NUTRITION, HORMONES, TRANSCRIPTIONAL REGULATORY NETWORKS AND DIVISION OF LABOR IN HONEY BEE COLONIES

BY

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DISSERTATION

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Abstract

Phenotypic plasticity – one genotype producing alternative phenotypes – is increasingly understood to be an important force in phenotypic evolution, but its mechanistic basis remains poorly understood. This thesis describes research into the molecular mechanisms underlying age-related behavioral and physiological plasticity in worker honey bees.

Many animals are able to alter their behavior and physiology in response to changes in the environment. At times, these changes in behavior and physiology are stable for long periods, a phenomenon known as phenotypic plasticity [1]. For instance, short periods of food deprivation stimulate feeding and the mobilization of stored nutrients to meet an individual's immediate energetic needs. But prolonged food deprivation can also lead to much longer-term effects, causing individuals to enter extended periods of inactivity, alter their reproductive strategy, or lose their position in a dominance hierarchy. In humans, chronic food deprivation early in life may lead to a propensity toward obesity and diabetes in later life (for an expanded and fully-referenced discussion of nutritionally-mediated phenotypic plasticity see Chapter 4). The mechanisms that enable and constrain plasticity in behavior and physiology are not well understood, but it is clear that they often involve coordinated and long-lasting changes in gene expression, brain circuitry, brain chemistry, and endocrine signaling [2]. My doctoral research has focused on understanding the molecular basis for nutritionally- and hormonally-mediated plasticity in the behavior and physiology of worker honey bees.

Honey bees are social insects, living together in colonies containing tens of thousands of individuals [3]. Colony life is organized by a complex and sophisticated division of labor. Each colony contains a single queen, who is specialized for reproduction and spends most of her time laying eggs. Males, called drones, are relatively rare, and their sole role is to mate. The vast majority of the individuals in the hive are sterile worker bees that are responsible for all of the other tasks performed by the colony. The tasks performed by worker bees are further divided up among individuals via a process of behavioral maturation that is the focus of this thesis. For the first 2-3 weeks of adult life, worker bees specialize on broodcare ("nursing"). They then switch for a few days to any of a number of more specialized tasks such as building honeycomb cells, storing food in honeycomb cells, or guarding the hive entrance against intruders. Finally, for the remaining 1-2 weeks of their life, worker bees forage outside the hive for nectar and pollen, the colony's sole sources of food.

The work presented in this thesis builds on previous findings demonstrating links between worker honey bee division of labor and nutrition (reviewed in Chapter 4). Behavioral maturation in worker bees is coupled to changes in nutritional physiology, including a dramatic and stable loss of abdominal lipid that occurs prior to the onset of foraging. Moreover, previous studies had demonstrated that nutritional status can have causal influences on the timing of behavioral maturation and manipulations of a few feeding- or nutritionally-related genes accelerates or delays the age at onset of foraging.

In the work described here, I first test the hypothesis that worker bee behavioral maturation, a highly derived trait, is regulated, in part, by conserved nutritionally-related hormones (Chapter

1). I demonstrate that genes related to insulin signaling are differentially expressed in the brains and fat bodies of nurses and foragers. Furthermore, I show that manipulation of the insulin-related TOR pathway influences the age at which bees initiate foraging. These results suggest that the evolution of honey bee social behavior involved new roles for ancient nutritionally-related pathways. However, my subsequent work shows that not all nutritionally-related pathways have been coopted in the same way. I describe a more complex, and less resolved, relationship between behavioral state, nutrition and brain gene expression for a second nutritionally-related hormone, Neuropeptide Y (Chapter 2).

Next, using transcriptomic experiments, I demonstrate that maturation, as well as age-related stable lipid loss, involve massive changes in gene expression in the fat bodies (Chapter 3). I show that these changes in gene expression involve age-related changes in the responsiveness of hormonally and metabolically related pathways to nutrition, and roles for two evolutionarily novel, non-dietary factors: the storage protein vitellogenin and Queen Mandibular Pheromone, each of which influenced many maturationally-related genes in the fat bodies. These results also suggest the involvement in the responses to all these factors of a single nutritionally-related hormone, juvenile hormone (JH), which had previously been shown to pace behavioral maturation.

In Chapter 4, I review my findings from chapters 1-2 of this thesis, and previous studies, and propose a molecular systems biology approach to understanding division of labor. Specifically, I propose that phenotypic plasticity in worker honey bees involves nutritionally- and hormonally-

driven changes in transcriptional regulatory networks in the fat bodies (as well in the brain), and I suggest methodologies for their elucidation.

Finally, in Chapter 5, I utilize the molecular systems biology approach outlined in Chapter 4 to show that a transcriptional regulatory network in the fat bodies underlies division of labor. I show that a juvenile hormone-related transcription factor, *Ultraspiracle (USP)*, influences the age at onset of foraging. I then use a combination of chromatin immunoprecipitation—genomic tiling microarrays, RNAi and deep mRNA sequencing to develop a model of the *USP* transcriptional regulatory network in fat cells. My results suggest that JH and *USP* function together to induce and maintain alternative states of a transcriptional regulatory network. These alternative states may well underlie the two basic phases of worker bee life, the in-hive and foraging phases.

Together, the studies presented in this thesis provide insights into the relationship between nutrition, hormones, transcriptional regulation, and phenotypic plasticity.

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Chapter 1

Insulin signaling is involved in the regulation of worker division of labor in honey bee colonies

Previously published work[1]

Abstract

It has been proposed that one route of behavioral evolution involves novel regulation of conserved genes. Age-related division of labor in honey bee colonies, a highly derived behavioral system, involves the performance of different feeding-related tasks by different groups of individuals. Older bees acquire the colony's food by foraging for nectar and pollen, while the younger "nurse" bees feed larvae processed foods. The transition from hive work to foraging has been shown to be socially regulated and associated both with decreases in abdominal lipid stores and increases in brain expression of genes implicated in feeding behavior in *Drosophila*. Here we show that division of labor is influenced by a canonical regulator of food intake and energy balance in solitary species, the insulin/insulin growth factor signaling (IIS) pathway. Foragers had higher levels of IIS gene expression in brain and abdomen than nurses, despite their low lipid stores. These differences are likely nutritionally mediated because manipulations that induced low lipid stores in young bees also upregulated these genes. Changes

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[1] Ament SA, Corona M, Pollock HS, and Robinson GE. 2008. Insulin signaling is involved in the regulation of worker division of labor in honey bee colonies. *Proc Natl Acad Sci USA* 105 (11): 4226-31.

in IIS also causally influenced the timing of behavioral maturation: inhibition of the insulin-related TOR pathway delayed the onset of foraging, in a seasonally dependent manner. In addition, pathway analyses of microarray data revealed that nurses and foragers differ in brain energy metabolism gene expression, but the differences are opposite predictions based on their insulin signaling status. These results suggest that changes in the regulation of the IIS pathway are associated with a novel role in social behavior.

Introduction

An important problem in biology is to understand the molecular basis for complex behavior. It has been proposed that one route of behavioral evolution involves new regulation of conserved genes (1). It is well established that orthologous sets of genes regulate the development of body plans across taxa (2), but this idea has only recently begun to be tested for behavior (3, 4).

Age-related division of labor in honey bee colonies involves the performance of different feeding-related tasks by different groups of individuals. Worker bees provision brood as "nurses" for the first 1-2 weeks of adult life, process and store food for another week, and then shift to foraging for nectar and pollen at about 2-3 weeks of age (5). This division of labor is socially regulated; bees speed up, slow down or reverse their maturation in response to colony needs (6). Although the mechanics of foraging in honey bees are similar to food-gathering in solitary bees, there are fundamental differences. Honey bees forage to improve the fitness of the colony rather than their own; they collect food when their colony needs it. Honey bees feed on honey before exiting the hive in order to fuel their foraging flights and most of the food obtained on a foraging trip is not for their own sustenance.

We hypothesized that the regulation of honey bee behavioral maturation involves novel roles for widely conserved nutrient-sensing or metabolic pathways, for the following reasons. Nutrition has an important role in honey bee age-related division of labor (5). The onset age of foraging is affected by experimentally induced changes in nutritional status (7, 8) and the expression of a nutritionally-related gene (9). Onset age of foraging also is affected by experimentally induced changes in the expression of genes related to feeding behavior in *Drosophila* (10, 11). Nurses have much larger lipid and protein nutrient stores than foragers (12). Large lipid stores may be functionally associated with nursing behavior because bees that are forced to revert from foraging to brood care do not regain large lipid reserves and are not as good at rearing brood as typical nurses (13). The striking loss of abdominal lipid that occurs prior to the onset of foraging (12) is thought to increase individual foraging performance (14). Nutritional differences between nurses and foragers occur even though all colony members are exposed to the same food stores inside the hive, further suggesting close coupling of nutritional status and behavior.

Insulin/insulin-like growth factor signaling (IIS) is a key regulator of both metabolism (15) and feeding-related behavior (16). Food intake or high levels of nutrient stores leads to enhanced synthesis of insulin (17) or (in insects) insulin-like peptides (ILPs) (18), and represses the synthesis of glucagon, or its insect equivalent adipokinetic hormone (AKH) (19). IIS also upregulates both the intracellular TOR pathway (20) and juvenile hormone (JH) (21, 22). JH is known to be involved in the regulation of honey bee behavioral maturation (23, 24).

We tested the hypothesis that behavioral maturation in honey bees entails novel regulation of IIS with gene expression analyses, behavioral analyses of foraging ontogeny following pharmacological manipulations, and pathway analyses of microarray data.

Results

Characterization of insulin-related neuropeptide and receptor genes. The honey bee genome contains genes encoding two insulin-like peptides (*AmIlp1* and *AmIlp2*), adipokinetic hormone (*AmAkh*), and the putative receptors for these peptides (*AmInR1, AmInR2,* and *AmAkhR*) (25-27). Several lines of evidence indicate a role for *AmIlp1* as a functional insulin propeptide gene. *AmIlp1* is positively regulated in larvae by good larval nutrition, and is much more highly expressed in larvae than *AmIlp2* (27). Phylogenetic analysis also indicates that *AmIlp1* is more closely related to other insulin-like peptides than is *AmIlp2* (Appendix A, Fig. A.1).

Experiment 1: Insulin signaling gene expression is higher in foragers than nurses. Experiments in *Drosophila* have shown that misregulation of ILPs and AKH in brain is sufficient to alter many IIS functions (19, 28), whereas insulin receptor signaling has distinct roles in central and peripheral tissues (29, 30). We therefore focused on the expression of ILPs and AKH in brain and expression of receptors in both brain and abdominal tissues. Abdominal gene expression represents a composite of several target tissues for these peptidergic systems (including gut and fat body).

Brain *AmIlp1* in brain and abdomen *AmInR1* and *AmInR2* were significantly more highly expressed in foragers than nurses, (Fig. 1.1A). *AmAkh* and *AmAkhR* expression did not differ

consistently between nurses and foragers (Appendix A, Fig. A.2). These results indicate that despite low adiposity relative to nurses, foragers have enhanced insulin production and responsiveness.

Experiment 2: Foraging-related upregulation of *ilp1* and *inR1* is due to nutritional status. Results from dietary manipulations support the hypothesis that differences between nurses and foragers in IIS gene expression are caused by the decline in nutritional status associated with behavioral maturation. Caged 4- and 6-day-old bees fed only sugar had significantly higher expression of brain (or head) *ilp1* (Fig. 1.1B) and both brain and abdomen *inR1* (Fig. 1.1B) than did bees fed sugar and (lipid- and protein-rich) pollen. Nutritional differences between the two diets are reflected by findings that the sugar-only diet resulted in significantly lower (forager-like) lipid stores and mRNA levels for *vitellogenin (vg)* (Appendix A, Fig. A.3). VG is a principal storage protein in bees that is typically more abundant in nurses than foragers (31).

Poor nutrition also increased brain *ilp1* expression in the field (Fig. 1.1C), but results varied with season and colony size. In trials performed in early summer using small colonies, chronic food deprivation increased brain *ilp1* in 5-day-old bees relative to same age bees from well-fed control colonies. The same effect was seen in late summer in (typical) large sized colonies (one trial), but in small colonies food deprivation decreased brain *ilp1*in late summer. These results indicate that poor nutrition can increase brain *ilp1* in the field as in the laboratory, but this effect depends on seasonal factors that are less potent in larger colonies. Given that foragers are less adipose than nurses, these results suggest that upregulation of *ilp1* and *inR1* in foragers is due to their decreased nutritional status.

Experiment 3: TOR nutrient-sensing pathway affects behavioral maturation. We tested whether insulin-signaling pathways influence honey bee behavioral maturation by determining the effect of oral treatment of rapamycin (a TOR inhibitor) on the age at onset of foraging. We hypothesized that rapamycin delays the onset of foraging because increased IIS up-regulates the TOR pathway (20).

Rapamycin delayed the onset age of foraging in a seasonally-dependent manner (Fig. 1.2A). Rapamycin caused a significant delay in foraging ontogeny in a combined analysis of 5 trials conducted during early summer. By contrast, rapamycin did not influence the age at first foraging in an analysis of 4 late summer trials. There was a significant interaction between the effects of rapamycin treatment and season.

The seasonal effect of rapamycin is consistent with other seasonal changes related to division of labor. Late-summer bees initiate foraging later in life than early-summer bees in temperate climates (32), as in our experiments (Fig. 1.2A); this is associated with maintenance of larger lipid stores later in life (33, 34) and lower blood titers of JH (35). These changes enable late-summer bees to overwinter inside the hive and survive several months longer than do bees emerging earlier in the summer (5).

Although the seasonal effect of rapamycin is consistent with the nutritional and endocrine seasonal changes, results from two 2x2 factorial experiments revealed that they are not causally related. These were conducted independently of the original rapamycin experiments, in a second

field season. As in the first field season, rapamycin delayed the onset of foraging in early summer but not late summer (Fig. 1.2B, C). Methoprene (a JH analog) caused precocious foraging as expected (24) but did not significantly interact with rapamycin to regulate the onset age of foraging and did not alter the seasonal change in response to rapamcyin (Fig. 1.2B). Similarly, bees fed a richer diet (honey and pollen) showed a trend toward a later onset of foraging relative to those fed sugar alone (a weaker effect than expected from ref. (7), but in the same direction), but diet did not significantly interact with rapamycin to regulate the onset of foraging and did not alter the seasonal change in response to rapamcyin (Fig. 1.2C). These results indicate that factors other than JH and nutrition mediate the seasonal differences in response to rapamycin.

We did detect a seasonal change in insulin signaling itself that might explain the rapamycin results. We performed additional qPCR experiments to examine trial by trial variation in brain gene expression between collections of nurses and foragers made early and late in the summer. In trials using small colonies (the same colonies as in Experiment 1) there was a late summer decline in *ilp1* expression in forager brains (Fig. 1.2D) but not in large colonies (Fig. 1.2E). Although these data come from only one field season, they suggest that insulin signaling is sensitive to seasonal factors, but as was true for food deprivation, large colonies are buffered from these seasonal changes. As our experiments with rapamcyin were performed with small colonies, the effect on foraging ontogeny may have disappeared late in the summer because IIS was already low in these colonies.

Experiment 4: Up-regulation of brain energy metabolism pathways in nurses despite low insulin signaling. We obtained additional evidence for novel regulation of insulin signaling by annotating energy metabolism pathways with results from three previously published microarray experiments (36, 37). These experiments produced three lists of genes that were: 1) differentially expressed in the brains of nurse bees and foragers; 2) in response to methoprene, which accelerates the onset age of foraging (23); and 3) in response to queen mandibular pheromone (QMP), which delays the onset age of foraging (38). Insulin signaling both regulates and is regulated by changes in energy metabolism, and generally there is a positive correlation between insulin signaling and energy metabolism gene expression in a variety of species and tissues (39-41). To test whether this positive relationship is present in the honey bee brain, we mapped genes to conserved energy metabolism KEGG pathways (42)) and performed statistical tests for enrichment and representational bias on each list of genes generated by the microarray experiments (37).

The list of genes differentially expressed in the brains of nurses and foragers was not significantly enriched for energy metabolism genes (37). However, differentially expressed energy metabolism genes were predominantly upregulated in nurse bees (Table 1.1), including 5 of 6 in the citrate cycle (Fig. 1.3). This suggests that the brains of nurses have higher capacity for energy metabolism than forager brains, in contrast to their lower IIS gene expression. By contrast, foragers have higher overall metabolic rate (43) and expression of oxidative phosphorylation genes in thorax and abdomen (44), and proteomic analyses show higher protein expression for several classes of energy metabolism enzymes in whole body samples of foragers

compared to nurses (45). These differences likely reflect the activity of tissues such as flight muscle that are more directly involved in increased forager metabolism.

Although circulating titers of JH are higher in foragers than nurses, treatment with the JH analog methoprene caused a nurse-like shift in brain energy metabolism gene expression. Energy metabolism genes were enriched among genes regulated by methoprene in the brain, but they were upregulated (Table 1.1). This is in contrast to the finding that methoprene causes forager-like changes in overall brain gene expression (36, 37). QMP causes nurse-like overall changes (36) but did not affect brain energy metabolism gene expression (Table 1.1).

Discussion

Molecular pathways that regulate hunger and food gathering behavior in solitary species influence the age at which worker honey bees shift from working in the hive to collecting food for their colony. This means that the regulation of honey bee behavioral maturation involves novel roles for widely conserved nutrient-sensing or metabolic pathways, in addition to previously implicated feeding-related (10, 11) and nutritionally-related genes (9).

The finding that IIS gene expression is upregulated in the brain by low nutrient stores and in foragers (previously reported in (22)) differs from commonly observed patterns of expression in other species in two ways. First, the direction of the response is reversed; high levels of nutrient stores typically lead to enhanced insulin signaling (17, 18). Second, whereas we found that *AmIlp1* and *AmInR1* expression were positively correlated, insulin signaling activity downregulates insulin receptor gene expression in *Drosophila* and in vertebrate cell lines by

inhibiting FoxO (46, 47). This feedback results in a homeostatic mechanism that ensures a rapid but brief response to nutritional changes.

Our results suggest roles for insulin signaling in brain and fat body. Increased *ilp1* production in the brain may influence behavior through local action on neuronal circuits that control foraging and may also affect non-brain targets, such as the fat bodies in the abdomen. High levels of *inR1* and *inR2* in abdomen should maximize the responsiveness of abdominal tissues to circulating ILPs. However, we cannot discern if the increase in insulin signaling during behavioral maturation is a cause or consequence of lipid loss. A few studies in other insect species suggest that insulin-like peptides can have catabolic functions in insects (48), so a causal relationship is possible. The nature of this speculative brain-abdomen communication system in bees is unknown but similar systems are well studied in vertebrates (49).

