by dietary nutrients.

To further test this idea, we examined the response of NPY-related genes in the brain to nutritional manipulations under more natural conditions. We placed one-day-old bees into small, experimental colonies in the field that were either well-fed with pollen and honey or food-deprived by providing only a small amount of honey. These conditions cause bees from food-deprived colonies to initiate foraging earlier than bees from well-fed colonies, beginning when they are around five-days-old (Schulz *et al.*, 1998). Again, we found that *npf* and *snpf* expression were insensitive to these nutritional conditions. However, colony food-deprivation caused a consistent upregulation of brain *snpfR* (Fig. 3B). These results suggest that the maturationally-related variability in *snpfR* expression may relate to nutrition.

Behaviorally associated changes in *snpfR* expression depend on nutrition

To more directly test the idea that maturation and nutrition interact to regulate the expression of *snpfR* and other NPY-related genes, we studied the effects of cage diet manipulations on bees at three different stages of maturation (young bees like those used in the experiments described above, nurses, and foragers). For this experiment, we added an additional diet -- a rich diet made with soy protein in place of pollen -- because foragers are known to digest pollen inefficiently (Crailsheim *et al.*, 1992).

Maturation-related changes in *npf* were observed regardless of cage diet (Fig. 4), despite several days of caging, indicating that the upregulation of this gene in foragers is stable and is not controlled by diet. *snpf* was expressed at similar levels across all groups, consistent with our other experiments. By contrast, *snpfR* was upregulated in foragers compared to younger bees (bees placed into cages as one-day-olds or nurses) only when they were fed a nutrient-poor sugar syrup diet. *snpfR* trended higher in foragers when bees were fed sugar and pollen, but this difference was not significant. When bees were fed the more easily digested soy protein, *snpfR* levels were indistinguishable between young and old bees. These results suggest that maturation-related differences in *snpfR* expression depend on nutrition.

NPF does not influence sugar intake

Studies in flies have shown that *npf* influences motivational aspects of feeding but not the consumption of very desirable substances (Wu *et al.*, 2005b; Lingo *et al.*, 2007). We tested whether injections of synthetic NPF peptide into the brains of foragers affected their short-term consumption of sugar syrup in cages. NPF had no effect on sugar syrup intake (Fig. 5). This result is consistent with the idea that in insects, unlike rodents, NPY-like peptides do not have direct influence on the consumption of desirable foods. Unfortunately, current

methodologies did not allow us to test more motivational aspects of foraging behavior in the field.

Discussion

Neuropeptide Y-like signaling is the best-studied molecular pathway involved in the regulation of hunger across multiple taxa. Because a variety of feeding- and nutritionally-related genes are involved in the regulation of worker division of labor in honey bees, NPY-like signaling is a prime candidate for a regulator of this process. We showed that workers specializing on nursing and foraging differ in the expression of NPY-related genes. However, the precise relationship between this pathway and division of labor remains puzzling because the functional relationships among the genes in the pathway are not entirely clear and each gene showed a unique expression pattern.

We found that genes encoding the two NPY-like peptides, *npf* and *snpf*, were expressed in separate populations of neurosecretory cells in the honey bee brain. The localization of these genes to neurosecretory cells is consistent with their predicted roles as neurohormones, likely with both paracrine and endocrine functions (Nijhout, 1994). These patterns are consistent with reports in other insects that NPF peptides are expressed in neurosecretory cells (Zhu *et al.*, 1998; Shen & Cai, 2001). Interestingly, sNPF is expressed in a much broader set of neural cell types in the *Drosophila* brain, as measured by either *in situ* hybridization (Lee *et al.*, 2004) or genetic methods (Nässel *et al.*, 2008). Therefore, our results suggest that the sNPF gene has shifted between neuropeptide-typical expression patterns in the bee and less traditional expression patterns in the fly over the course of evolution. The broader expression of sNPF in *Drosophila* might imply that this gene is involved in more diverse functions in the fly than in the honey bee.

In addition to their localization to different neural circuits, *npf* and *snpf* also responded differently to maturational and nutritional cues. We found that *npf* was stably upregulated in the brains of foragers compared to nurses. By contrast, *snpf* was expressed at nearly identical levels across all experiments in this study. These results suggest that *npf* but not *snpf* has functions that change during maturation.