It is possible that the combination of high brain *ilp1* and high abdominal *inR1* in foragers reflects a change in the adipostatic set point relative to nurses, rather than the traditional homeostatic mechanism associated with insulin signaling. In this view, the combination of high insulin synthesis and high insulin sensitivity maintains, or perhaps causes, a shift from high to low adiposity during behavioral maturation (and in response to experimental nutritional manipulations). Similar reasoning has been used to explain relationships between nutrient-sensing pathways and variation in nutrient stores in the contexts of mammalian torpor (50, 51) and insect diapause (52).

"Reversed" IIS gene expression and the suggested set point regulation do not occur in all contexts in honey bees. More typical homeostatic regulation is seen during larval development; *ilp1* in honey bee larvae is upregulated by good nutrition (27). It is not known why these differences in IIS in honey bees appear to be limited to behavioral maturation. Perhaps this is because the system of social foraging in honey bees requires that they forage when they are not personally hungry.

There were seasonal changes in IIS brain gene expression and the effects of IIS on behavioral maturation, but these were limited to small, not large, colonies. We speculate that this might have been because large colonies are able to maintain more stable levels of food stores (5), and that the seasonal effects we detected in late summer in small colonies would have been detected in large colonies sampled later in the fall than we did. It is possible that our use of small colonies made it easier to expose the seasonal effects of IIS in honey bee colonies.

A surprising result was that the transition from in-hive tasks to foraging is associated with a decrease in whole brain energy metabolism gene expression that does not appear to be caused either by insulin or by JH, two hormones that have causal effects on behavioral maturation. Alternatively, insulin might regulate these changes, but in the opposite direction to other tissues and species. Perhaps high levels of brain energy metabolism are required in nurses for energy-intensive processes such as brain plasticity that are not necessarily correlated with metabolism in other tissues. Changes in brain morphology occur throughout the lifespan of worker honey bees, but are more intense in young bees (53).

Another explanation for the high levels of brain energy metabolism in nurse bees is that whole brain analyses of energy metabolism pathways do not adequately reflect what is going on in specific brain regions. In most insect brains insulin-like peptides are produced primarily in a small cluster of neurosecretory cells (48) and the distribution of insulin receptors in the bee brain is not known.

Insulin signaling influences diverse aspects of phenotypic plasticity in honey bees. Insulin signaling has been implicated in the regulation of caste (queen vs. worker) determination in honey bees (27, 54), and insulin signaling genes are among the more promising candidate genes located in quantitative trait loci associated with genetic variation for honey bee foraging behavior (55). Several models have been proposed to explain how insulin signaling can influence diverse aspects of phenotypic plasticity in honey bees (22, 55, 56). Our experiments confirm a specific prediction of Corona *et al.* (22) by showing that low nutrient stores can increase insulin signaling. However, the context-specificity of this effect implies that interactions between insulin signaling, nutrition, JH, Vg, and the environment are more complicated than had previously been imagined.

Our results support the notion that molecular pathways that govern nutritional state and feeding behavior in solitary animals represent one "toolkit" that can be used in the evolution of division of labor in social insects (4). Learning how and why some components of insulin signaling pathways are more evolutionarily labile than others will help understand the molecular basis of behavior.

Methods

Behavioral collections. Honey bees (*Apis mellifera*) were collected from four small colonies (~10,000 bees; Experiment 1, Trials 1-4) and two large colonies (~30,000 bees; Trials 5-6) while performing nursing or foraging behaviors, observed and identified in typical fashion (10). Nurses were collected after they repeatedly placed their heads into honeycomb cells containing larvae (10) and foragers, as they returned to the hive with visible loads of pollen on their legs. In Experiment 4, nurse bees were identified by age and location in the hive, not behavior; these also are robust identification methods (57). Upon capture, bees were flash-frozen in liquid nitrogen, and qPCR analyses were performed on insulin signaling and AKH signaling genes in brain and abdomen. Collection timing in Results.

Nutritional and pharmacological treatments. One-day-old bees were obtained by removing frames of pupae from typical field colonies and placing them in an incubator (34°C and 80% relative humidity). For behavioral analyses, bees were marked with a dot of colored paint (Testor's PLA) on the thorax. Groups of 35 (lipid analyses) or 50 bees (behavioral and molecular analyses) were placed into Plexiglas cages (36), caged for 3-5 days in constant darkness, and fed *ad libitum* a sugar diet (sugar syrup: 50% sucrose/water w/v; or sugar bee candy: 80% confectioner's sugar, 20% sugar syrup) or pollen paste (45% pollen/45% honey/10% water). Rapamycin (LC Labs, Woburn, MA) was delivered chronically during the entire caging period and delivered orally, 10mg/g in food. Methoprene was delivered chronically for the entire caging period and delivered orally, 4mg/g food (58). For analyses of foraging ontogeny, bees were placed into colonies after 3 days. For lipid and RNA analyses, bees were flash frozen in liquid

nitrogen (10). qPCR analyses were performed on the genes that showed the most consistent differences between nurses and foragers, *ilp1* and *inR1*.

Food-deprivation. Paired single-cohort colonies were established (7) each with a queen and 1200 one-day-old bees derived from the same source colonies. Food-deprived colonies were fed honey for 2 days, then food-deprived completely for 2 days. Well-fed colonies were provided excess honey and pollen for the entire trial. For food-deprivation of colonies with typical age demographies, one-day-old bees were marked with paint for identification and placed into a pair of size-matched colonies. Two days later, all honeycomb frames that contained food were removed from one colony and replaced with empty frames, while the paired colony was mock manipulated. After 1-2 days of food deprivation, focal bees were collected. One trial was performed using small colonies (~10,000 bees, occupying one Langstroth hive box) and one trial was performed using large colonies (~30,000 bees, occupying 3 hive boxes). Bees were collected by flash freezing at dawn when they were 5-days-old, prior to the onset of foraging, and qPCR analyses were performed on *ilp1* and control gene expression in brains or heads.

RNA extraction and qPCR. Total RNA was extracted from dissected brains (44), whole heads, or whole abdomens. cDNA was synthesized from 200ng total RNA. qPCR was performed using an ABI Prism 7900 sequence detector using specific primers (Table S1). Results for experimental genes were normalized to a validated control gene, *rp49*, using a standard curve or ddCt method or to an exogenous RNA spiked into a master mix prior to cDNA synthesis (22, 44) (Table 1.2).

Quantification of abdominal lipid. Lipid from abdominal fat bodies was extracted in chloroform/methanol and quantified using a colorimetric assay with vanillin/phosphoric acid (8).

Behavioral analyses of age at first foraging. Methods were slightly modified from (11). After 3 days of treatment in cages, all surviving bees (>90% survival) were placed into a single-cohort colony made with 1000 one-day-old (untreated) bees and a queen. Each trial included 1-3 cages (50-150 bees) per group. Colonies were observed for at least 3 h/day for the following 5-7 days, including the first 5 days on which bees foraged. Bees were captured briefly as they returned from their first foraging flight, identified by treatment group, and marked with an additional dot of paint on the abdomen so that they could be identified as experienced foragers. At the conclusion of the experiment, colonies were killed by flash-freezing with liquid nitrogen, and all focal bees remaining in the hive were censored. We performed 9 independent trials with rapamycin-treated bees and untreated controls fed sugar bee candy, 4 trials with combinatorial rapamycin and methoprene treatments fed pollen paste, and 2 trials with rapamycin treatments in combination with sugar bee candy or pollen paste diet. Dates of trials in Results.

Statistical analyses. Following normalization procedures, qPCR and lipid data were analyzed using 1-, 2-, or 3-factor ANOVA in SAS (PROC MIXED). ANOVA was followed by pairwise comparisons with Tukey post-hoc corrections for multiple comparisons. Age at first foraging results were treated as survival data and analyzed using Cox Proportional Hazards (PROC PHREG). Unless otherwise indicated, the main effect of trial was confounded with variation between qPCR runs so is not shown. Main effects of group, treatment or diet for pooled trials are shown.

Pathway analyses of microarray data. Lists of differentially expressed genes (ANOVA; nurse vs. forager: $p < 0.001$; methoprene vs. untreated: $p < 0.05$; QMP vs. solvent: $p < 0.05$) were obtained from three previously conducted microarray experiments (36, 37). These lists were annotated with a revised set of *Apis-Drosophila melanogaster* orthologs kindly provided by C. Elsik, Texas A&M University (unpublished data). Based on these orthologs, we mapped the gene lists to the following KEGG energy metabolism pathways: oxidative phosphorylation (map00190); citrate cycle (TCA cycle; map00020); glycolysis (map00010); fatty acid metabolism (map00071) (42)). We did this with online GeneMerge (59), and visualized these maps by manually annotating KEGG pathways with honey bee orthologs (www.genome.jp/KEGG). We performed two statistical tests on the results. First, in GeneMerge, we tested whether differentially expressed genes were enriched for energy metabolism pathways relative to the reference population of genes on the array (hypergeometric test). Second, we tested whether there was a representational bias among differentially expressed genes in each pathway towards higher expression in hive bees or foragers (chi-square test).

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Tables for Chapter 1

Table 1.1. Tests for enrichment and directional biases of energy metabolism genes on cDNA microarrays. Published expression data (37) were mapped to KEGG energy metabolism pathways. Enrichment (over- or under-representation) of differentially expressed genes in each pathway was determined using a hypergeometric test, and directional biases were determined using a chi-square test. Ratios in bold differed significantly from null hypotheses: ** $P < 0.01$; *** $P < 0.001$.

Gene Set	*Nurse vs. Forager*	*Methoprene vs. Control*	*QMP vs. Control*
Enrichment: (up + down) / all genes			
All genes	1086/2094	659/2128	323/2044
Oxidative phosphorylation	15/23	24/32***	2/22
Citrate cycle	6/8	5/10	2/7
Glycolysis	6/11	5/14	2/11
Fatty acid metabolism	4/11	3/10	3/11
Combined energy metabolism	30/51	37/63***	8/49
Directional bias:	nurse-high : forager-high	down : up	up : down
All differentially expressed genes	546:540	269:390	123:200
Oxidative phosphorylation	13:2**	0:24***	1:1
Citrate cycle	5:01	0:05	1:1
Glycolysis	5:01	0:05	2:0
Fatty acid metabolism	3:01	0:03	2:1
Combined energy metabolism	25:5***	0:37***	5:3

Table 1.2. Primer sequences used for qPCR. Sequences in a 5'-3' order. Forward primer, F; Reverse primer, R; TaqMan probe, P. The length of the PCR products is shown between parentheses..qPCR in Experiments 1 and 3 was performed using Sybr Green tehnology and normalized to *Rp49* using a standard curve method. Experiments 2A (Trials 1-2), 2B, 2D, and 2E (Trials 1-3) were performed using Sybr Green technology and normalized to *rcp1.* Experiments 2A (Trials 3-5), 2E (Trials 4-5), and 2F were performed using TaqMan technology and normalized to *Rp49* using the ddCt method. Primer sequences for *Rp49* are the same as in Grozinger *et al* (2003), and primer sequences for *rcp1*, *ilp1*, *inR1*, *InR2*, and *Vg* are the same as in Corona *et al* (2007).

***AmRp49-GB10903* qPCR (100)**
F: GGGACAATATTTGATGCCCAAT
R: CTTGACATTATGTACCAAAACTTTTCT
***RCP1* qPCR (65)**
F: TCAATTAACTCGGAATCGGA
R: CCTGGATTTCCCTGCTGAT
***AmILP1-GB17332* qPCR (68)**
F: GCTCAGGCTGTGCTCGAAAAGT
R: CGTTGTATCCACGACCCTTGC
P: FAM6-TCAGCGACGCCCTGTACCTCG-TAMRA
***AmILP2-GB10174* qPCR (87)**
F: AAAAAGTAATCAAGAAATGGAGATG
R: TTCGCATTTTTAATAGATTTATAAGG
***AmILP2-GB10174* Standard Curve PCR (188)**
F: TTAAAAAAAGTAATCAAGAAATGGAGATGGA
R: AACGGGCACCGCAATAGGAG
***AmInR1-GB15492* qPCR (71)**
F: ACGGGATGGCCTACTTGGAG
R: GGAAACCATGCAATTCCTCG
***AmInR2-GB30192* qPCR (83)**
F: ACGAGGTCGGCCAGATCTC
R: AGTACCAGGAATAGGAACAAAATGGT
***AmAKH-GB30028* qPCR (75)**
F: CAATTGTTCCACTGAAGGTTTGG
R: CAAAGGATCGGAGTTGTCGAA
***AmAkhR-GB16857* qPCR (87)**
F: TTGGGCGATCACTGTTTCCT
R: GATGATAAGTACAGGCCAAACATTCTAA
***Vg-GB13999* qPCR (63)**
F: AGTTCCGACCGACGACG
R: TTCCCTCCCACGGAGTCC

Figures for Chapter 1

Figure 1.1. Upregulation of insulin signaling genes in brain and abdomen of worker honey bees during behavioral maturation and in response to poor nutrition (qPCR). **A**) IIS gene expression in brains and abdomens of nurses and foragers. Data pooled from four independent trials. ANOVA; brain *ilp1:* $P_{group(df=1,68)}<0.0001$, $P_{group\ x\ trial(df=3,68)}<0.0001$. brain *ilp2:* $P_{group(df=1,67)}<0.05$, $P_{group\ x\ trial(df=3,67)}<0.0001$; brain *inR1:* $P_{group(df=1,67)}>0.05$, $P_{group\ x\ trial(df=3,67)}>0.05$; brain *inR2:* $P_{group(df=1,67)}>0.05$, $P_{group\ x\ trial(df=3,67)}>0.05$; abdominal *inR1:* $P_{group(df=1,71)}<0.0001$, $P_{group\ x\ trial(df=3,71)}=0.05$; abdominal *inR2:* $P_{group(df=1,71)}<0.0001$, $P_{group\ x\ trial(df=3,71)}>0.05$. Data for individual trials are shown in Fig. A.2. **B**) IIS gene expression in brains/heads and abdomens of 4- and 6-day-old bees caged and fed pollen and sugar or a sugar-only diet. Data for brain and head *ilp1* pooled from five independent trials (ANOVA: $P_{diet(df=1,74)}<0.05$, $P_{trial\ x\ diet(df=4,74)}>0.05$). Data for brain *inR1* pooled from four independent trials ($P_{diet}<0.05$, $P_{diet\ x\ trial}>0.05$). Two independent trials for abdomen *inR1* are shown. Data for individual trials are shown in Fig. A.3. **C)** *ilp1* expression in brains/heads following field diet manipulations. Data are pooled for brain *ilp1* from three early-summer trials using single-cohort colonies (1200 bees; ANOVA: $P_{diet(df=1,45)}<0.0001$, $P_{trial(df=2,45)}<0.001$; $P_{diet\ x\ trial(df=2,45)}<0.05$). Data are shown for head *ilp1* from two individual late-summer trials using single-cohort colonies (combined analysis: $P_{diet(df=1,37)}<0.01$, $P_{trial(df=1,37)}<0.0001$, $P_{diet\ x\ trial(df=1,37)}>0.05$). Single trials were performed for head *ilp1* with small typical colonies (~10,000 bees) and large typical colonies (~30,000 bees) in late summer. Main effect of group or diet for pooled trials, and Student's t-tests for individual trials: * P<0.05; ** P<0.01; *** P<0.001.

Figure 1.2. Delayed behavioral maturation caused by rapamycin. **A)** Proportion of bees that initiated foraging at 5-9 days of age after rapamycin or control treatments. Data for early summer (July, 2006) and late summer (August-September, 2006) are pooled from 5 and 4 trials, respectively. Cox Proportional Hazards; All trials, 2006: (foragers/total) n_{rapa}=120/587, $n_{control}$=109/547, $P_{trt}<0.05$, $P_{date\text{-}of\text{-}trial}<0.05$, $P_{trt*date}<0.01$, $P_{trial}<0.05$, $P_{trt*trial}>0.05$, $P_{date*trial}<0.01$, $P_{trt*date*trial}<0.01$; Early summer 2006: n_{rapa}=47/217, $n_{control}$=56/173, $P_{trt}<0.05$, Ptrial<0.001 ($P_{trt*trial}>0.05$, removed from model); Late summer 2006: n_{rapa}=73/370, $n_{control}$=53/374, $P_{trt}>0.05$, $P_{trial}<0.05$, $P_{trt*trial}<0.01$. **B,C)** Similar results were obtained in a second year. Early summer 2007: $P_{rapa}<0.01$, $P_{trial}<0.001$, $P_{meth*diet}<0.05$ (interactions P>0.05 removed); Late summer 2007: $P_{rapa}>0.05$, $P_{trial}<0.05$, $P_{meth*diet}<0.05$ (interactions P>0.05 removed). **B)** Proportion of bees that foraged before 10 days of age following combinatorial treatments with rapamycin and the juvenile hormone analog methoprene. Data from individual trials shown. Statistical analyses on data pooled from two early summer trials and two late season trials: $P_{meth}<0.01$, $P_{rapa}>0.05$, $P_{season}<0.01$, $P_{rapa*season}=0.05$ (interactions P>0.05 removed) **C)** Proportion of bees that foraged before 10 days of age following combinatorial treatments with rapamycin and adult diet manipulations (sugar only or pollen and honey). Data are from single early and late summer trials. $P_{diet}=0.08$, $P_{rapa}<0.05$, $P_{season}>0.05$, $P_{rapa*season}<0.01$ (interactions P>0.05 removed). **D)** Expression of *ilp1* in brains of nurses and foragers collected from small colonies in early and late summer. ANOVA, followed by paired contrasts: $P_{early\text{-}F\ v.\ late\text{-}F} < 0.0001$, $P_{early\text{-}N\ v.\ late\text{-}N} > 0.05$. **E)** Expression of *ilp1* in brains of nurses and foragers collected from large colonies in early and late summer. * P<0.05, *** P<0.001.

Figure 1.3. Citrate cycle genes are upregulated in the brains of nurse bees. Gene expression in whole brains of nurse bees and foragers was measured on cDNA micoarrays (37). Expression data were mapped to pathway diagrams compiled by the Kyoto Encyclopedia of Genes and Genomes (KEGG). P values are based on ANOVA described in (37). Abbreviations for gene names are based on SwissProt naming conventions.