We found that *npf* and *snpf* transcription was not influenced by nutrition. However, a limitation of this analysis is that peptides undergo extensive post-transcriptional processing that could lead to functional differences in activity without transcriptional changes. In particular, nutritionally-related changes in peptide release may occur on a shorter timescale than is reflected by gene expression. In support of this, we recently found using mass spectrometry proteomics that sNPF peptide levels in the brain vary with two aspects of foraging performance: between foragers collecting nectar vs. pollen, and between foragers arriving and departing a feeder over a timescale of just minutes (Brockmann *et al.*, 2009). In the same study, sNPF peptide was found at similar levels in the brains of nurses and of foragers consistent with the transcriptional data reported here. NPF peptide was not detected by the mass spectrometry approach used by Brockmann *et al.* (2009). If NPF and sNPF are involved in regulating feeding-related processes, it is puzzling that this does not lead to expression differences after several days of chronic nutritional manipulations, especially because nutritional changes do induce expression changes in NPY-like genes in the fly (Shen & Cai, 2001).

We found that *snpfR*, the only NPY-family receptor identified in the sequenced honey bee genome, is upregulated in the brain by poor nutrition, especially in foragers. Foragers are known to be more responsive to nutritional cues than nurses (Scheiner *et al.*, 2002), and the foraging task in general can be construed as an extremely motivated response to the

nutritional needs of the colony, compared to the option of feeding inside the hive. The increased sensitivity of *snprR* to nutrition in the brains of foragers might be one reason that foragers respond more strongly than nurses to nutritional stimuli.

While NPY-family receptors have been shown to have prominent roles in the control of behavior (Wu *et al.*, 2005a; de Bono & Bargmann, 1998), to our knowledge the expression response of NPY-family receptors to nutritional manipulations has been studied in only one other invertebrate species, the fire ant *Solenopsis invicta*, in which several days of starvation reduced transcript abundance of an *snprR* homolog (Chen & Pietrantonio, 2006). These results suggest that plasticity in NPY-family receptor gene expression is a recurring phenomenon and a candidate mechanism underlying plasticity in feeding-related behaviors.

The interpretation of these results is complicated by the lack of an identified honey bee ortholog to *npfr1*, the *Drosophila* receptor for long NPF peptides. Our analyses suggest that while the *npfr1* gene was lost early in hymenopteran evolution the *npf* gene has remained intact. This evolutionary conservation suggests that the NPF peptide is able to function through a different receptor. We speculate that binding of hymenopteran NPFs to this unknown receptor was facilitated by the sequence changes we observed in important receptor-binding sites at the C-terminus of the peptide. Only -RXRF forms of NPF have been identified so far in insect lineages basal to Hymenoptera and Lepidoptera such as Hemiptera and Coleoptera, but non-insect arthropods contain both Y-terminal and F-terminal forms (Roller *et al.*, 2008; Huang *et al.*, 2010). These results suggest that sequence changes in the receptor binding residues of the NPF/NPY peptide have occurred multiple times during evolution but that hymenopterans are unique among the insects whose genomes have been sequenced in having only a Y-terminal form of the NPF peptide. Future studies should address the question of what gene serves as the receptor for NPF in hymenopteran insects.

We focused primarily on brain gene expression because we are interested in understanding behavior. However, NPY-like signaling also influences physiology through its effects outside the brain. We found that *npf* expression in the abdomen did not differ between nurses and foragers, suggesting that some of the patterns we observed are specific to the brain. However, we have recently shown that *snprR* is upregulated in the fat bodies of foragers compared to nurses and in fat, as in the brain, becomes more sensitive to nutritional stimuli in foragers compared to younger bees (Ament and Robinson, unpublished results). These results provide validation for the patterns we observed in the brain and suggest that similar changes in NPY-like signaling underlie maturational changes both in a bee's behavior and physiology.

We were unable to show a direct effect of NPF peptide on sugar consumption. This result is consistent with previous findings in *Drosophila*, but an important goal for future research will be to characterize aspects of feeding-related behavior that might be influenced by the expression changes we observed. Also, although we found no evidence for maturation-related functions of sNPF, this peptide remains interesting in light of its dynamic peptide levels in the bee brain (Brockmann *et al.*, 2009), and its functional role should be examined in future studies.