Figure 1.1

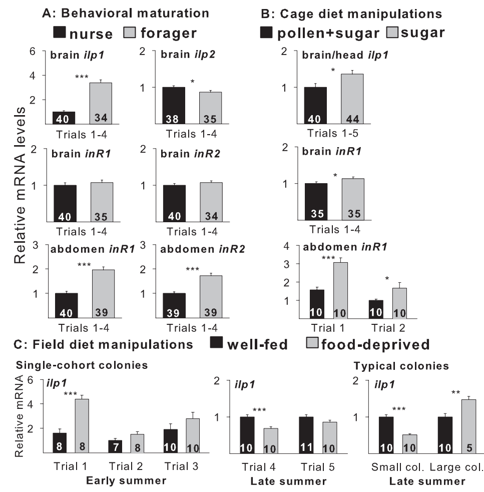


Figure. 1.2

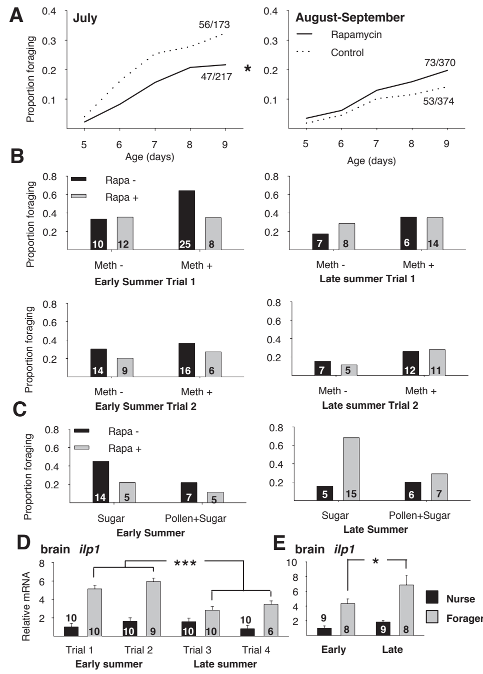
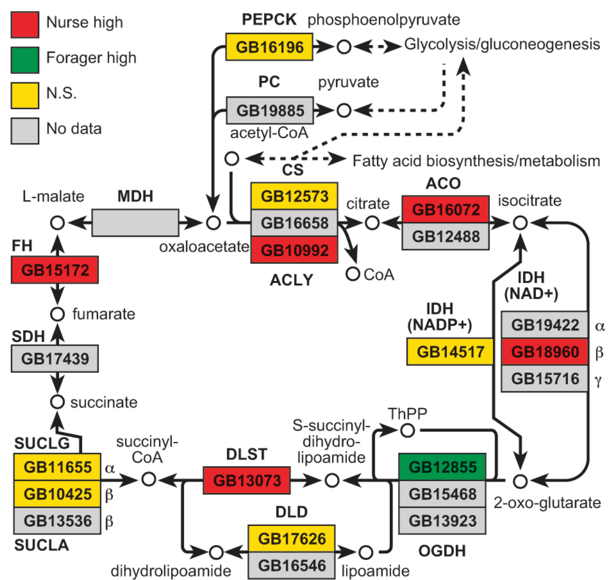


Figure 1.3



Chapter 2

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

Co-authored with Rodrigo A Velarde, Mira Kolodkin, Daniel Moyse, and Gene E. Robinson [2]

Abstract

It has been proposed 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 NPF 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.

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[2] This chapter has been formatted for *Insect Molecular Biology*.

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. 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 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 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, 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 rigorous 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 were recently shown to change during the period between when they arrive at a feeder and when they depart it a few minutes later (Brockmann *et al.*, 2008).

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 brain in 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

Evolution of NPY-like peptides and NPY-family receptors in hymenopteran insects. The sequenced honey bee genome contains orthologs to genes encoding two NPY-like peptides, *AmNPF* and *AmsNPF* (Hummon *et al.*, 2006). The predicted NPF peptide has considerable homology to other insect NPF peptides (Fig. 2.1A) and retains key attributes of NPY-family peptides, such a proline-rich N-terminus that is likely to form the characteristic polyproline-like helix (Blundell *et al.*, 1981). However, *Apis* NPF differs from other known insect NPF peptides in that the –RXRF-amide C-terminus that is common to most insect NPFs, and which includes the most critical residues for receptor-binding (Lindner, *et al.* 2008), has been modified to -KARY-amide in the honey bee. To determine whether these sequence modifications occurred recently in hymenopteran evolution we compared the sequence of a putative NPF prepropeptide gene in the genomic sequences from the jewel wasp, *Nasonia vitripennis* (Hauser *et al.* 2010). The predicted *N. vitripennis* and *Apis* NPF peptides are 79% identical and share the unusual -KARY-amide C-terminus (Fig. 2.1A). Jewel wasps and honey bees diverged approximately 150-

200 million years ago, shortly after the divergence of hymenopterans from other holometabolous insects (Dowton *et al.*, 2009), so this sequence is likely to have been stable through most of hymenopteran evolution. The honey bee sNPF peptide sequence differs little from sNPF peptides found in other insects (Hummon et al., 2006; Vanden Broeck, 2001).

In *Drosophila*, the NPY-family receptors *npfr1* and *sNPFR (npfr-76F)* are activated by NPF and sNPF peptides, respectively (Garczynski *et al.*, 2002; Mertens *et al.*, 2002). Orthologs to both of these genes have also been identified in mosquito and beetle genomes (Hill *et al*., 2002; Hauser *et al.*, 2008). However, only an ortholog to *sNPFR* was identified in the honey bee genome (Chen & Pietrantonio, 2006; Hauser *et al.*, 2006). The absence of an identified *npfr1* gene in the honey bee genome sequence could be either a technical artifact due to limitations in the genome annotation or in the depth of sequencing, or it could be a true gene loss. We searched the *N. vitripennis* genome and brain Expressed Sequence Tag databases from 11 additional bee species and found orthologs of *sNPFR* but not of *npfr1* (data not shown), suggesting that hymenopteran genomes lack orthologs of *npfr1*. However, in the genome of the pea aphid, *Acyrthosiphon pisum* (International Aphid Genomics Consortium 2010), which is basal to all holometabolous insects, we identified orthologs to both NPY-family receptors. These results suggest that the *npfr1* gene was present in the ancestors of bees and wasps but was lost early in hymenopteran evolution. Overall, our phylogenetic analyses indicate that a number of changes in NPY-like signaling genes occurred early in hymenopteran evolution, but we find less evidence for more recent changes within the Hymenoptera.

***npf* and *snpf* are localized to separate populations of neurosecretory cells in the honey bee brain.** Although the honey bee orthologs to NPY-like signaling genes in the bee had been identified in surveys of neuropeptide signaling systems (Hummon *et al.*, 2006; Hauser *et al.,* 2006), these genes had not previously been characterized experimentally. As an initial measure of these genes' functions in the bee we used *in situ* hybridization to determine where *npf* and *snpf* 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. 2.2A). *snpf* was localized bilaterally in 4-6 pairs of lateral neurosecretory cells (Fig. 2.2B). These results are consistent with the predicted endocrine functions for these genes, and they suggest that *npf* and *snpf* function in independent circuits.

***npf* is upregulated in the brains of foragers.** If NPY-like signaling is involved in division of labor, NPY-related genes are likely to be differentially expressed between behavioral groups in the hive. To test this hypothesis, we used quantitative, real-time PCR to measure the expression of the two NPY-like peptides and of the bee's single NPY-family receptor, *sNPFR*. We measured transcript abundance in whole brains of nurses and foragers from each of four typical honey bee colonies.

A subset of NPY-related genes were more abundant in the brains of foragers than nurses (Fig. 2.3). *npf* was upregulated in forager brains in 3 of 4 individual trials and in a combined analysis (Fig. 2.3, Mixed Model ANOVA: $P_{group} < 0.0001$, $P_{group\ x\ colony} < 0.0001$). s*NPFR* 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 ($P_{group(1,}$

$_{68)} > 0.05$, $P_{group\ x\ colony\ (3,\ 68)} < 0.001$). *snpf* was similar in nurse and forager brains in all four trials. These results suggest that only *npf* expression is consistently associated with behavioral state in the brains of honey bee workers.

***sNPFR* is upregulated by food deprivation.** One explanation for the observed expression patterns of *npf* and *sNPFR* is that they may be responding to nutritional stimuli. Nutritional changes may influence expression over the course of maturation, which is more likely for *npf* given its consistent association with behavioral state, but nutrition also varies between colonies and over relatively short timescales, which could explain the variability in *sNPFR* expression. We used a series of nutritional manipulations of caged and colony-reared bees to determine whether the expression of NPY-related genes was regulated by nutrition.

We first examined the effects of diet quality on gene expression in young, caged bees. Bees in this experiment were fed either a nutrient-poor diet of 50% sugar syrup or a nutrient-rich diet containing both sugar syrup and pollen paste. Both diets were supplied *ad libitum*, but bees fed the poor diet have smaller lipid and protein stores than bees fed the rich diet (Ament *et al.*, 2008). Therefore, this treatment affects diet quality but not diet quantity. Diet quality did not affect the expression of *npf, snpf,* or *sNPFR* in brain (Fig. 2.4A). This lack of effect was not due to a treatment failure because bees in the same cages differed in their lipid stores and in the expression of brain *insulin-like peptide-1* (these were some of the same bees used in Ament *et al.,* 2008).

We next examined the response of NPY-related genes in the brain to nutritional manipulations under more natural conditions. We created small, single-cohort colonies with only one-day-old bees and fed them either an *ad libitum* diet with both pollen and honey ("well-fed"), or limited their consumption by providing only honey for two days after which they had no food at all ("food-deprived"). 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). We collected bees from these colonies after four days, when they were the same age as the young, caged bees described above, and prior to the onset of foraging. Colony food-deprivation caused a consistent upregulation of brain *sNPFR* but did not cause consistent changes in *npf* or *snpf* (Fig. 2.4B).

Behaviorally associated changes in *sNPFR* expression depend on nutrition. Our results suggest that *sNPFR* expression is related to both nutritional status and behavioral state. To examine the relationship between nutritional sensitivity and behavior, we performed nutritional manipulations on bees at different stages of behavioral maturation and looked for interactions between behavioral state and diet. This experiment used similar manipulations of diet quality to those shown in Fig. 2.4A, except that we added a third diet, a nutritionally rich diet containing soy protein in place of pollen. This condition was added because foragers are known to digest pollen inefficiently (Crailsheim *et al.,* 1992).

Maturation-related changes in *npf* were observed regardless of cage diet (Fig. 2.5), 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 syrup intake. Our results suggest that some aspects of NPY-like signaling in the brains of foragers are responsive to nutrition. In order to determine whether NPF influences food intake, we tested whether injections of synthetic NPF peptide into the brains of foragers affected their short-term consumption of sugar syrup in cages. We injected 1ug NPF in water directly into the brain via the ocellar tract, a dose determined to be 10% of an LD50 dose (data not shown). NPF injections had no effect on sugar syrup intake (Fig. 2.6A). These results suggest that, as in flies (Wu *et al.* 2005b; Lingo *et al.* 2007) but unlike vertebrates, the relationship in the honey bee between NPY-like signaling and nutritional status does not involve a simple effect on food intake.

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. Here we present evidence 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 neuroepeptide-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 but was unresponsive to nutritional manipulations. By contrast, *snpf* was expressed at nearly identical levels across all experiments in this study. These results suggest that *npf* but not of *snpf* has functions that change during maturation. The lack of transcriptional responses of *npf* and *snpf* to nutritional manipulations does not necessarily mean that these genes have no involvement in nutritional processes. Rather, 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.*, 2008). 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. (2008). If NPF and sNPF are involved in regulating feeding-related processes, it is interesting 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 they fly (Shen & Cai, 2001). Perhaps, despite short-term, feeding-related, changes in release, the demand for NPF and sNPF is fairly constant over longer timescales that affect transcription. If so, this implies that NPY-like signaling has an active role in the behaviors of nurses, perhaps in regulating food-related behaviors such as brood feeding and nectar and pollen consumption inside the hive.

We found that NPF does not directly effect the consumption of sugar syrup. Because we measured these effects using only one concentration of injected peptide, it remains possible that NPF effects sugar feeding when administered at a different dose. However, our results are consistent with earlier work in *Drosophila* showing that genetic manipulations of NPY-like signaling influence the acceptability of undesirable foods but not the consumption of sugar syrup (Wu *et al.*, 2005a; Wu *et al.*, 2005b; Lingo *et al.*, 2007). Together, these results suggest that in insects, unlike vertebrates, consumption of highly desirable foods is not directly influenced by NPY-like signaling.

In order to predict changes in the sensitivity of bees to NPY-like peptides, we examined the expression of *sNPFR*, the only NPY-family receptor identified in the sequenced honey bee genome, an ortholog of the fly gene that binds sNPF peptides (Hauser *et al.*, 2006). Our results suggest that *sNPFR* expression is upregulated in the brain by poor nutrition, and is most sensitive to nutritional changes in the brains of foragers. The interpretation of our 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 the *npfr1* gene was present in basal insect lineages but was lost early in Hymenopteran evolution. Our results also show that the NPF peptide underwent fairly radical changes in Hymenoptera, shifting from the –RXRF-amide C-terminus found in most invertebrates to the novel –KARY-amide C-terminus found in the bee and wasp. However, the stability of the NPF sequence within the Hymenoptera indicates that the NPF signaling system remains functional in the absence of *npfr1,* presumably through interactions with a different receptor. The closest paralog to *npfr1* is *sNPFR*, so perhaps the receptor for NPF is *sNPFR* itself. However, in the absence of functional data on ligand-receptor interactions this remains speculative. Although the functional relationships between NPY-like peptides and *sNPFR* are not known, it is clear that as bees mature from hive work to foraging, their brains produce more of an NPY-like peptide and become more sensitive to nutritional cues, via increased plasticity in NPY-family receptor gene expression. Although we have not tested the functional consequences of these maturational changes in NPY-like signaling in the bee, our results suggest that NPY-like signaling is a candidate regulator of some aspects of foraging behavior.

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 *sNPFR* homolog (Chen & Pietrantonio, 2006). These results suggest that plasticity in NPY-family receptor gene expression could be a common phenomenon and a candidate mechanism underlying plasticity in feeding-related behaviors.

An important next step will be to evaluate the functional roles for NPY-like signaling and its interactions with other feeding- and nutritionally-related pathways in the bee. 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* controls 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.*, 2005). 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 is surprising, given that in the fly *npf* and IIS signaling are opposed, but we speculate that the simultaneous upregulation of these pathways is important for foraging tasks. The combination of high NPY-like signaling and high IIS could increase sensitivity to both hunger and satiety cues. Interactions between these pathways could be involved in the exaggerated responses of foragers to the need for food – i.e., flying far from the hive to collect nectar and pollen rather than eating stores inside the hive.

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 group. 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.

Experimental Procedures

Phylogenetic analyses. The sequences of NPF peptides from *Drosophila melanogaster* (Brown *et al.*, 1999), *Apis mellifera* (Hummon *et al.*, 2006), *Daphnia magna* (Christie *et al.* 2008), and *Nasonia vitripennis* (Hauser *et al.* 2010) were retrieved from published sources. *npf* genes from*,* *Acyrthosiphon pisum* and *Bombyx mori* were identified as reciprocal best BLAST hits to *Apis* and *Drosophila npf* genes among automatically annotated genes predicted in the respective genome sequencing projects for these species. Predicted peptide sequences were determined by manually assessing cleavage sites and by comparing the sequences to previously characterized peptides.

Similarly, sequences of NPY-family receptors from *D. melanogaster* (Garzynski *et al.* 2002; Mertens *et al.*, 2002), *A. mellifera* (Hauser *et al*, 2006) and *T. castaneum* (Hauser *et al.*, 2008) were previously published. We searched for orthologs to *sNPFR* and *npfr1* in *A. pisum* and *N. vitripennis* using tBLASTN searches of their genomic sequences available in GenBank. Sequences were aligned using CLUSTALW.

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. DIG-labeled probes were hybridized to cryo-sectioned brains of foragers as previously described (Velarde *et al.*, 2006).

Cage diet manipulations. For manipulations of cage diet quality, groups of bees of the desired age or behavioral group were collected directly into Plexiglas cages (11 x 11 x 7 cm). They were subsequently fed one of three diets: a) 50% sucrose w/v in water; b) 50% sucrose and a second feeder containing pollen paste (45% ground pollen / 45% honey / 10% honey); or c) 50% sucrose and a second feeder containing soy paste (45% soy protein / 45% honey / 10% water). Diets were *ad libitum* and were replaced daily. Food consumption was measured on a per cage basis by weighing each feeder before it was placed into the cage and again after it was removed one day later. We conducted two trials using young bees, one-day-old at the start of the experiment and groups of 35 in each cage, reported in Fig. 2.3. These methods are the same as described in (Ament e*t al.*, 2008) and include bees from two of the same trials. We performed a third trial

using bees that were collected as nurses and foragers in addition to young bees (Fig. 2.4); in this trial cage density was reduced to 15 bees / cage to reduce forager mortality. After 3 days (Trials 1 and 2) or 5 days (Trial 3), bees were flash-frozen in liquid nitrogen to preserve RNA.

Tissue handling, RNA extraction, and quantitative RT-PCR (qPCR). Quantification of brain RNA was performed according to standard methods in our laboratory (Ament *et al*., 2008). Heads were partially lyophilized in a vacuum freeze-dryer for 1h. Brains were then dissected from head capsules in a dissection dish containing 95% ethanol on a bed of dry ice. Brains or whole abdomens were then homogenized in lysis buffer, and RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA). cDNA was synthesized from 200ng total RNA using random hexamers as primers and ArrayScript reverse transcriptase (Ambion). Additionally, 100pg of a plant RNA (*root-cap protein 1*) was spiked into the RT reaction as a loading control. Quantitative PCR was performed using an ABI Prism 7900 sequence detector with specific primers (Table 1) and Sybr Green technology. Transcripts for each experimental gene were quantified using a genomic DNA standard curve, and *rcp1* was quantified using a standard curve derived from a plasmid clone of that gene. For nurse and forager brains and for the colony food-deprivation experiment, we report expression of each gene relative to *Rp49*, a validated endogenous "control" gene. For manipulations of cage diet quality, we report expression of each gene relative to *rcp1* (Trials 1 and 2) or normalized to total RNA (Trial 3). 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 EPEPMARPTRPEIFTSPEELRRYIDHVSDYYLLSGKARY-NH_2 was synthesized by Global Peptide (Fort Collins, CO). Peptide was dissolved in deionized H_2O. 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 10ug was determined to cause approximately 50% mortality (data not shown). We subsequently used a dose of 1ug 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 diet quality 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.

References for Chapter 2

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Figures for Chapter 2

Figure 2.1. Evolution of NPY-like signaling genes in the Hymenoptera and other insects. Sequences of NPF peptides in insects and in the water flea, *Daphnia magna.* Phylogenetic relationships shown are based on species divergence rather than on the alignment of peptide sequences.

Figure 2.2. in situ hybridizations with npf and snpf. DIG-labelled antisense RNA probes were used to localize transcripts for *npf* (A) and *snpf* (B) in the brains of forager honey bees.