Previous studies provide a rich precedent for the association of NPY-like signaling with both short- and long-term plasticity in feeding-related behaviors. In addition to its best-established role in the short-term regulation of hunger, NPY-like signaling in invertebrates and vertebrates has been shown to be involved in the regulation of more stable changes in feeding-related behaviors over both organismal and evolutionary time. In *Drosophila*, NPF signaling controls the maturational transition from a feeding- to a non-feeding larval stage

(Wu *et al.*, 2003). Changes in NPY-related genes are also associated with the day-length induced onset of torpor in Siberian hamsters (Day *et al.*, 2005). On an evolutionary timescale, naturally occurring sequence variation in a NPY-family receptor of the nematode worm *Caenorhabditis elegans* influences the formation of aggregations on food (de Bono & Bargmann, 1998). Our results appear to show very stable changes in *npf* expression during maturation but more dynamic expression of *snpfR*, so expression data do not provide a unified answer as to the timescale on which NPY-like signaling is important.

Among the feeding-related pathways that have been previously studied in the bee, insulin/insulin-like growth factor signaling (IIS) is most clearly interrelated with NPY-like signaling. In flies, sNPF signaling stimulates the synthesis of insulin-like peptides in the fly brain (Lee *et al.*, 2008). Insulin signaling in *npfr1* neurons also is thought to block the effects of NPF signaling on food intake (Wu *et al.*, 2005b). Therefore, aspects of NPY-like signaling act both upstream and downstream of IIS to regulate feeding-related behaviors in flies, and the two NPY-like peptide systems appear to interact with IIS in opposite directions. Our results in the bee show that IIS-related genes are often regulated by both maturation and nutritional manipulations (Ament *et al.*, 2008), whereas NPY-related genes were regulated in one context but not the other. Therefore, insulin signaling seems to be more closely linked than NPY-like signaling to nutritionally-mediated behavioral maturation.

Interestingly, we found that *npf* and *ilp1*, the insulin-like peptide dominantly expressed in the bee brain, were both upregulated in forager brains compared to nurses. This may be surprising, given that in the fly *npf* and IIS signaling are opposed. However, we speculate that the simultaneous upregulation of these pathways makes sense in the context of foraging tasks. The combination of high NPY-like signaling and high IIS could make foragers sensitive to both hunger and satiety cues, contributing to their exaggerated responses to nutritional stimuli.

A growing body of evidence suggests that division of labor among workers in social insect colonies is regulated by conserved feeding-related genes that have taken on novel roles during the course of honey bee evolution. However, this framework does not necessarily imply that all such genes are involved in division of labor itself. Rather, it is likely that some genes regulate maturation while others participate in short-term food-related processes within each behavioral state. In the case of insulin signaling (Ament *et al.*, 2008) we found that the same genes could respond to both maturational stimuli and shorter-term nutritional changes. By contrast, NPY-like signaling genes responded to maturational stimuli or nutritional stimuli, but not both. Apparently, when it comes to the evolution of social behavior not all nutritionally-related pathways are created equal. Our results suggest that some elements of molecular pathways associated with feeding behavior may be more evolutionarily labile than others.

Experimental Procedures

Phylogenetic analyses

We searched for orthologs to *npf*, *snpf*, *snpfR* and *npfr1* in *A. mellifera*, *A. pisum* and *N. vitripennis* using tBLASTN searches of their genomic sequences available in GenBank using the sequences from *D. melanogaster*. Sequence orthology was determined based on reciprocal best BLAST hits and was further examined using CLUSTALW alignments. In addition, we searched for these genes in EST databases from 10 bee species (Woodard, Fischman, and Robinson, unpublished results) using the same methods.

Bees

Honey bees were collected from typical colonies headed by naturally mated queens at the University of Illinois Bee Research Facility, Urbana, IL, USA. One-day-old bees were obtained using standard methods (Ament *et al.*, 2008) by placing honeycomb frames with emerging brood into specially designed emergence cages overnight in a 34°C incubator and collecting bees from these cages the following day. Nurses and foragers were also identified by standard behavioral assays (Ben-Shahar *et al.*, 2002). Nurses were identified placing their heads into honeycomb cells containing larvae. Foragers were identified returning to the hive entrance with visible loads of pollen.