Figure 2.3. Expression of NPY-like signaling genes in nurses and foragers. qRT-PCR was used to measure *npf*, *snpf*, and *sNPFR* in whole brains (A) and 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:* $P_{group} < 0.0001$, $P_{group\ x\ colony} < 0.0001$; *snpf:* $P_{group} > 0.05$, $P_{group\ x\ colony} > 0.05$; *sNPFR:* $P_{group} > 0.05$, $P_{group\ x\ colony} < 0.0001$. Student's t-test for individual trials: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 2.4. Effects of nutritional manipulations on NPY-related 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"). (B) Expression of *npf, snpf*, and *sNPFR* in brains of worker honey bees following colony food deprivation. One-day-olds

were placed into single-cohort 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. Mixed Model ANOVA for food-deprivation: *npf:* $P_{trt(1,42)} > 0.05$, $P_{trt\ x\ trial(2,\ 42)} > 0.05$; *snpf:* $P_{trt(1,27)} > 0.05$, $P_{trt\ x\ trial(2,27)} < 0.01$; *sNPFR:* $P_{trt(1,44)} < 0.0001$, $P_{trt\ x\ trial\ (2,44)} > 0.05$. Student’s t-test for individual trials: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 2.5. Maturational 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. 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 2.6. Effects of synthetic NPF on food intake. Sugar syrup intake of foragers after injection with 1ug NPF peptide into the brain via the ocellar tract; controls injected with vehicle only (water). Results represent the aggregate food intake of 10 bees placed together in a cage. n=5 trials / group.

Figure 2.1

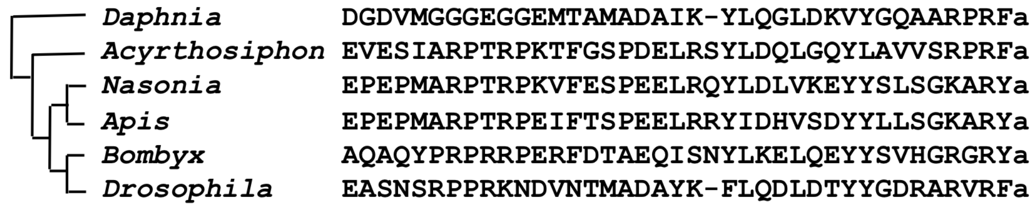


Figure 2.2

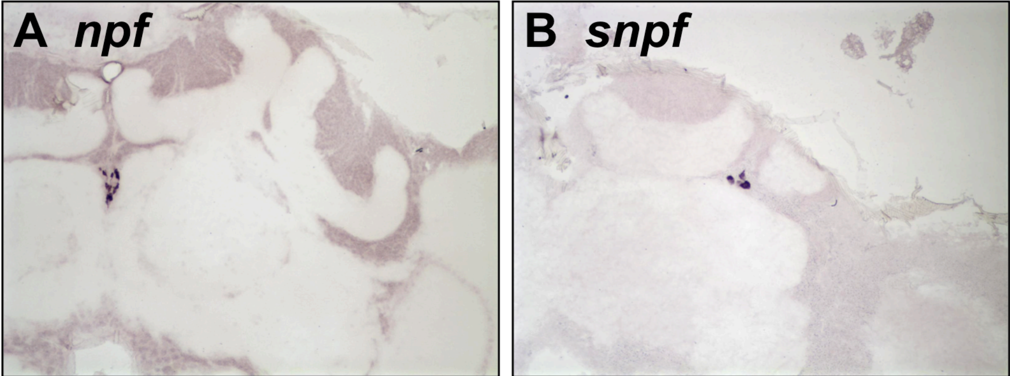


Figure 2.3

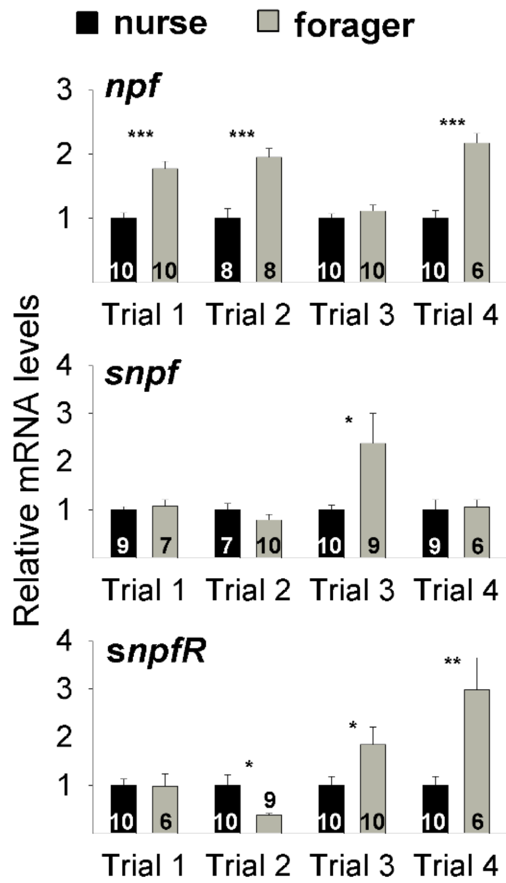


Figure 2.4

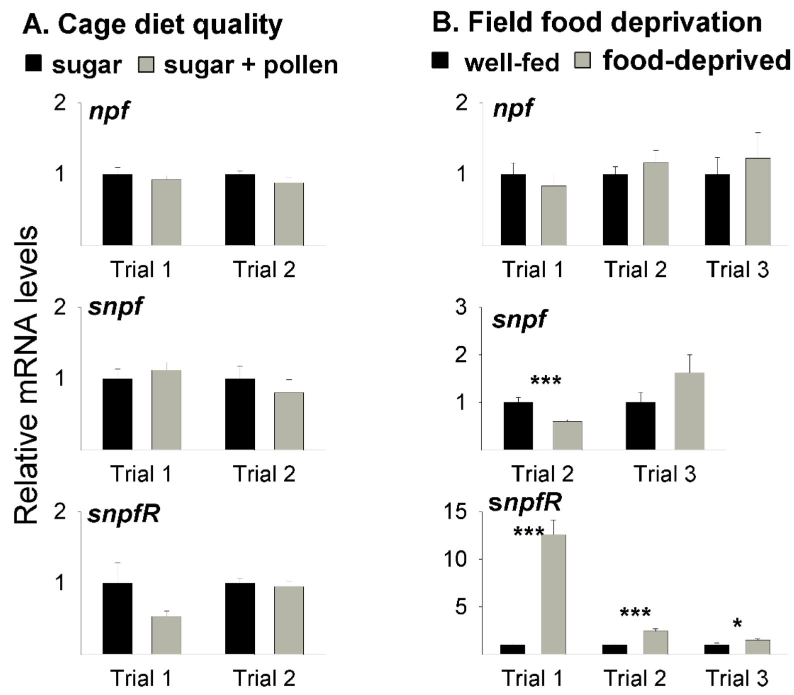


Figure 2.5

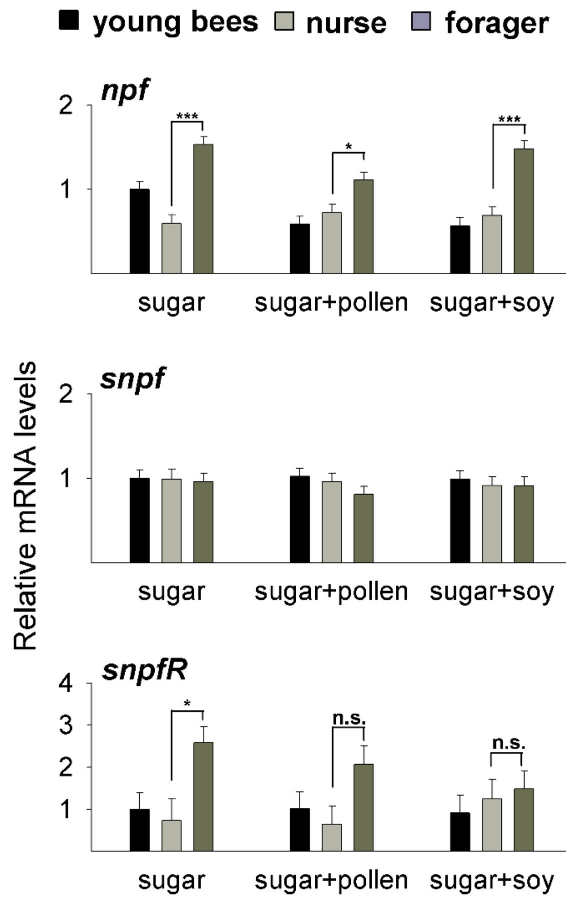
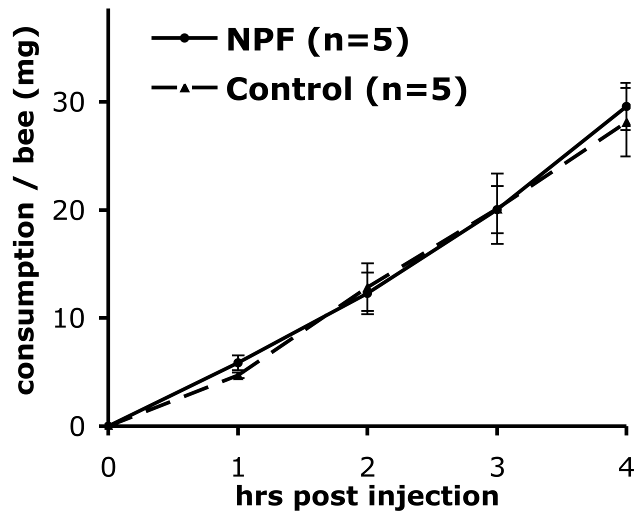


Figure 2.6



Chapter 3

Mechanisms of stable lipid loss in a social insect

Co-authored with Queenie Chan, Marsha Wheeler, Scott E. Nixon, S. Peir Johnson, Sandra Rodriguez-Zas, Leonard J. Foster, and Gene E. Robinson [3]

Abstract

Humans that become obese often struggle to achieve stable weight loss. By contrast, recent work has shown that adult worker honey bees build up large lipid stores early in life, then undergo a dramatic and stable lipid loss as part of their normal maturation. We used a combination of physiological, transcriptomic, and proteomic experiments to explore the mechanistic basis for stable lipid loss in bees. We show that this lipid loss involves massive changes in gene expression in the abdominal fat bodies, including many changes in core macronutrient and energy metabolism pathways; quantitative proteomics provided independent support for many of these changes. Many of these same genes and pathways were also regulated in young bees by diet, but diet-regulated components of hormonal and fatty acid metabolism pathways did not respond to nutritional manipulations in older bees, suggesting that stable lipid loss involves the regulation of these pathways by non-dietary factors. These factors include the yolk protein vitellogenin and Queen Mandibular Pheromone, each of which had maturationally-related effects on fat body gene expression that were independent of nutrition.

[3] This chapter is formatted for *PLoS Biology*.

Introduction

A challenge in combating the obesity epidemic in developed countries is that most people who become obese have difficulty losing weight and keeping it off over the long term [1]. A growing literature from studies with human subjects and animal models suggests that stable weight loss is difficult because hormonal signaling pathways and metabolic networks quickly adapt to elevated adiposity as a norm and respond to subsequent decreases in adiposity as though to wasting or food deprivation [2]. This problem is compounded by the complex regulation of body weight, which is influenced by interactions among hundreds if not thousands of genes [3]. Such elaborate mechanisms to preserve body weight may have been adaptive during periods of famine, but they pose a challenge to the development of treatments to alleviate obesity.

One strategy for understanding how to achieve stable weight loss in humans is to study mechanisms by which this occurs naturally in other species, including hibernation in mammals and diapause in insects. An intriguing example of stable lipid loss occurs in honey bees (reviewed in [4]). Worker bees begin their adult life lean but develop large lipid stores after a few days of consuming a nutrient-rich diet of pollen and honey. After 1-2 weeks of elevated adiposity, they undergo a dramatic loss of abdominal lipid and subsequently remain lean for the remaining 1-2 weeks of their life [5]. These changes in adiposity are embedded in a broader process of behavioral and physiological maturation and relate to the social roles of worker bees within their colony [6]. Young (adipose) bees perform brood care and other tasks inside the hive; these "nurse" bees use their large internal stores of lipids and proteins to produce glandular secretions, called brood food, that they feed to larvae and to other workers [7]. Stable lipid loss occurs prior to a lifestyle transition from in-hive tasks to foraging outside the hive for nectar and

pollen; small lipid stores are thought to have adaptive functions in the performance of foraging behavior [8].

Insects are of potential relevance to the regulation of body weight in humans because many of the underlying mechanisms are deeply conserved. The regulation of adiposity in insects and vertebrates involves many of the same hormonal systems, which in both taxa are sensitive to nutrient abundance and which direct tissue-specific responses in metabolism, nutrient storage, and feeding. These homologous functions are particularly well established for insulin/insulin-like growth factor signaling (IIS) and neuropeptide Y-like signaling [9-11]. Analogous functions have also been described for signaling systems with more ambiguous homology, such as the shared functions of vertebrate glucagon and an insect equivalent, adipokinetic hormone [12], and similarities between the functions and mode of action for vertebrate thyroid hormone and a structurally-related insect hormone, juvenile hormone [13]. The complement of macronutrient and energy metabolism enzymes is virtually unchanged between vertebrates and insects.

Anatomical features are less well conserved than the molecular components, but analogous functions are easy to discern and new studies are uncovering surprisingly deep conservation. Insects store fat in the fat bodies that line the body walls, most thickly in the abdomen [14]. Insect fat bodies have analogous functions to liver as well as adipose tissue in that they are critical for carbohydrate metabolism in addition to lipid storage [15]. Although a homolog of the vertebrate adipocyte hormone leptin has not been identified in insects, insect fat bodies, like vertebrate adipose tissue, serve as nutrient sensors and send signals to the brain to influence behavior [16]. There is also conservation in the developmental specification and transcriptional

control mechanisms within insulin-producing cells in vertebrates and insects [17, 18], despite the fact that these cells are located in the brains of insects but in the pancreas of mammals. These results support the idea that insects and vertebrates control body weight through conserved mechanisms.

Changes in body weight result from an imbalance between the rate at which an animal ingests nutrients and the rate at which these nutrients are utilized for energy [1]. However, energy imbalance can result from diverse changes in behavior, physiology, hormones, and molecular signaling and metabolic pathways. In the bee, changes in energy balance during stable lipid loss likely arise from differences both in the intake and in the utilization of nutrients. Although no thorough studies have been done on caloric intake of individual bees in the hive, young and old bees differ in their source of dietary protein and lipid; young bees mostly receive these nutrients by ingesting pollen, whereas foragers cannot digest pollen efficiently and receive dietary protein and lipid primarily in secretions fed to them by nurses [7, 19]. Foragers are known to have a faster resting metabolism than nurses, and the foraging task requires them to perform energy-intensive flights far from the hive [20]. These metabolic differences are likely to contribute to greater total nutrient utilization in foragers and thus to the maintenance of small lipid stores [5].

Previous work suggests that the regulation of these maturational differences between nurses and foragers involves many conserved nutritionally-related mechanisms. The age at which a bee begins to forage is influenced by her internal lipid stores [21, 22], suggesting that nutrition itself could be an important regulator of lipid loss. Moreover, several conserved, nutritionally- and metabolically-related signaling pathways have been implicated in the behavioral maturation

process (reviewed in [4]), including insulin and target of rapamycin signaling [23], cGMP-dependent protein kinase signaling [24], and juvenile hormone (JH) signaling [25-27]. These pathways might therefore also regulate stable lipid loss.

However, the bee's unusual pattern of stable lipid loss cannot be explained simply by invoking conserved mechanisms since these mechanisms do not produce stable lipid loss in other species. We hypothesized that stable lipid loss involves two kinds of regulatory differences between the bee and species that do not have stable lipid loss. First, stable lipid loss might involve age-related changes in how conserved, nutritionally-related processes are regulated. This hypothesis is supported by previous work showing that maturation involves unusual, non-homeostatic regulation of genes related to insulin signaling [23]. Second, stable lipid loss might involve evolutionarily novel signals that are incorporated into the control of lipid storage. In support of this idea, previous work has shown that worker honey bees utilize the storage protein vitellogenin (vg) in novel ways, including a causal role in maturation [28-30]. In addition, bees have evolved intricate mechanisms by which social signals influence physiology; Queen Mandibular Pheromone (QMP), a blend of chemicals produces by the queen, has its primary function in inhibiting worker reproduction [31], but it also delays the transition of worker bees from hive work to foraging [32] and causes them to have increased lipid stores [33], both of which may increase the colony's capacity to rear brood at times when the queen is actively laying eggs.

Here we explore the molecular basis for stable lipid loss in honey bees, using quantitative transcriptomic and proteomic techniques. Our results demonstrate that stable lipid loss involves

age-related changes in the responses of hormonal and metabolic pathways to nutritional stimuli, as well as robust genomic responses in the fat bodies to evolutionarily novel regulators of nutrient stores, vg and QMP. We discuss implications of these results for the evolution of nutritional physiology and for research into human obesity.

Results

Gene expression changes in fat body tissue during natural lipid loss.

To measure transcriptional differences in the fat bodies during stable lipid loss, we used a previously described, custom microarray with 13440 unique, oligonucleotide probes designed primarily based on gene predictions from the sequence of the honey bee genome [34, 35]. Fat body tissue from nurses and foragers differed in the expression of 2641 transcripts (ANOVA, False Discovery Rate [FDR] < 0.05, 21% of the quantified transcripts in this tissue). Mapping differentially expressed transcripts to Gene Ontology (GO; [36]; Table 3.1; Appendix B) and to pathways from the Kyoto Encyclopedia of Genes and Genomes (KEGG; [37], Fig. 3.1) suggested dramatic changes in core macronutrient and energy metabolic pathways. In particular, nurses had higher expression of genes related to lipid and protein metabolism, consistent with their large lipid and protein stores. By contrast, foragers had higher expression of genes related to glycolysis/gluconeogenesis and the metabolism of glycogen and trehalose (a disaccharide used as an important energy store in insects; [38]; Fig. 3.1), as well as energy metabolism pathways (e.g., “oxidative phosphorylation”, “cofactor metabolic process”). These results suggest that the fat bodies of nurses are specialized for the synthesis and utilization of lipids and proteins, whereas the fat bodies of foragers are specialized for carbohydrate metabolism and for the generation of energy. This maturational shift in the metabolic specialization of the fat bodies

makes sense given the known differences in nutrient stores and the energetic costs of nursing vs. foraging and could be either a cause or consequence of stable lipid loss.

We also identified changes in genes with potential regulatory functions. Several genes known to be involved in nutritionally-related signaling pathways were upregulated in the fat tissue of nurses, including *juvenile hormone epoxide hydrolase* (a JH-degrading enzyme) and *insulin-like peptide 2*. In addition, GO categories containing regulatory genes (e.g., "regulation of developmental process", "cellular structure morphogenesis", "protein kinase cascade", "cellular localization") were more highly expressed in foragers than nurses. It is not known if any of these "developmental" genes are causal for stable lipid loss, but their reuse during maturation suggests commonalities between development and adult plasticity [39].

We used quantitative mass-spectrometry proteomics to validate the gene expression differences discovered using microarrays. Despite relatively low power to detect significant differences in the proteomics study (n=3; 14 proteins differentially expressed, $P < 0.05$), RNA and protein measurements for 104 genes quantified by both microarrays and mass spectrometry were strongly correlated (Pearson correlation, $r = 0.71$, $p << 2.2e-16$; Fig. 3.2), suggesting that most transcriptional differences are reflected by differences in protein levels within the fat bodies. The proteins quantified in this experiment included storage proteins and many enzymes involved in macronutrient and energy metabolism, further supporting our finding that metabolic processes are remodeled during maturation.