In situ hybridization

In situ hybridization was performed essentially as described previously (Velarde *et al.*, 2006). Probes were prepared from PCR products using specific primers with T3 and T7 promoters attached to the 5'- and 3' primers, respectively (primer sequences are shown in Table 1). Synthesis of riboprobes and digoxigenin-labelling were performed by means of *in vitro* transcription using Roche RNA Labeling Mix (Roche 1277073, Indianapolis, IN). Probes spanned the entire coding sequence of each of these short genes, and were 432 and 321 bases in length, for *npr* and *snpf*, respectively. Brains to be used in hybridization studies were dissected from the head capsule of cold-anaesthetized bees in a small drop of bee saline (Huang *et al.*, 1991). Dissected brains and/or whole pupae were immediately transferred to Cryo-M-Bed embedding compound (Bright Instrument Company Ltd, Huntingdon, UK), frozen on to cryostat chucks using powdered dry ice, sectioned at 10 µm, and thaw-mounted on to FisherPlus slides (Fisher Scientific, Pittsburgh, PA). After overnight air-drying, sections were fixed in 4% paraformaldehyde, deproteinized with proteinase K (Sigma P5568 St Louis, MO), and treated with acetic anhydride prior to hybridization with a digoxigenin-labelled riboprobe (1000 ng/ml) at 50 °C overnight in 50% formamide. Following posthybridization rinses, sections were incubated with a sheep antidigoxigenin-alkaline phosphatase antibody (Roche 1093274), treated with levamisole to block endogenous alkaline phosphatase activity, and developed in NBT/BCIP (Vector Laboratories, Burlington, CA). Developed slides were coverslipped with CrystalMount (Biomed, Foster City, CA) or glycerol. Sense strand probes were used as controls. All solutions used prior to hybridization were RNase-free.

Cage diet manipulations

Groups of bees of the desired age or behavioral group were collected directly into Plexiglas cages (11 × 11 × 7 cm). They were fed one of three diets: a) “poor diet”: 50% sucrose w/v in water; b) “rich diet”: 50% sucrose and a second feeder containing pollen paste (45% ground pollen / 45% honey / 10% water); or c) “alternate rich diet”: 50% sucrose and a second feeder containing soy paste (45% soy protein / 45% honey / 10% water). Diets were *ad libitum* and were replaced daily. We conducted two trials using “young bees”, which were one-day-old at the start of the experiment, reported in Fig. 3. In these trials, each cage contained 35 bees. These methods are the same as described in (Ament *et al.*, 2008) and include bees from two of the same trials. We performed two additional trials using nurses and foragers in addition to young bees (Fig. 4); in these trials cage density was reduced to 15 bees / cage to reduce forager mortality. After 3 days (Trials 1 and 2) or 5 days (Trials 3 & 4), bees were flash-frozen in liquid nitrogen to preserve RNA.

Tissue handling

Heads and abdomens were separated from other body parts on dry ice. Frozen heads were partially lyophilized in a vacuum freeze-dryer for 1h (300mTor, -80°C). Brains were then

dissected from head capsules in a dissection dish containing 95% ethanol on a bed of dry ice. Dissected brains were stored at -80°C for less than two weeks.

RNA extraction

Total RNA was extracted from brains and abdomens using the RNeasy Mini kit (Qiagen, Valencia, CA), including an on-column DNase digestion (RNase-free DNase kit, Qiagen). Total RNA yields were determined using a NanoDrop 1000 spectrophotometer and were ca. 1–2 μg from brains and ca. 25–50 μg from abdomens. All samples had very low contamination ($\text{OD } 260/280 > 2.0$). Total RNA in ddH₂O was stored at -80°C .