Overall, these results suggest that stable lipid loss during worker honey bee maturation is a highly regulated process involving transcriptional changes in a large number of genes. We next set out to understand the mechanistic basis for these changes by comparing the transcriptional changes during maturation to transcriptional responses induced by factors that are known to influence nutrient stores in the bee.

Gene expression responses of fat body tissue to diet quality.

We first hypothesized that stable lipid loss is caused by nutritional differences between nurses and foragers. As an initial test of this, we studied gene expression in the fat bodies of pre-nursing-age bees that were removed from the hive and fed a lipid- and protein-rich diet (ground pollen, honey, and sugar-syrup, fed *ad libitum* for four days after adult emergence) or a nutrient-poor diet (sugar-syrup only, *ad libitum*). This experimental paradigm mimics the lipid gain during the bee's early life [23] and allowed us to characterize the transcriptional changes induced by nutrition in isolation from social stimuli in the hive. We predicted that rich diet would cause bees to have nurse-like patterns of gene expression.

Diet quality caused differential expression of 3372 transcripts in fat tissue (27%), including broad changes in metabolic processes. Many (1305; i.e., 39%) of these diet-responsive transcripts were also differentially expressed between nurses and foragers, significantly more than expected by chance (Table 3.2). However, the direction in which genes responded to diet was only a modest predictor of the direction in which genes responded during maturation – while there was a significant bias for genes to be regulated concordantly with the effects of maturation and diet on lipid stores, only 56% of genes showed this pattern (Table 3.3). A similar proportion

of genes were concordantly regulated by maturation and diet when we applied more and less stringent statistical cutoffs in defining gene lists, supporting the conclusion that shared responses to maturation and diet include a mix of concordant and discordant changes (Figure C.1). These results suggest that stable lipid loss involves both diet-dependent and diet-independent changes in gene expression.

Analysis of functional categories differentiating bees fed a rich vs. poor diet suggested, similarly, that there was a strong overlap between processes regulated by maturation and diet, but that these processes were not always regulated in concordant directions in the two experiments. Impressively, 15 out of 21 GO categories with biased representation between nurses and foragers also had biased representation between rich and poor diet (Table 3.1; Appendix B). Like nurses, bees fed a nutrient-rich diet had increased expression of genes related to lipid and protein metabolism; like foragers, bees fed a poor (carbohydrate-only) diet had higher expression of glycolytic enzymes (Appendix B). We note that the upregulation of carbohydrate metabolism in bees fed a sugar-only diet differs from the genomic responses to food deprivation reported in *Drosophila* [40, 41], during which all major macronutrient metabolism pathways are tuned down to preserve nutrient stores. This suggests that both foragers and bees fed a sugar-only diet are responding specifically to the nutrients available to them rather than merely to the absence of dietary lipids and proteins.

In addition, maturation and diet quality influenced many of the same categories of regulatory genes (e.g., "regulation of developmental process", "protein kinase cascade"), further suggesting that there is a shared regulatory underpinning for the effects of maturation and diet. The shared

effects of maturation and diet on metabolic and developmental processes suggested that nutrition could be responsible for the maturational differences in these processes. Alternatively, maturation and diet may regulate these processes independently, but via shared intermediate mechanisms.

By contrast, whereas foragers had higher expression than nurses of genes related to energy metabolism, bees fed a poor diet had lower expression of many of these genes. Conserving energy (both at a behavioral and physiological level) is a common response to poor nutrition in many species and is one of the mechanisms making it difficult for obese humans to lose weight [1]. These results suggest that the increased energy metabolism associated with stable lipid loss is distinct from the energy metabolism responses to poor nutrition found both in bees and in other organisms and is likely caused by factors other than diet.

Diet quality also influenced the expression of a number of genes and processes that were not differentially expressed during maturation (Appendix B). For instance, genes related to amino acid metabolism (58 genes) were upregulated in rich diet, but this process was not enriched among behaviorally-responsive genes. Notably, a number of genes with conserved roles in nutritional regulation, including the insulin-related transcription factor *FoxO* [42], the cholesterol sensor *Hr96* [43], and the cGMP-dependent protein kinase *foraging* [44], were differentially expressed in the fat bodies in response to diet quality but not during maturation. These results suggest that maturation is not simply a result of nutritional differences between nurses and foragers. Rather, stable lipid loss likely involves a mix of nutritionally-dependent and

independent changes, including the regulation of some nutritionally-related genes by non-dietary factors.

We used quantitative proteomics to determine whether diet-induced transcriptional changes were reflected at the protein level. There was a strongly significant, positive correlation between transcript abundance and protein abundance for the 390 genes measured by both techniques ($r = 0.34$, $p = 8.2e-12$, Fig. 3.2).

Maturational changes in responsiveness to nutritional stimuli

To more directly test the idea that maturation involves diet-independent regulation of nutritionally-related genes, we examined the expression of metabolic and signaling-related genes under a broader set of conditions using quantitative RT-PCR (qPCR). Bees at three different stages of maturation – nurses, foragers, and pre-nursing bees like those used in the microarray experiment – were removed from the hive and fed either a rich or a poor diet for four days, after which we measured gene expression in the fat bodies. We included diet- and maturation-regulated genes (based on microarray studies or previous qPCR studies in [23]) that were involved in protein storage (*vitellogenin; vg)*, lipid storage (*lipid storage droplet 2; lsd2*), and fatty acid β-oxidation (*carnitine O-palmitoyl transferase 1, CPT1,* and *thiolase*), as well as components of the juvenile hormone signaling pathway -- *JH esterase (JHE), JH epoxide hydrolase (JHEH)* -- and of the insulin signaling pathway -- *insulin-like peptide 2 (ilp2)*, *insulin-related receptor 1 (inR1)*. We also included two additional genes related to well-known, nutritionally-related peptide signaling pathways – the *adipokinetic hormone receptor (akhR)* and

the *neuropeptide F receptor (npfR)* – for which we did not have previous evidence of differential expression between nurses and foragers or in response to diet.

We confirmed that all 10 genes were differentially expressed between nurses and foragers (Fig. 3.3A). 8 of 10 genes (all except *akhR* and *npfR*) differed between pre-nursing bees fed a nutrient-poor sugar syrup diet compared to the nutrient-rich pollen/honey diet. 9 out of 10 genes (including *akhR* but not *npfR*) differed between the sugar-syrup diet and a different nutrient-rich diet made from a mixture of royal jelly and honey (we used two rich diets here to facilitate comparisons with foragers below--foragers cannot digest pollen and are fed jelly from workers [19]). These results provide validation for maturational and nutritional expression patterns discovered in our microarray studies. In addition, the similar responses to the two rich diet regimes suggest that these differences relate to diet quality in general rather than being a specific response to pollen.

Older bees responded very differently than pre-nursing bees to the same nutritional manipulations. Rich vs. poor diets in nurses did not cause differential expression of any of the 10 genes. Instead, most genes retained expression levels similar to nurses in the hive, regardless of the diet they were fed. These results suggest that nurses are "buffered" against changes in their diet. Foragers were selectively responsive to rich vs. poor diet. Genes related to fatty acid metabolism and JH signaling, *vg*, *ilp2*, and *lsd2* responded weakly or not at all to diet, and generally were fixed at low levels similar to foragers in the hive. However, the three peptide signaling receptors -- *inR1*, *akhR,* and*, npfR* -- had equal or greater sensitivity to nutrition compared to pre-nursing bees.

These maturational differences in responsiveness to diet lead to three intriguing inferences about stable lipid loss. First, only young bees, not nurses or foragers, are similar to humans and previously studied model organisms in that they adjust nutrient storage and utilization to match what is available in their diet. Nurses maintain large nutrient stores despite several days without dietary protein and lipid, while foragers remain lean despite four days consuming a rich diet and being prevented from flying. Second, maturational changes in responsiveness to diet reflect the actions of different signaling pathways regulated independently. Third, since diet does not appear to influence lipid metabolism in nurses and foragers, dietary changes on their own are insufficient to cause stable lipid loss during maturation. Our results suggest that stable lipid loss in the bee involves regulation of nutritionally-related pathways by both dietary and non-dietary factors.

As a first step toward understanding what non-dietary factors might be involved, we studied co-expression among the 10 genes in our qPCR study (Fig. 3.3B). Co-expression does not prove causal relationships, but if genes in a signaling pathway are co-expressed with genes in effector processes such as metabolic pathways it is reasonable to hypothesize that the signaling pathway acts upstream. We found that the fatty acid oxidation genes, *cpt1* and *thiolase*, were tightly correlated with *jhe, jheh, vg, and ilp2* ($r = 0.38 - 0.55$) but not with *inR1, akhR,* or *npfR* ($r = -0.00 - -0.21$). JH, Vg, and insulin signaling have coordinated actions in the bee [45], and JH has well-known effects on lipid metabolism in other insects (e.g., [46]) and energy metabolism in the bee [47]. Based on these results, we hypothesized that non-dietary processes involving JH, Vg and insulin signaling mediate stable lipid loss.

Effects of *vitellogenin* RNAi on fat body gene expression

To explore the molecular mechanisms influenced by Vg and to gain insight into how this gene, with already known novel regulatory functions in the bee [29, 30], influences maturation and nutrient storage, we measured fat body gene expression following RNAi knockdown [29, 30] of *vg* expression in the peripheral tissues of young bees. *vg* RNAi influenced the expression of 2136 transcripts in the fat bodies, including changes in diverse metabolic pathways (Appendix B). 1030 (48%) of these transcripts were also differentially expressed between nurses and foragers, more than twice as many as expected by chance (Table 3.2). In addition, responses to *vg* RNAi were a significantly better predictor of the direction of maturational changes than were responses to diet quality (*vg*: 70% concordant vs. diet 56% concordant; Fisher's Exact Test, $P < 4.5e\text{-}11$; Table 3.3), suggesting a tighter relationship with maturational state for *vg* than for diet. This strong, maturationally-related, response to *vg* RNAi is consistent with the idea that *vg* has taken on signaling-like roles in the bee [58, 59] and could have causal influences on lipid loss.

Despite the generally strong relationship between the effects of maturation and Vg, *vg* RNAi did not influence as many of the maturationally-regulated metabolic and developmental processes as did diet (Table 3.1). Consistent with its effects on behavior, *vg* RNAi caused forager-like decreases in the expression of genes related to lipid metabolism. However, these changes in lipid metabolism were embedded in a metabolic response in which carbohydrate metabolism and energy metabolism pathways were also tuned down (Table 3.1; Appendix B). These food deprivation-like responses occurred even though bees were fed an *ad libitum* pollen/honey diet. This suggests that disruption of a single storage protein can cause dramatic changes in the ability

of bees to store and utilize nutrients; perhaps Vg titer acts independent of nutrient availability *per se* as a signal of nutritional status. Consistent with this speculation, *vg* expression declines naturally by a similar or greater proportion as they undergo stable lipid loss during maturation (Fig. 3A) but do not initiate these starvation-like responses. These results suggest that Vg on its own is not sufficient to induce the metabolic or regulatory changes involved in lipid loss without inducing compensatory responses to preserve nutrients and energy.

Vg RNAi also influenced a number of pathways that were not altered during maturation (Appendix B). Many aspects of the translational machinery were upregulated after *vg* knockdown (translation: 42 genes), including genes related to translational initiation (11 genes) and components of both the cytosolic and mitochondrial ribosome. However, *vg* knockdown also increased the expression of genes related to proteolysis (32 genes) and protein localization (40 genes; primarily components of the endosome). These changes suggest that knockdown of *vitellogenin* led to increased protein turnover in the fat bodies.

Effects of Queen Mandibular Pheromone on lipid stores, food intake, and fat body gene expression

Previous results have shown that nutrition and social factors have independent effects on maturation [22]. We investigated a role for one such factor, QMP, in stable lipid loss and explored interactions between QMP and dietary factors. We first confirmed (following [33]) that QMP exposure caused young bees fed a rich diet to have larger lipid stores (Fig. 3.4A). In addition, we found that bees also had larger lipid stores after exposure to QMP when they were fed a poor diet, and that these effects were largely additive, suggesting that the effects of QMP

do not depend on a particular component of the diet. Bees fed both a rich diet and exposed to QMP had lipid stores similar to or even larger than age-matched, 5-day-old bees reared in a hive and nurse bees, whereas bees fed a poor diet and not exposed to QMP had levels similar to 1-day-old bees and foragers. It was thus possible by manipulating diet and QMP to reproduce in young caged bees the full range of naturally occurring lipid stores for bees in the hive.

Furthermore, we found with cage experiments that QMP exposure caused bees fed a rich diet to consume more pollen/honey paste (Fig. 3.4B) and bees fed a poor diet to consume more sugar syrup (Fig. 3.4C). These results indicate that changes in food consumption may be one mechanism by which QMP causes bees to build up larger lipid stores.

These results were obtained using bees from genotypes highly responsive to QMP in a standard behavioral assay; QMP had weaker effects on lipid stores and food consumption for bees from genotypes with lower responsiveness (Fig. C.2). Similar genetic variability has been seen previously in lab assays using QMP [48]. Using bees from highly-responsive genotypes, we found that QMP had much more subtle effects on fat body gene expression than did the other factors tested, influencing the expression of only 309 transcripts. QMP, like diet and vg, disproportionately influenced genes that were also influenced by maturation (Table 3.2). These overlapping genes tended to be differentially expressed in the direction predicted by the effects of QMP on lipid stores and maturation (57% concordant), but this trend had only marginal significance (chi-square test: $p = 0.05$; Fisher's Exact Test: $p = 0.04$).

QMP did not cause significantly biased expression in many maturationally-related processes (Table 3.1). And although QMP increased pollen consumption, it appeared to cause bees to degrade proteins (Appendix B). Genes upregulated by QMP were enriched for categories related to proteasomal degradation, whereas QMP downregulated genes related to amino acid biosynthesis and metabolism. These seemingly contradictory effects of QMP to enhance protein degradation while increasing pollen consumption and nutrient stores may reflect tradeoffs between its numerous functions in the colony. It is in the interest of the queen for workers to have large nutrient stores so that they are effective at nursing, but not so large that workers might themselves become reproductively active. Perhaps our results reflect a subtle balancing of physiological processes by QMP to promote efficient colony functioning.

Our results confirm an effect of QMP on lipid stores and suggest that one mechanism for this is an effect on food consumption. However, the relatively subtle effects on fat body gene expression suggest that the actions of QMP are mostly mediated by changes outside this tissue, with the brain the most likely target because pheromone detection occurs primarily through receptors in olfactory neurons. Nonetheless, our results support the idea that social signals act as novel regulators of nutrient storage in the bee. Similarly, a recent study showed that a second pheromone, brood pheromone, influences Vg titers [49]. Despite the relatively weak effects of QMP on its own, it is possible that the added effects on nutritional physiology of other pheromones could be quite significant. Insect societies are well known for extensive use of pheromones to regulate diverse aspects of colonial life [71].

Shared and unique responses to diet, *vg*, and QMP

Our results support the idea that diet, *vg* and QMP all contribute to stable lipid loss during maturation. However, diet, *vg*, and QMP differed in the extent to which they "tracked" metabolic and regulatory processes that differed between nurses and foragers. We therefore performed additional bioinformatic analyses to explore relationships between the responses to diet, *vg*, and QMP, in order to determine whether they reflect shared or independent mechanisms for the control of lipid stores in the bee.

The sets of genes regulated by maturation, diet, *vg*, and QMP all overlapped significantly more than expected by chance (Table 3.2). This result suggests that these factors influence shared mechanisms. In addition, as described above, the directional responses to maturation and to each of the other factors were biased in a way that matched the effects of treatments on the pace of maturation and on lipid stores. Therefore, the effects of diet, *vg*, and QMP are aligned with respect to their shared effects on maturation.

By contrast, the directional relationships between the effects on gene expression of diet, *vg*, and QMP (Table 3.3) were either non-significant (diet vs. *vg*, *vg* vs. QMP) or biased in the direction opposite of predictions (diet vs. QMP). These results may reflect the differing roles of diet, *vg*, and QMP outside the context of maturation [31, 50] or differences between physiological, genetic, and social manipulations, respectively.

Since diet, *vg*, and QMP have concordant effects primarily in the context of maturation, genes that are influenced concordantly by all of these factors may represent particularly integral aspects of stable lipid loss. We identified (using FDR < 0.2 in order to reduce false negatives) 25

transcripts that were concordantly upregulated in all four conditions with large nutrient stores (nurse > forager, rich diet > poor diet, control > *vg* RNAi, QMP > control) and 11 transcripts that were concordantly upregulated in conditions with small nutrient stores (Fig. 3.5). Some of the genes associated with large lipid stores were related to lipid biosynthetic processes (5 genes), and, more generally, oxidation/reduction (8 genes). Several genes associated with small lipid stores were related to the breakdown of glycogen, including glycogen synthase kinase 3 and phosphorylase kinase – hormonally-regulated enzymes that inhibit glycogen synthase and activate glycogen phosphorylase, respectively – as well as the glycogen degrading enzyme α-glucosidase. These results support the idea that a shift from lipid metabolism to carbohydrate metabolism is important to stable lipid loss.

The JH-degrading enzyme *JH epoxide hydrolase* was upregulated across all four lipogenic conditions, suggesting a strong association between low JH signaling and large lipid stores. In addition to the evidence cited earlier linking JH to nutrition and metabolism, the stimulatory effects of JH on behavioral maturation are well documented [25-27], as are its antagonistic relationships with *vg* [29, 51, 52] and QMP [53, 54]. These results provide further evidence of a role for endocrine signaling as a shared mechanism that mediates the effects of diet, *vg*, and QMP on lipid storage even though these three factors also have distinct effects on gene expression. Perhaps this contrast reflects the diversity of functions performed by the fat bodies in insects.

Maturational and diet-related changes in blood protein content.

Some fat body proteins are secreted into the hemolymph (insect blood) for nutrient storage purposes and as part of communication between the fat bodies and other tissues. Using mass spectrometry, we identified 47 proteins (out of 212 quantified) that were differentially abundant in hemolymph from nurses and foragers. We studied the relationship between fat body transcription and hemolymph protein abundance in order to identify proteins that were likely secreted from the fat bodies. Overall, there was a positive correlation between the effects of maturation on fat body RNA and hemolymph protein (Pearson correlation; $r = 0.35$, $P = 3.7e-7$; Fig. 3.2), and we identified proteins with storage, transport, and signaling functions among those with the most concordant differences between nurses and foragers in the two tissues. These proteins are candidate molecules for maturationally-related communication between the fat bodies and other tissues. However, these changes likely under-represent the complement of signaling molecules secreted by the fat bodies into the hemolymph because small peptides were not quantified by the technique we used. In addition, we identified many carbohydrate and energy metabolism enzymes that were more abundant in hemolymph from foragers but were not differentially expressed in fat. These hemolymph-specific differences likely reflect maturation-related changes in the function of hemocytes (the intrinsic cells of the hemolymph).

We identified 52 proteins (out of 281 quantified) that differed in abundance between the hemolymph of pre-nursing bees fed a rich vs. poor diet, and these included a mix of fat-related and hemolymph-specific differences. Overall, the responses to diet quality of fat body RNA and hemolymph protein were weakly correlated ($r = 0.26$, $p = 2.7e-5$, Fig. 2). Several of the same storage and signaling-related proteins that responded similarly in fat and hemolymph during maturation were concordantly responsive to diet as well. Therefore, just as the responses to

maturation and diet have many similarities within the fat bodies, there are similarities in how these factors influence the proteins that are secreted out of the fat bodies. In addition, hemolymph from bees fed a poor diet contained an increased abundance for genes related to energy metabolism, carbohydrate metabolism, and immune functions. The former category is somewhat surprising given that energy metabolism enzymes were downregulated by poor diet in the fat bodies. Perhaps these changes in energy and carbohydrate metabolism reflect the increased reliance of bees fed a sugar-only diet on the utilization of carbohydrate stores in the hemolymph for energy. JH esterase, the principal JH degrading enzyme in the hemolymph, was more abundant in bees fed a rich diet, supporting the idea that good nutrition represses JH signaling in adult worker bees.

Together, these results identify maturation- and diet-related proteins that are secreted out of the fat bodies and could potentially serve functions in communication between the fat bodies and other tissues. In addition, maturation and diet induce a number of hemolymph-specific changes in carbohydrate and energy metabolism, which may relate to the functions of the hemolymph in nutrient storage and utilization.

Discussion

We found that stable, maturational lipid loss in the honey bee involves massive changes in gene expression in the abdominal fat bodies, including many changes in core metabolic and signaling pathways. Our results support an integrated hypothesis about the evolutionary and mechanistic basis for stable lipid loss. First, stable lipid loss involves the regulation of nutritionally-related pathways by both dietary and non-dietary factors, most likely including evolutionarily novel,

hormonally-related signals, i.e., vitellogenin and Queen Mandibular Pheromone. Second, there are maturational changes in responsiveness of nutritionally-related signaling and metabolic pathways to the nutritional environment. Together these findings appear sufficient to explain the phenomenon of stable lipid loss in the bee.

We originally proposed novel regulation of conserved nutritionally-related pathways and novel nutritionally-related signals as separate hypotheses for the control of stable lipid loss in the bee. However, if vitellogenin is a causal factor in the nutritionally-independent regulation of nutritionally-related genes, then the two hypotheses converge on an integrated mechanism for stable lipid loss.

Previous studies had shown that nurses and foragers differ in a number of nutritionally-related physiological and behavioral traits in addition to their differences in lipid stores. For instance, foragers respond more strongly than nurses to weak sugar solutions by extending their proboscis to drink [55]. Moreover, the foraging task itself, flying at long distances to collect food for the colony, can be construed as an extraordinarily vigorous response to nutritional stimuli compared to the in-hive feeding behaviors of nurses. However, it had been mysterious why these differences persist, given that foragers are not obviously food-deprived and consume food inside the hive prior to their foraging flights [6]. Diet-independent regulation of nutritionally-related pathways provides a reasonable explanation for how these differences between nurses and foragers are established and maintained.

Here we report maturational changes in responsiveness to nutrition, but previous work has shown that maturation also entails changes in responsiveness to social signals. For instance, as bees mature, they become decreasingly responsive to QMP [48]. In addition, Brood Pheromone has age-dependent effects on behavior; it inhibits young bees from initiating foraging, but stimulates existing foragers to collect more pollen [56, 57]. Therefore, maturationally-related changes in responsiveness to environmental signals may be a general mechanism that stabilizes the behavioral and physiological differences between nurses and foragers, including differences in lipid stores.

Our results, together with previous work, allow us to propose a model for how stable lipid loss is achieved in the bee (Fig. 3.6). We suggest that relationships between JH, IIS, and metabolism are largely conserved between bees and other species, but that novel repressors of JH and IIS contribute to stable lipid loss. In particular, our findings extend previous work demonstrating inhibitory relationships between Vg and JH and between QMP and JH [54, 58] and suggest new repressive relationships between nutritional status and JH. An interesting feature of this proposed mechanism for stable lipid loss is that it features multiple, largely independent mutually repressive relationships between JH/IIS and external and internal signals associated with maturation. Because mutual inhibition between two factors can establish a simple bistable switch [59], we speculate that changes in any of these signals could be sufficient to cause bees to begin the process of stable lipid loss. Further work is needed to validate some of these proposed relationships and to establish what maturational factors are responsible for inhibition of diet and QMP.

In what ways do the mechanisms of stable lipid loss in the bee inform us about the potential for stable weight loss in humans? Our studies confirm that stable lipid loss is unlikely to succeed based simply on nutritional interventions, given the differences we found between the regulation of stable, maturational lipid loss vs. diet-induced lipid loss in the bee. However, our results suggest that stable lipid loss in the bee largely results from novel regulation of pathways that also control body weight in humans. Therefore, a promising approach might be to use comparative studies to identify specific aspects of nutritional physiology that could be targeted in humans to facilitate weight loss.

Such comparative studies need not be limited to the bee. Although stable lipid loss is a highly derived trait in honey bees, likely having evolved during or after the evolution of worker division of labor, other insects undergo related physiological and behavioral life transitions. Studies of diapause in flies and other species [60, 61] and oogenesis in mosquitoes [62] indicate that these traits, like stable lipid loss in the bee, involve novel regulation of conserved, nutritionally-related metabolic and signaling pathways. Combining the insights from studies in all these species will reveal the degrees of freedom around which these pathways can be modified and recombined during evolution to produce novel phenotypes. Our work suggests that large-scale, genomics approaches are a particularly promising route to developing and testing these hypotheses.

Materials and Methods

Bees. Bees were maintained according to standard practices at the University of Illinois Beekeeping Facility in Urbana, IL, and experiments were performed during the summers of 2007 and 2008. Colonies were of a mix of European genotypes. For microarray experiments, we used

exclusively queens each inseminated with a single, different drone (SDI) to reduce genetic variability among worker bees. In other experiments, we used a mix of SDI and naturally-mated queens. For microarray and proteomics experiments, nurses and foragers were identified based on standard behavioral assays [23]. Nurse bees used for nutritional manipulations were instead collected based on their age (8-9-days-old) and proximity to the brood; this is also a reliable assay for nursing. Age-matched bees were obtained by placing a brood frame into an incubator (34°C) and collecting bees that emerged as adults over a 24-hour period. Bees were collected immediately (1-day-olds) or aged in a host colony to the desired age (5-day-olds, 9-day-olds). For behavioral assays, bees were collected into cages using soft forceps. For molecular analyses, bees were killed by flash freezing in liquid nitrogen or on dry ice.

Diet manipulations. Young bees were caged and fed rich diet (pollen/honey) or poor diet (sugar syrup) as previously described [23]. The alternate royal jelly / honey diet was as described by Hoover et al. (2008). We modified conditions slightly to improve survival of older bees. 1-day-old bees and nurses were maintained in groups of 35 at 34°C; foragers were maintained groups of 25 at 27°C. Cumulative mortality was <10% for one-day-olds and nurses and 20-30% for foragers. We verified that all groups consumed the diets by weighing and replacing feeders daily. After four days, bees were collected by brief CO_2 anaesthetization and flash freezing in liquid nitrogen.

***vitellogenin* RNAi.** Previously described *dsVg* probes [63] were synthesized by *in vitro* transcription with T7 RNA polymerase and gel purified. We diluted dsRNA to a concentration of 10ug/ul in buffered saline solution and injected 1ul intra-abdominally using a microinjection

system equipped with a 34G beveled needle. Control bees were injected with 1 ul of buffered saline alone. After injection, bees were painted with an identifying mark on the thorax and placed into cages with equal numbers of RNAi-injected, saline-injected, and uninjected bees. They were fed a rich diet of pollen/honey and sugar syrup for four days then collected by flash freezing in liquid nitrogen. Mortality was 10-20% over 4 days. We validated knockdown by qPCR and selected 5 (out of 10) individuals / group from each of two trials for microarray analysis based on the strongest knockdown. Knockdown among selected bees was 50-70% relative to saline-injected controls.

Queen Mandibular Pheromone. QMP (0.1 queen equivalents synthetic QMP [Pherotech, Trail, British Columbia], in 90% isopropanol/10% water dried onto a microscope coverslip) was administered as previously described [64] to groups of 35 caged bees fed a rich or poor diet. Control cages were administered solvent alone. We measured total daily food consumption for each cage every 24 hours by weighing and replacing feeders. After 4 days bees were collected by flash freezing.

To account for genetic variation in responsiveness to synthetic QMP, colonies were screened using a retinue assay (modified from [48] based on recommendations from C.M. Grozinger [personal communication]). Nurse bees were captured from each colony and caged overnight (15 bees / cage) without QMP, fed sugar syrup. A QMP lure and a control lure were placed simultaneously into a cage, and retinue behavior was quantified by observing the number of bees contacting each lure at 30 s intervals for 5 m. The retinue response score was calculated as the total number of contacts to the QMP lure, minus contacts to the control lure observed over this

interval. Observations of the same bee contacting the lure at different time points were counted as separate lure contacts. An average retinue response score for each of 19 colonies was determined by averaging 3 independent assays with different groups of bees.

Hemolymph extraction. Hemolymph was extracted by making a small incision at the neck and drawing clear hemolymph using a microcapillary tube. Hemolymph from 5-7 bees was combined for each sample and diluted in 100 ul 50mM NH4HCO3, pH 8, containing protease inhibitors. Samples were then centrifuged and stored at -80°C.

Lipid extraction and quantification. Lipid from abdominal fat bodies was extracted in chloroform/methanol and quantified by using a colorimetric assay with vanillin/phosphoric acid [5, 23].

Sample preparation for microarrays and qPCR. Abdomens of frozen bees were soaked overnight in 0.6mL RNAlater-ICE (Ambion). We then removed the gut and extracted total RNA from the remaining fat body and annealing cuticle using RNeasy kits (Qiagen, Valencia, CA).

Microarray Procedures. The microarray has been characterized in previous studies [34, 65] and contains 28,800 spotted oligos, including 13,440 duplicately-spotted experimental probes based largely on annotations from the honey bee genome sequencing project and 2000 control sequences. We used loop designs, with a total of 161 microarrays used to profile abdominal fat bodies from 127 individual bees. Effects of maturation, diet quality, and QMP were measured in one integrated study with n=16-17 samples per group (Fig. C.3), and the effects of *vg* RNAi were

measured separately with n=10 samples per group (Fig C.4). Microarray procedures were slightly modified from [34]. To quantify gene expression from individual fat bodies, RNA was amplified according to manufacturer instruction with the MessageAmp II aRNA Amplifcation kit (Ambion) starting with 500 ng RNA. Dye coupling and labeled aRNA cleanup were performed with the Kreatech Universal Labeling System (Open Biosystems), using 2ug aRNA in each reaction. Paired aRNA samples having incorporated 60pmol Cy3/Cy5 dye were hybridized to microarrays overnight. Slides were scanned with an Axon 4000B scanner, and images were analyzed using GENEPIX software (Agilent Technologies).

Microarray Data Analysis. Analysis was performed as in [34]. A Loess transformation was performed using Beehive (http://stagbeetle.animal.uiuc.edu/Beehive) to normalize expression intensities. A linear mixed-effects model implemented with restricted maximum likelihood was used to describe the normalized log2 transformed gene intensities values, including the effects of dye, treatment, bee, and microarray. Effects were evaluated with an F-test statistic and the P values were adjusted for multiple testing by using a FDR criterion. We evaluated two separate statistical models for the maturation-diet-QMP loop. The first analysis was a one-factor model, and the second was a two-factor model with Diet and QMP (nurses and foragers were excluded from this model). The Maturation gene list was derived from probes that were significant in a post-hoc nurse vs. forager contrast using the one-factor model. The Diet and QMP gene lists are based on the main effects of Diet and QMP, respectively, in the two-factor model, excluding probes that showed a significant *Diet x QMP* interaction (FDR < 0.05). The Vg RNAi loop was evaluated using a one-factor model with three levels (dsRNA-injected, saline-injected,

uninjected), and the Vg RNAi gene list consists of probes that were significant in the post-hoc dsRNA-injected vs. saline-injected contrast.

Functional enrichment analysis. GO directional bias analysis was performed with *Drosophila* orthologs to bee genes [65]. Statistical bias was determined using the DAVID Bioinformatics Resources 2008 Functional Annotation tool [66], based on the ratio of up- vs. down-regulated genes in each GO category, compared to that ratio among all genes with annotated *Drosophila* orthologs. Differentially expressed genes were mapped to pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG; [37]), primarily for visual purposes, using the Color Objects in Pathways tool on the KEGG website. We visualized the union of KEGG annotations for honey bee genes and the KEGG annotations for *Drosophila* orthologs.

Statistical Analysis to Determine Relationships Among Gene Lists. To determine whether the number of genes that overlapped on two gene lists (maturation, diet, *vg* RNAi, QMP) was statistically significant, a "representation factor" was calculated [65]. This factor is the number of observed overlapping genes divided by the expected number of overlapping genes. The denominator is calculated as the product of the number of oligos differentially expressed in each experiment divided by the total number of oligos analyzed. We tested statistical significance by using an exact hypergeometric test (1-tailed) for the overlap between two gene lists sets.

To determine whether pairs of treatments influenced the expression of genes in the same direction, we assembled 2x2 contingency tables for up vs. down-regulated genes that were differentially expressed in both treatments and computed significance using chi-square tests.

RT-qPCR. cDNA was synthesized from 200 ng of total RNA. qPCR was performed by using an ABI Prism 7900 sequence detector using specific primers. Results for experimental genes were normalized to a validated control gene, *eIFIII-S8 (GB12747)*, using a standard curve method. Statistical differences were determined using fixed-effects linear models implemented in SAS PROC MIXED (Cary, NC). Data were combined from three independent trials using different colonies.

Quantitative LC/MS. For proteomics, we thawed abdomens briefly (without RNAlater-ICE); after the gut was removed we additionally dissected the fat bodies away from the cuticle, and used this tissue for analysis. After washing in PBS three times, 100 μl of lysis buffer (1% NP-40, 150mM NaCl, 20mM Tris-HCl pH 7.5) including a protease inhibitor cocktail (Roche, at eight times the suggested concentration) was added and homogenized by ten strokes through a syringe tipped with a 25 G needle. The sample was clarified for 10 min at 16,100 r.c.f. at 4°C and the pelleted debris was discarded, while the supernatant proteins were precipitated by adding ethanol and sodium acetate as described in [68]. Hemolymph was processed as described previously [69] and hemolymph and fat body proteins were prepared for mass spectrometry, isotopically labeled and analyzed on an LTQ-OrbitrapXL (ThermoFisher) exactly as described [70]. The reported relative protein expression average was calculated by averaging across at least two of the three biological replicates, provided that the total number of measured peptides for that protein was not less than three.

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Tables for Chapter 3

Table 3.1. Functional categories showing directionally biased responses during stable lipid loss, and their responses to diet quality, *vg* RNAi, and Queen Mandibular Pheromone. We identified Gene Ontology Biological Process categories for which there was a directional bias such that genes in that category were more likely to be regulated in a particular direction within each experiment. Numbers of up- and down-regulated genes are shown for categories in which there was a directional bias in the maturation experiment. Statistical significance ($P < 0.01$) was determined relative to the total number of up- vs. down-regulated genes in each experiment that had unambiguous *Drosophila* orthologs ("All genes"), based on a modified version of Fisher's Exact Test [66]. Data for these same "behaviorally-related" GO categories are also shown for the diet, *vg* RNAi, and QMP experiments if an equivalent statistical test indicated a directional bias (using a more lenient statistical cutoff, $P < 0.1$, in order to show as many relationships as possible). Asterisks (*) indicate that the statistical bias in the diet, vg RNAi, or QMP experiment was in an opposite direction to that predicted by the directional bias for that category in the maturation experiment. Appendix B contains complete lists of GO terms showing directional bias for all experiments.

Table 3.1

Biological Process	***Maturation***	***Diet Quality***	***Vg RNAi***	***QMP***	
	Nurse ↑ / Forager ↑	Rich ↑/ Poor ↑	Ctrl. ↑ / Vg RNAi ↑	QMP ↑/ Ctrl. ↑	
All genes	413 / 591	658 / 511	419 / 336	56 / 50	
translation	79 / 33 (p=9.83e-11)	-	32 / 42 (p=3.05e-02)*	-	
fatty acid metabolic process	20 / 4 (p=1.44e-04	27 / 2 (p=1.04e-04)	-	-	
cell. macromol. metab. proc.	157 / 168 (p=1.5e-03)	-	92 / 118 (p=7.7e-05)*	26 / 3 (p=9e-6)	
lipid metabolic process	41 / 31 (p=7.47e-03)	59 / 15 (p=4.10e-05)	46 / 13 (p=4.83e-04)	-	
biological regulation	60 / 147 (p=7.91e-05)	114 / 165 (p=6.2e-09)	84 / 89 (p=3.21e-02)	-	
localization	84 / 182 (p=2.51e-04)	-	-	-	
compound eye development	3 / 28 (p=5.98e-04)	10 / 29 (p=3.08e-04)	-	-	
protein kinase cascade	0 / 18 (p=9.20e-04)	5 / 16 (p=9.32e-03)	-	-	
response to stimulus	39 / 99 (p=1.11e-03)	70 / 73 (p=5.61e-02)	-	-	
reg. of developmental proc.	2 / 22 (p=2.35e-03)	12 / 26 (p=4.61e-03)	-	-	
cell. comp. org. & biogenesis	81 / 165 (p=2.71e-03)	152 / 173 (p=6.58e-05)	94 / 112 (p=9.20e-04)	-	
developmental process	75 / 155 (p=2.89e-03)	121 / 182 (p=7.05e-11)	-	-	
cofactor biosynthetic proc.	3 / 24 (p=3.03e-03)	21 / 1 (p=4.84e-04)*	14 / 2 (p=2.91e-02)*	-	
phosphate metabolic process	26 / 71 (p=3.22e-03)	-	-	-	
response to chem. stimulus	7 / 33 (p=3.56e-03)	19 / 26 (p=7.19e-02)	-	-	-
primary metabolic process	284 / 354 (p=3.8e-03)	428 / 308 (p=8.7e-02)*	247 / 216 (p=9e-02)*	-	
oxidative phosphorylation	6 / 30 (p=4.61e-03)	33 / 1 (p=9.74e-07)*	-	-	
cellular localization	26 / 67 (p=8.21e-03)	-	33 / 43 (p=3.02e-02)	-	
regulation of boil. quality	7 / 30 (p=9.50e-03)	-	-	-	
cell. Struct. morphogenesis	14 / 44 (p=9.82e-03)	25 / 68 (p=9.09e-09)	-	-	

Table 3.2. Overlap of differentially expressed genes between experiments. The number of overlapping genes between pairs of experiments, representation factor (RF) indicating fold enrichment for the overlap between lists relative to random, and the statistical likelihood of overlap based on a hypergeometric distribution.