Reverse transcription

200ng total RNA was converted to cDNA with ArrayScript reverse transcriptase (RT; Ambion), using random hexamers as primers. 20 μl reactions containing RNA, 40U RT, 625ng primers, RNase Out (Invitrogen), and RT buffer were incubated 1h at 42°C . In most experiments, 100pg of an exogenous RNA (*Arabidopsis thaliana XCP1*) was added into the RT reaction to monitor the efficiency of RNA-cDNA conversion (Ament *et al.*, 2008). cDNA was diluted with ddH₂O to a total volume of 200 μl and stored at -20°C or -80°C .

Quantitative PCR (qPCR)

Primers specific to *npf* (GB16364), *snpf* (GB16965), *snpfR* (GB30377), the endogenous control *Rp49* (GB10903), and the exogenous control (*Arabidopsis thaliana XCP1*) were designed to amplify 60–100bp regions of each gene (Table 1). We used an ABI Prism 7900 sequence detector and triplicate 10 μl reactions for each sample. Each reaction contained 3 μl diluted cDNA, 0.25 μM forward and reverse primers, and Sybr Green qPCR master mix (Applied Biosystems or Roche). 35 PCR cycles were performed using standard two-step cycling parameters. Transcripts for each *A. mellifera* gene were quantified relative to a genomic DNA standard curve, and *A. thaliana XCP1* was quantified using a standard curve derived from a plasmid clone of that gene.

Normalization procedures varied slightly between experiments, depending on the performance of the endogenous and exogenous controls. For nurse and forager brains and for the colony food-deprivation experiment, we report expression of each gene relative to *Rp49*. In the diet quality experiments, we report expression of each gene relative to *XCP1* (Trials 1 and 2) or normalized to total RNA (Trials 3 and 4). All of these normalization procedures have been previously shown to yield high-quality, replicable results (Corona *et al.*, 2005; Ament *et al.*, 2008; Toth *et al.*, 2007).

Injections of synthetic NPF into the brain

Custom honey bee NPF peptide with the sequence EPEPMARPTRPEIFTSPEELRRYIDHVSDDYLLSGKARY-NH₂ was synthesized by Global Peptide (Fort Collins, CO). Peptide was dissolved in ddH₂O. Peptide solutions were injected into the brain via the central ocellus using a 35 G beveled needle and a syringe attached to a controlled volume microdispenser and a micromanipulator device. In preliminary experiments, a dose of 10 μg was determined to cause approximately 50% mortality (data not shown). We subsequently used a dose of 1 μg delivered in a volume of 200 or 400 nL.

Effects of NPF injections on food intake

These experiments were performed in early summer using bees from 3 typical honey bee colonies. Bees were collected at midday foraging at a pollen feeder. They were collected into Plexiglas cages and fed 50% sugar syrup overnight. The feeder was removed in the

morning, and bees were food deprived 4–6 h. Bees were then injected with NPF peptide. After injection, groups of 10 bees injected within 15 minutes were placed into Plexiglas cages and fed 50% sugar syrup. Food intake was recorded on a per cage basis every hour for 4 hours from paired cages of bees injected with NPF or a control water injection. Data were analyzed from 5 trials. Two additional trials were excluded because 2 or more bees died in one of the cages.

Statistics

After the normalization procedures described above, qPCR data were analyzed using Mixed Model ANOVA (PROC MIXED, SAS Institute, Cary, NC). For most experiments, all pairwise comparisons were computed and statistical significance was assessed using Tukey's method. In the experiment involving diet manipulations of both young and old bees we performed a Mixed Model ANOVA including all groups in the experiment then computed only planned contrasts between nurses and foragers within each diet group, with Bonferroni corrections for multiple comparisons. Food intake data were analyzed using a paired t-test.

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Figure 1. *npf* and *snpf* are expressed in distinct populations of neurosecretory cells in the honey bee brain

In situ hybridization of digoxigenin-labeled probes specific to *npf* (A) and *snpf* (B) to 10µm coronal cryosections of forager brains. The mushroom body calyces are shown at top in 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 = 400µm.