	Maturation (2641)			***Diet (3372)***			***Vg RNAi (2136)***		
	Overlap	RF	P	Overlap	RF	P	Overlap	RF	P
Diet (3372)	1305	1.76	6.39e-158						
Vg RNAi (2136)	1033	2.20	1.83e-198	939	1.57	7.82e-67			
QMP (308)	176	2.60	3.96e-42	206	2.39	8.20e-48	147	2.69	3.53e-34

Table 3.3. Directional relationships of differentially expressed genes between experiments. We compared the distribution of up- and down-regulated genes between each pair of experiments. Chi-square tests were used to determine if there was significant directional bias in these relationships. Numbers represent the intersection between the conditions indicated.

	Maturation			***Diet***			***Vg RNAi***		
	F>N	N>F	Significance	P>R	R>P	Significance	V>C	C>V	Significance
Poor > Rich Diet	346	281	$\chi2 = 20.55$						
Rich > Poor Diet	288	390	$P = 5.88e-6$						
vg RNAi > Ctrl.	370	105	$\chi2 = 170.12$	207	175	$\chi2 = 2.56$			
Ctrl. > vg RNAi	208	350	$P < 2.2e-16$	271	286	N.S.			
Ctrl. > QMP	60	49	$\chi2 = 3.45$	34	104	$\chi2 = 5.16$	19	80	$\chi2 = 0.09$
QMP > Ctrl.	26	41	$P = 0.05$	28	40	$P = 0.02$	11	37	N.S.

Figures for Chapter 3.

Figure 3.1. Maturational changes in fatty acid and carbohydrate metabolism gene expression. Transcripts differentially expressed between nurse and forager fat bodies (FDR < 0.05) were mapped to metabolic pathways based on the union of direct annotations from the Kyoto Encyclopedia of Genes and Genomes (KEGG, [37]) and the KEGG annotations for *Drosophila* orthologs of honey bee genes. Pathway diagrams are modified from portions of KEGG maps for “glycolysis/gluconeogenesis” (00010), “starch and sucrose metabolism” (00500), and “fatty acid metabolism” (00071).

Figure 3.2. Correlations of transcriptional responses in fat bodies to maturation and diet with protein differences in the fat bodies and hemolymph. *log2* expression differences are shown for mRNA (microarrays) and protein (LC/MS) for all genes quantified in both platforms. Labels are shown for selected genes (*lsp2: larval serum protein 2; vg: vitellogenin; obp13: odorant binding protein 13*) and categories (energy metabolism: GO biological processes “tricarboxylic acid cycle”, “ATP synthesis”, or “oxidative phosphorylation”); carbohydrate metabolism (GO: “carbohydrate metabolic process”).

Figure 3.3. Age-related changes in responsiveness of metabolic and hormonal signaling pathways in fat bodies to nutritional stimuli. A. Fat body gene expression (RT-qPCR) for nurses and foragers collected directly from the hive and for pre-nurses, nurses, and foragers that were caged and fed either a poor or rich diets. Asterisks indicate significance in paired contrasts following mixed-model ANOVA (* $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$, *N.S.* $P > 0.05$).

Expression (mean +/- s.e.m.) is shown relative to nurses in the hive. Genes: *vitellogenin (vg), JH Esterase (jhe), JH epoxide hydrolase (jheh), insulin-like peptide 2 (ilp2), thiolase, carnitine O-palmitoyl transferase I (cpt1), lipid storage droplet 2 (lsd2), insulin-related receptor 1 (inR1), adipokinetic hormone receptor (akhR),* and *neuropeptide F receptor (npfR)*. N=19-30 bees/group B. Pearson correlation matrix for co-expression between genes shown in (A) (qPCR, N≈250) with hierarchical clustering based on Euclidean distance.

Figure 3.4. Effects of Queen Mandibular Pheromone (QMP) on abdominal lipid stores and food consumption. A. Abdominal lipid stores of hive-reared one-day-old bees (1D), five-day-old bees (5D), nurses (N) and foragers (F), and of cage-reared five-day-old bees fed either rich or poor diet in combinations with exposure to QMP or a solvent control. Mean +/- s.e.m. N = 30 bees. ANOVA for Diet x QMP factorial: P_{Diet} = 1.0e-8, P_{QMP} = 0.006, $P_{Diet*QMP}$ = 0.20. B. Effects of QMP on food consumption (total consumption over 4 days for cages containing 35 bees) by bees fed both pollen paste and sugar syrup and exposed to either QMP or a solvent control. *N = 6 cages*. C. Effects of QMP on food consumption by bees fed sugar syrup only. *N = 8 cages*. In (B) and (C): Bars indicate least square means and their standard errors based on ANOVA for QMP exposure and trial. * $P_{QMP} < 0.05$.

Figure 3.5. Genes with concordant responses to maturation, diet, *vg* RNAi and QMP. Genes are shown that responded significantly (FDR < 0.2) in all four experiments concorant directions relative to the effects of each factor on a bee's lipid stores. Heatmap shows the *log2* transformed difference estimate in each experiment. Dots indicate annotation of a gene to the Gene Ontology biological processes listed or manually annotated to glycogen breakdown. Gene names are listed

according to the *A. mellifera* Official Gene Set 2 [35] and their orthology to *Drosophila melanogaster* genes based on Reciprocal Squared Distance or reciprocal BLAST. Oligos corresponding to unannotated ESTs are listed according to the EST name [67].

Figure 3.6. Verbal model for the regulation of stable lipid loss and its coordination with behavioral maturation. We propose that stable lipid loss occurs through a shift from the utilization of nutrients for lipid biosynthesis to their utilization via energy metabolism pathways. These metabolic pathways are regulated by juvenile hormone (JH) and insulin signaling (IIS), much as in other species, and by novel regulatory inputs to JH and IIS signaling whose efficacy depends on a bee's maturational state. Lipid gain early in life is likely controlled primarily by diet, but an unknown maturational signal decreases the responsiveness of JH and IIS to diet once bees become nurses. Later, maturational signals related to the transition from hive work to foraging repress the inhibition of JH/IIS by QMP, as part of the declining sensitivity of older bees to QMP [54]. In addition, there is a well-established mutually repressive relationship between JH and Vitellogenin (Vg) that is thought to contribute to the timing of maturation [29, 58, 59]. Communication between the brain and fat are implicit in this model because of the localization of IIS and JH synthesis to the brain and the adjacent retrocerebral complex, respectively, whereas metabolic changes and stable lipid loss occur in the fat bodies. Mutually repressive relationships in general are thought to act as bistable switches, so changes in any of these JH-related repressors during maturation could trigger stable lipid loss. Activating connections are shown by lines ending in arrows; inhibitory connections are shown by lines ending in ovals.

Figure 3.1

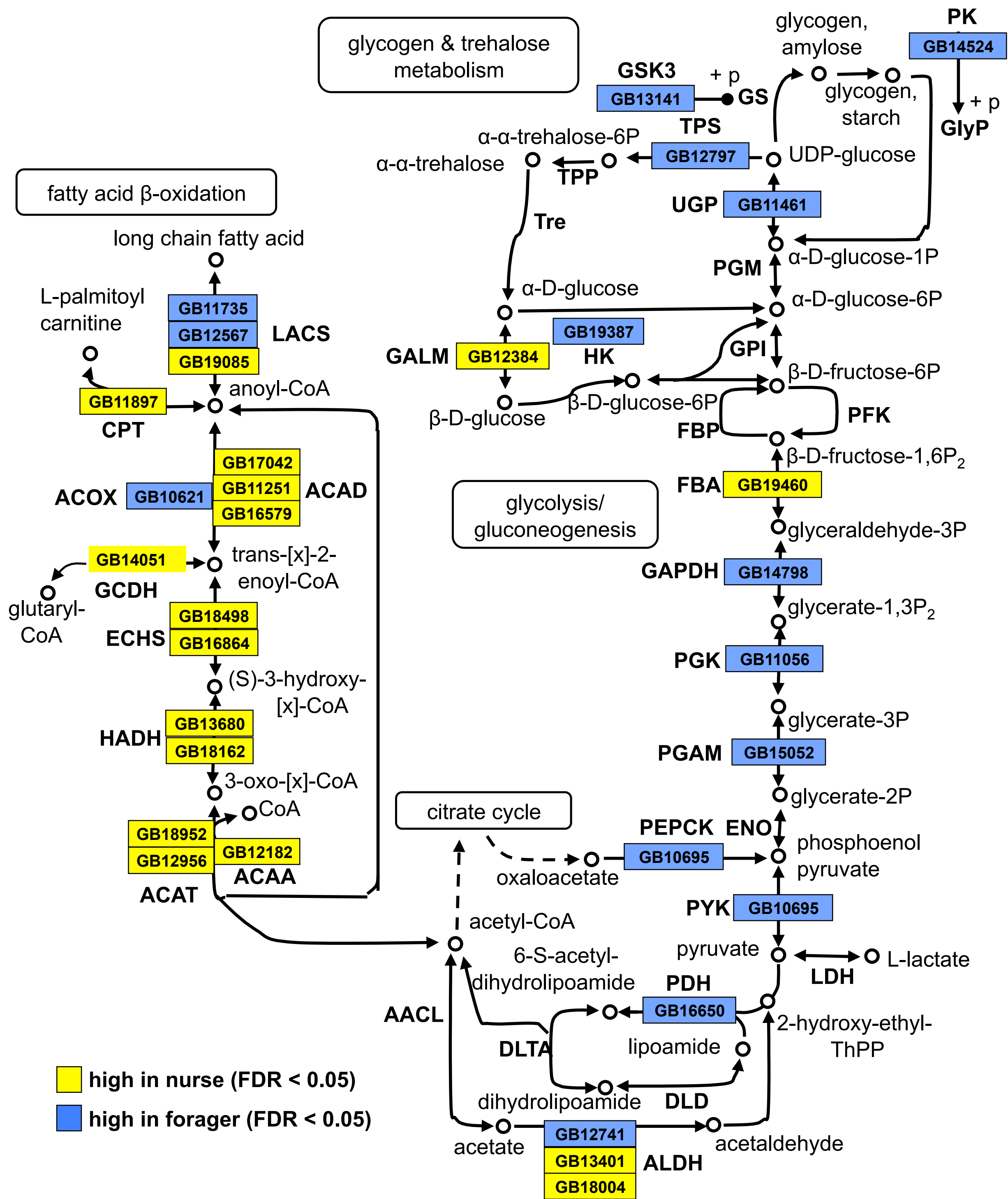


Figure 3.2

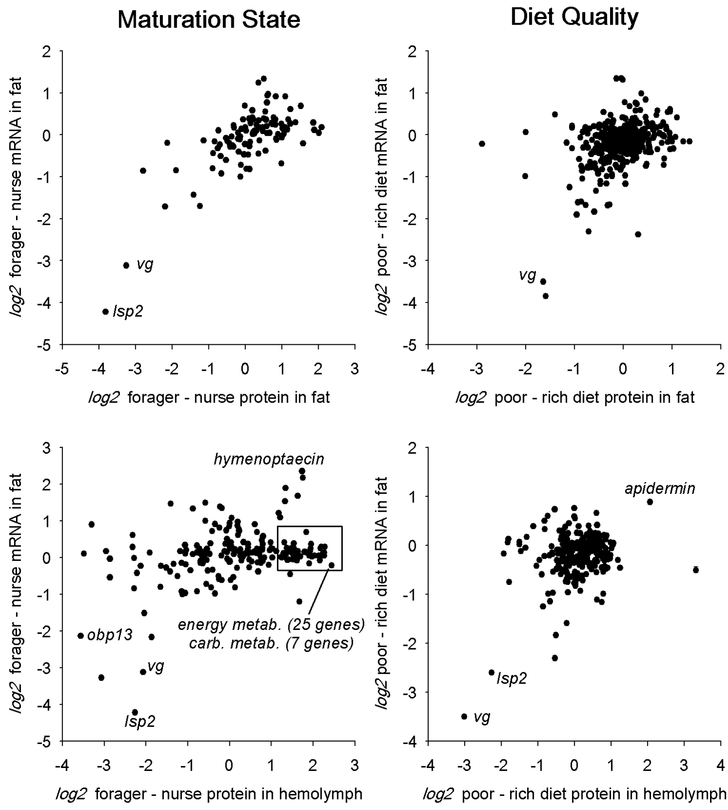


Figure 3.3

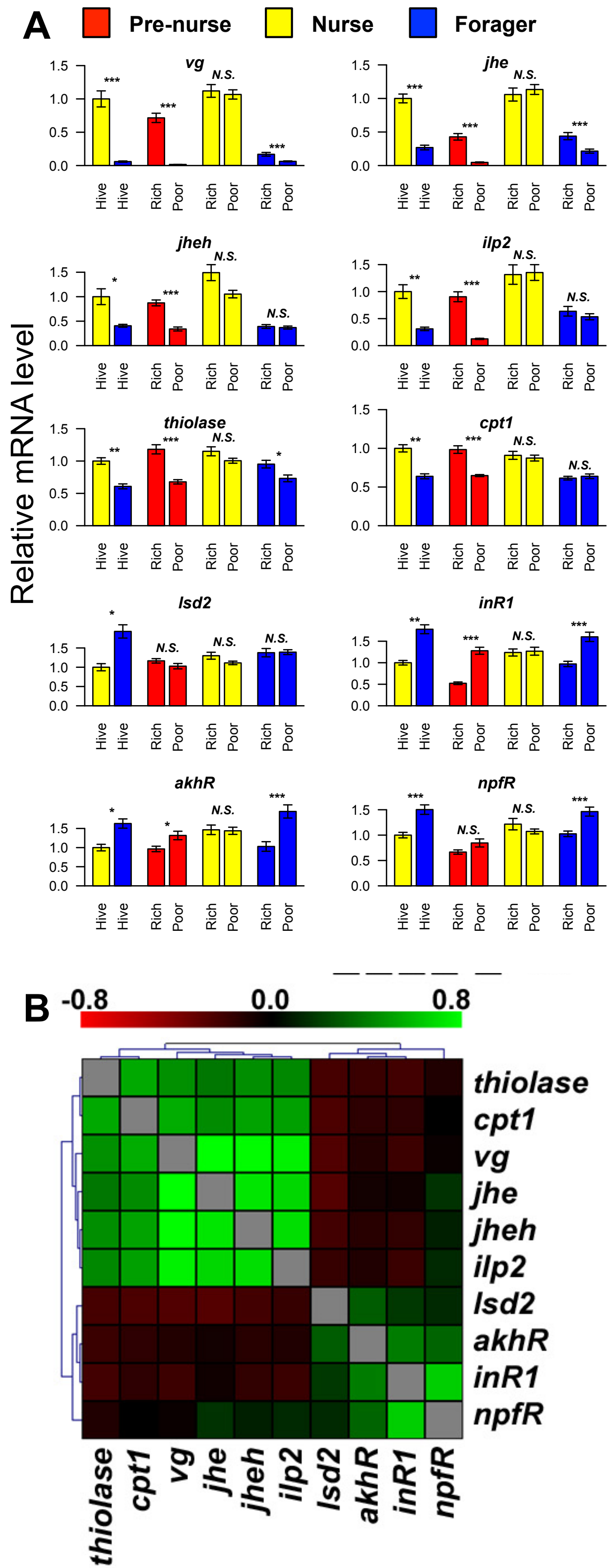


Figure 3.4

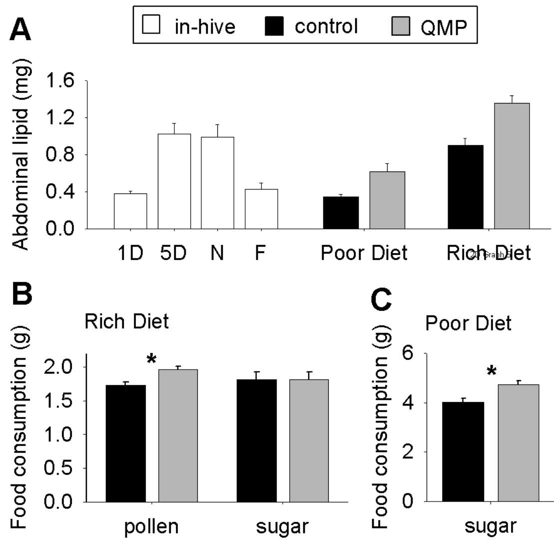


Figure 3.5

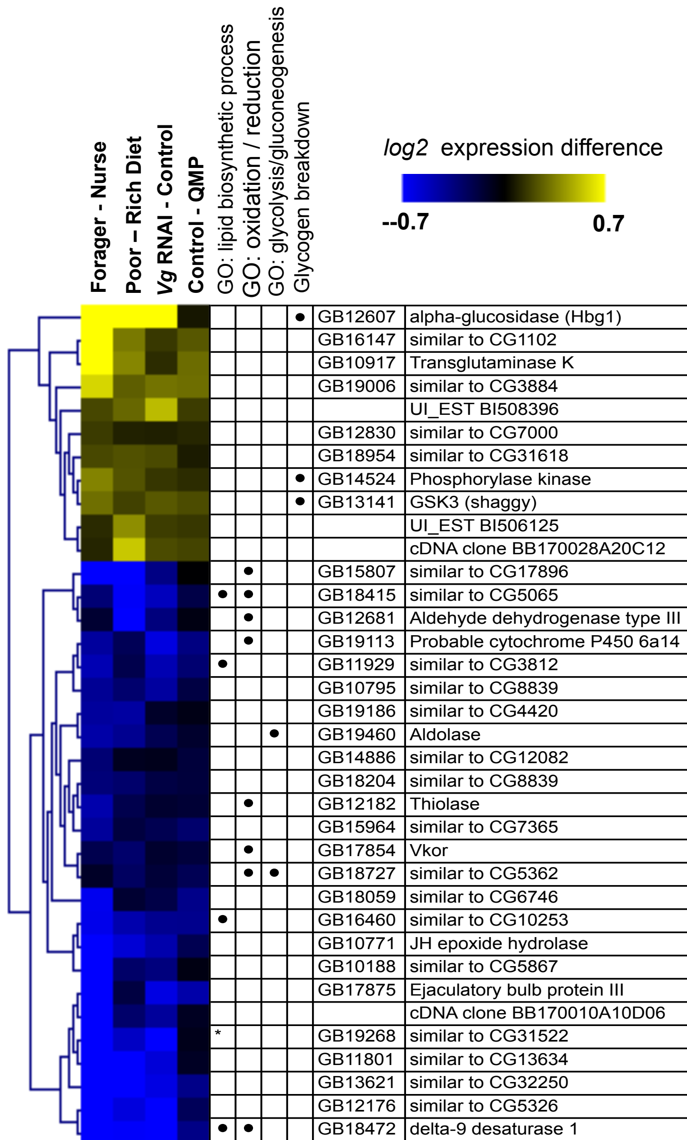
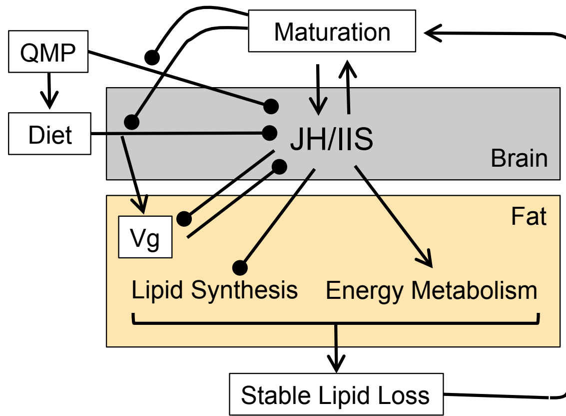


Figure 3.6



Chapter 4

Nutritional regulation of division of labor in honey bees: toward a systems biology perspective

Previously published work[4]

Abstract

Organisms adapt their behavior and physiology to environmental conditions through processes of phenotypic plasticity. In one well-studied example, the division of labor among worker honey bees involves a stereotyped yet plastic pattern of behavioral and physiological maturation. Early in life, workers perform brood care and other in-hive tasks and have large internal nutrient stores; later in life, they forage for nectar and pollen outside the hive and have small nutrient stores. The pace of maturation depends on colony conditions, and the environmental, physiological, and genomic mechanisms by which this occurs are being actively investigated. Here we review current knowledge of the mechanisms by which a key environmental variable, nutritional status, influences worker honey bee division of labor. These studies demonstrate that changes in individual nutritional status and conserved food-related molecular and hormonal pathways regulate the age at which individual bees begin to forage. We then outline ways in which systems biology approaches, enabled by the sequencing of the honey bee genome, will allow researchers

[4] Ament SA, Wang Y, and Robinson GE. 2010. Nutritional regulation of division of labor in honey bees: toward a systems perspective. *Wiley Interdiscipl Rev: Systems Biol Med*. 2(5): 566-576.

to gain deeper insight into nutritional regulation of honey bee behavior, and phenotypic plasticity in general.