Figure 2. Expression of NPY-like signaling genes in nurses and foragers

RT-qPCR was used to measure the expression of *npf*, *snpf*, and *snpfR* in whole brains (A) and of *npf* in whole abdomens (B) of nurses and foragers from typical honey bee colonies. Expression values for nurse and forager in each trial are shown as fold changes relative to nurse (mean set at 1 for each trial). Mixed Model ANOVA for combined trials: *npf*: $P_{\text{group}} < 0.0001$, $P_{\text{group} \times \text{colony}} < 0.0001$; *snpf*: $P_{\text{group}} > 0.05$, $P_{\text{group} \times \text{colony}} > 0.05$; *snpfR*: $P_{\text{group}} > 0.05$, $P_{\text{group} \times \text{colony}} < 0.0001$. Student's t-test for individual trials: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 3. Effects of nutritional manipulations on NPY-like signaling genes

(A) Expression of *npf*, *snpf*, and *snpfR* in brains of worker honey bees following manipulations of diet quality. Bees were placed into cages at one day of age and fed either a nutrient poor diet (“sugar”) or a nutrient-rich also containing pollen/honey paste (“sugar +pollen”). $N = 9-10$ bees / trial. (B) 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 two days followed by two days without food. $N = 9-10$ bees / trial. Mixed Model ANOVA for food-deprivation: *npf*: $P_{\text{trt}(1,42)} > 0.05$, $P_{\text{trt} \times \text{trial}(2, 42)} > 0.05$; *snpf*: $P_{\text{trt}(1,27)} > 0.05$, $P_{\text{trt} \times \text{trial}(2,27)} < 0.01$; *snpfR*: $P_{\text{trt}(1,44)} < 0.0001$, $P_{\text{trt} \times \text{trial}(2,44)} > 0.05$. Student's t-test for individual trials: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 4. Maturation differences in brain *snpfR* expression depend on nutrition

One-day-olds (“young bees”), nurses, and foragers were collected from a typical colony and placed into cages. Bees were fed for five days with one of three diets: sugar syrup (“sugar”), sugar syrup and pollen/honey paste (“sugar+pollen”), or sugar syrup and soy protein/honey paste (“sugar+soy”). Transcript abundance for *npf*, *snpf*, and *snpfR* was measured in brains. Pooled data are shown from two independent trials. $N = 20$ bees. Mixed-model ANOVA for all groups, followed by planned contrasts between nurses and foragers with Bonferroni corrections for multiple comparisons: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 5. Injection of NPF peptide into the brain does not influence food intake
Sugar syrup intake of foragers after injection with 1 μ g NPF peptide into the brain via the ocellar tract; controls were injected with vehicle only (water). Results represent the aggregate food intake of 10 bees placed together in a cage. n=5 trials / group.

Table 1
Primer sequences for *in situ* hybridization and qPCR

The following primers were used to amplify PCR products for *in situ* hybridization and during qPCR. “GB” gene names refer to *A. mellifera* Official Gene Set 2 annotations. The position of each primer relative to the 5'ATG and the strand it matches (Forward, Reverse) are indicated in the primer name.

Gene	Primer	Sequence
<i>npf</i> (GB16364)	T7-NPF-1F	ATTAGCGGCCGCATGCAATCGTATTC
<i>npf</i> (GB16364)	T7-NPF-432R	ATTAGGTACCTTATTGCACATCATCAT
<i>snpf</i> (GB19695)	T7-sNPF-1F	ATTAGCGGCCGCATGCCAATCAAGTT
<i>snpf</i> (GB19695)	T7-sNPF-321R	ATTAGGTACCTTTATCATCGAATTTG
<i>npf</i> (GB16364)	NPF-319F	GAACCTTTGGGTGAGCACGAA
<i>npf</i> (GB16364)	NPF-392R	ACGTGACAAGGTCGAGTATCGA
<i>snpfR</i> (GB30377)	snpfR-232F	AATTTGGCATTGAGCGATGTT
<i>snpfR</i> (GB30377)	snpfR-302R	CCGAGGAACGTGTACAATGGT
<i>Rp49</i> (GB10903)	Rp49-147F	GGGACAATATTGATGCCCAAT
<i>Rp49</i> (GB10903)	Rp49-246R	CTTGACATTATGTACCAAACTTTTCT
<i>A. thaliana XCP1</i>	XCP1-11F	TCAATTAACCTCGGAATCGGA
<i>A. thaliana XCP1</i>	XCP1-75R	CCTGGATTTCCTGCTGAT