Introduction

Many animals are able to alter their behavior and physiology in response to changes in the environment. At times, these changes in behavior and physiology are stable for long periods, a phenomenon known as phenotypic plasticity [1]. For instance, short periods of food deprivation stimulate feeding and the mobilization of stored nutrients to meet an individual's immediate energetic needs. But prolonged food deprivation can also lead to much longer-term effects, causing individuals to enter extended periods of inactivity [2], alter their reproductive strategy [3], or lose their position in a dominance hierarchy [4]. In humans, chronic food deprivation early in life may lead to a propensity towards obesity and diabetes in later life [5, 6]. The mechanisms that enable and constrain plasticity in behavior and physiology are not well understood, but it is clear that they often involve coordinated and long-lasting changes in gene expression, brain circuitry, and physiology (e.g., [7, 8], reviewed in [9]). We are developing a systems biology approach to studying the mechanisms that underlie phenotypic plasticity in the honey bee, *Apis mellifera*.

The adult worker honey bee exhibits phenotypic plasticity as part of the division of labor that defines the roles of each individual in the colony (Box 1). For the first 2-3 weeks of their adult life, bees perform brood care ("nursing") and other tasks inside the hive. They then transition to foraging for nectar and pollen outside the hive for the final 1-2 weeks of their life [10].

Nursing and foraging define the two most distinct and highly stable states in the life of a bee. Under ordinary circumstances, once a bee transitions into the foraging state she ceases to perform other tasks; although some foragers can be forced to perform brood care by removing all of a colony's younger bees, these reverted nurses are inefficient at rearing brood, suggesting that some aspects of the transition are irreversible [11]. Microarray experiments show that the brains of nurses and foragers differ in the expression of hundreds to thousands of genes, up to 40% of the genes expressed in the brain [12, 13]. This finding, together with results from neuroanatomical and neurochemical studies [14], demonstrates that honey bee behavioral states are defined by major changes in the brain.

The behavioral states of nursing and foraging also are associated with a number of physiological changes outside the brain. A honey bee loses half its abdominal lipid stores during the transition from working in the hive to foraging. The lipid loss is stable; a forager's lipid stores subsequently remain low even when food is plentiful [15]. The coordination of these changes in the brain and periphery and the importance of nutritional changes to division of labor will be described in detail below.

Although the stages of behavioral maturation are stereotyped and stable in honey bees, their timing is plastic. When older bees are removed from the hive, young bees rapidly establish a division of labor; some individuals begin to forage as young as five days of (adult) age [11], over two weeks earlier than normal. The pace of behavioral maturation also depends on the needs of the colony. For instance, when a colony lacks stored pollen and honey, bees also become precocious foragers, helping to replenish these essential provisions [16]. Plasticity in the age at

onset of foraging makes it possible with the honey bee to dissociate age and behavior, something usually impossible to do when studying maturation. Most of the maturational changes in physiology [15] and brain gene expression [12, 13] discovered in honey bees are associated with a bee's behavior, and not her age.

The stability of nursing and foraging, along with the ability to influence the transition between these states through a variety of techniques, has made honey bee division of labor an important model for understanding phenotypic plasticity [7, 14]. Here we review current knowledge of the mechanisms by which a key environmental variable, nutritional status, influences division of labor. We then outline ways in which systems biology approaches, enabled by the sequencing of the honey bee genome, will allow researchers to gain deeper insight into nutritional regulation of honey bee behavior, and phenotypic plasticity in general.

Nutritional regulation of division of labor in honey bee colonies

Given that many of the tasks performed by honey bees relate to collecting, handling, and distributing food, it is perhaps not surprising that their division of labor is regulated by nutritional stimuli. A number of studies have now elucidated mechanisms by which changes in colony nutrition lead to changes in individual behavior, including roles for well-conserved signaling pathways that regulate hunger and the more straightforward food-related behaviors of solitary species.

Honey bees differ from non-social species in that food-related tasks are performed in an intrinsically social context. Because a colony of social insects is widely thought to be the unit

upon which natural selection acts, tasks performed by workers are typically intepreted in terms of how they benefit the whole colony, rather than the individual worker. For instance, foragers directly consume very little of the food they collect [10]. Instead, they initially store the nectar they have collected in their foregut, sometimes called the honey stomach, then regurgitate it to food storing bees, who are responsible for converting it into honey and storing it. Much of the actual food consumption occurs via sharing among adult bees of processed nectar and "jelly," a proteinaceous glandular secretion produced primarily by nurses and subsequently passed to foragers [17, 18]. Moreover, a forager consumes honey inside the hive before leaving the hive for a foraging trip [10], so a bee is not food-deprived when she leaves the hive to forage. For these reasons, while division of labor revolves around the performance of food-related tasks, foraging itself is not an appetitive or consummatory behavior in the traditional sense. It has therefore been all the more exciting to learn over the past decade that nutritionally-related mechanisms within individuals play an important role in the regulation of honey bee social behavior. Some of the key findings are described in the following paragraphs.

In the first paper describing an effect of nutrition on honey bee division of labor, Schulz et al. [16] monitored the age at first foraging for bees in matched colonies that were either well-fed with excess honey and pollen, or food-deprived with no pollen and only enough honey to last 2-3 days. Bees from food-deprived colonies began foraging earlier than bees from well-fed colonies, establishing that food availability inside the hive was involved in behavioral maturation. The important factor affecting behavioral maturation appears to be intake of nutrients by individuals. To establish this, the authors uncoupled the usually linked factors of food availability and food storage. They created well-fed bees in starved colonies by providing a feeder inside the hive to

nurture individual bees, but bees were prevented from storing food in honeycomb cells by use of a vacuum pump mounted on the back of the honeycomb frame. Under these conditions, bees foraged as though they were well-fed, indicating that individual food intake rather than assessment of stored food caused precocious foraging in food-deprived colonies.

The most obvious means by which individual nutrition could lead to long-term changes in behavior is through changes in physiology. Nurses and foragers differ dramatically in several aspects of nutritional physiology. Toth and Robinson [15] studied the relationship between a bee's lipid stores and her behavioral state. Bees reach adulthood with low lipid stores and gain lipid mass during the first few days of adult life. They maintain large lipid stores for as long as they nurse brood, but during the transition to foraging they lose half of their abdominal lipid. Interestingly, this lipid loss does not depend on the performance of energy-intensive flights outside the hive; experimentally hive-restricted bees lose almost as much lipid mass as controls during the days leading up to foraging. In a follow-up experiment, Toth et al. [19] treated bees with a fatty acid synthesis inhibitor to artificially reduce lipid stores of bees in an otherwise well-fed colony. Individuals with reduced lipid stores initiated foraging earlier than controls, suggesting that changes in lipid stores can accelerate behavioral maturation.

Independent evidence for the causal effects of stored nutrients on honey bee behavioral maturation comes from work on the storage protein Vitellogenin (Vg). Like lipid stores, Vg titers and *vg* transcription are high in nurses and decline prior to the onset of foraging [20, 21]. Inhibition of *vg* synthesis using RNAi causes precocious foraging [22, 23]. This result indicates that storage proteins, like stored lipids, are causal for the timing of behavioral maturation. In the

colony environment, the availability of food to each individual is influenced both by the availability of forage outside the hive and of stored nectar and pollen, and by explicitly social factors such as the sharing of food among individuals [17]. We can summarize that the availability of food to each individual within the colony leads to changes in her ability to store nutrients inside her body, and that these changes in physiology cause changes in behavior.

Bees at different stages of behavioral maturation also differ in their basal metabolism. Harrison [24] demonstrated that foragers have greater capacity for flight than nurses due to a higher capacity for energy metabolism in flight muscle. Interestingly, although energy metabolism in flight muscle and in the body as a whole increases during behavioral maturation, the metabolic capacity of the brain is actually lower in foragers than nurses. This was initially discovered through pathway analysis of microarray experiments [25] (Fig. 1.3) and later confirmed by assays of mitochondrial function [89]. These results suggest that the bee brain has specific metabolic needs that are distinct from those of the rest of the animal, as is well established in other species [26, 27]. Nonetheless, behavior is responsive to the nutritional and energetic state of the organism as a whole. This is accomplished in both vertebrates and invertebrates by changes in the activity of brain circuits that are both uniquely exposed to the conditions of the circulatory system and signaled to change their activity via endocrine signals [28-30].

Roles for conserved food-related pathways in division of labor in honey bee colonies

The recent sequencing of the honey bee genome [31] has made it possible to explore the roles of nutritionally-related molecular pathways in the regulation of honey bee division of labor. The mechanisms that regulate food intake and nutritional physiology are under active investigation in

many laboratories, in part because of the clinical need to develop treatments for obesity [32]. In insects, the synthesis and metabolism of lipids, proteins, and carbohydrates are accomplished by the fat bodies, which are most prevalent lining the walls of the abdomen and have functions comparable to both the liver and adipose tissues of vertebrates [29, 33]. As in vertebrates, there is active communication between these peripheral tissues and the nutrient-sensing brain circuits that regulate both behavior and physiology [29], and conserved signaling pathways have roles in this process across vertebrate and invertebrate taxa [29, 30].

Many insights on nutritional regulation have come from traditional genetic model systems such as the fruit fly *Drosophila melanogaster* and the mouse *Mus musculus*. Conserved food-related pathways have generally been studied in the context of homeostasis, and changes in nutrient stores (either wasting or obesity) are viewed as dysfunctions. In the honey bee the situation is different – the extreme changes in behavior that occur during behavioral maturation are associated with striking changes in nutritional physiology and food-related behaviors, namely the loss of abdominal lipid that precedes the onset of foraging. It might thus be possible to use the honey bee to identify mechanisms that regulate stable lipid loss. Studying these mechanisms in the bee also provides an opportunity to explore the adaptation of conserved, nutritionally-related signaling pathways to the derived, social context, perhaps providing insights into the evolution of behavior.

Insulin signaling and the related target of rapamycin (TOR) pathway are the best understood, most well conserved pathways linking nutrition to changes in physiology and behavior [28, 30]. In *Drosophila* and rodents, insulin and TOR act as satiety signals in the brain, decreasing food

intake in response to acute or chronic increases in circulating nutrients, such as after a meal or in the context of elevated adiposity [28, 34, 35]. Recently, it has been shown in the honey bee that insulin-related transcripts are upregulated in the head and fat bodies of foragers compared to nurses [25, 36]. Moreover, pharmacological manipulations of the TOR pathway led to a delay in the initiation of foraging in a seasonally-dependent fashion [25]. These results suggest a causal role for conserved nutrient-sensing pathways in the regulation of honey bee division of labor.

In both vertebrates and invertebrates, insulin-like peptides are typically secreted in response to high levels of circulating nutrients and synthesized at greater levels in response to high levels of stored nutrients, acting in both cases as negative feedback signals that inhibit further food consumption [28]. Interestingly, while stored nutrients influence insulin signaling gene expression in the honey bee, they do so in the opposite direction: bees with larger lipid stores have lower expression of insulin-like peptides, whether in the context of natural maturation (nurses vs. foragers), manipulations of colonies (well-fed vs. food-deprived colonies), or manipulations of individuals (caged bees fed a rich or poor diet) [25].

It is possible that this reversed polarity in the relationship between insulin signaling and nutrition reflects a change in the adipostatic set point of foragers relative to nurses, rather than the traditional homeostatic mechanism associated with insulin signaling [25]. In this view, the combination of high insulin synthesis and high insulin sensitivity reflects, or perhaps causes, a shift from high to low adiposity during behavioral maturation (and in response to experimental nutritional manipulations). Similar reasoning has been used to explain relationships between nutrient-sensing pathways and variation in nutrient stores in the contexts of mammalian torpor

[37, 38] and insect diapause [39]. “Reversed” IIS gene expression and the suggested set point regulation do not occur in all contexts in the life of a bee. Injections of insulin peptides acutely reduce blood carbohydrates in the honey bee, as in other species [40]. And a more traditional (homeostatic) relationship between nutrition and insulin signaling is seen during larval development [41]. These results suggest that the cooption of insulin signaling for the regulation of adult worker division of labor entailed changes in some aspect of the signaling system, but that the system is intact in its fundamentals within the species.

The discovery that nutrient-sensing pathways are involved in the regulation of worker division of labor helps to understand earlier results implicating feeding-related genes in this process. The gene known as *foraging (for)* encodes a cGMP-dependent protein kinase (PKG) [42]. Variant alleles of *for* were originally shown in *Drosophila* to underlie naturally-occurring variation in larval foraging strategies, and brain expression differences in its bee homolog were subsequently shown to regulate behavioral maturation [43]. The effect of *for* on behavioral maturation in the bee was initially linked to age-related changes in phototaxis (both foragers and bees treated with a membrane-permeable form of cGMP have increased attraction to light) [44]. However, since these initial studies, *for* has been shown in *Drosophila* to regulate nutrient storage and nutrient utilization [45, 46]. Perhaps *for* also has a role in regulating similar physiological processes in the context of worker honey bee division of labor.

Malvolio (*mvl*) is another gene involved in regulating feeding-related behavior in *Drosophila* shown to also be involved in regulating honey bee division of labor. This gene was initially identified in *Drosophila* in a screen for mutants with defects in taste behavior [47] and is

involved in the transport of manganese (Mn^{2+}) ions [48]. The bee homolog of *mvl* is highly expressed in the brains of foragers compared to nurses, and Mn^{2+} supplementation causes both precocious foraging and increased responsiveness to sugar [49]. Together, the involvement of insulin signaling, *for*, and *mvl* suggest that there may be a general role for feeding-related pathways in the regulation of worker division of labor.

The role of nutritionally-related pathways in honey bee division of labor also provides an important new perspective on the role that the multifunctional insect hormone juvenile hormone (JH) plays in this process. A role for JH in division of labor was first shown more than 30 years ago [50], and a variety of subsequent studies established that JH paces behavioral maturation [51, 52] and underlies age-related changes in energy metabolism [53] brain neurochemical levels [54], and, perhaps, brain structure [55]. In adult worker bees, JH and the key storage protein vitellogenin are mutually inhibitory, and it has been proposed that this feedback loop serves as a timing mechanism in behavioral maturation [56]. Recent studies in a variety of insect species have shown that JH and insulin signaling often have overlapping functions, and there appears to be extensive crosstalk between the two signaling pathways [30, 36, 57]. Consequently, it has been possible to link nutrient-sensing mechanisms to JH signaling [36, 58]. Elucidating the precise interactions between these signaling pathways and their effects on target genes would be greatly facilitated by systems biology approaches.

The results reviewed here on conserved feeding-related pathways provide context for the role of the neuromodulator octopamine (OA) in both worker division of labor [59] and in the assessment of food rewards during honey bee foraging [60, 61]. OA has well-established roles in the

behavioral and physiological responses to nutritional cues in a variety of insect species [62, 63], and OA is known to interact with JH in honey bees [54, 64]. Thus, the regulation of division of labor involves a web of interconnected feeding- and nutritionally-related pathways.

Division of labor as nutritionally-mediated plasticity

Based on the studies reviewed above, it is now clear that nutritionally-related pathways regulate division of labor in honey bee colonies. We stress three characteristics of this system that are likely to inform future studies. First, the regulation of behavioral maturation involves the coordination of changes occurring in multiple tissues, in part via hormonal signaling pathways. Insulin and JH are synthesized in glandular cells, in or associated with the brain, and target both brain circuits and peripheral tissues. Vitellogenin and lipids are released from the fat bodies and are thought to directly or indirectly modulate the activity of brain circuits. Some octopaminergic neurons are also involved in transmitting peripheral nutritional signals into the brain [62]. The importance of these cross-tissue communication signals, as well as the strong coordination between nutrient stores and behavioral state, suggests that any model for the regulation of division of labor should explicate the changes induced by these signals in both the periphery and brain, with an emphasis on the fat bodies and on specific nutrient-sensing circuitry.

Second, behavioral maturation involves massive changes in gene expression. As described above, as many as 40% of genes are differentially expressed in the brains of nurses and foragers. Unpublished data show that a similarly large number of genes change expression in the fat cells of these bees (Ament and Robinson, unpublished data). These results suggest that transcriptional control is an important part of the regulation of division of labor.

Finally, the behavioral and physiological states of nursing and foraging are highly stable, and these two groups of bees typically have different responses to similar conditions. When a colony's nutrient stores run low, existing foragers immediately leave the hive in search of nectar and pollen, but it takes at least a day for bees that had not previously been foraging to enter the foraging force [16]. This delay suggests that the transition into foraging requires a maturational process. The regulation of physiology seemingly also involves a stable state change since a forager's lipid stores remain low in well-fed colonies, even in bees that are forced to revert from foraging back to nursing [15]. Together, these results suggest that the hormones and related signals that regulate behavioral maturation are involved in setting target cells into distinct regulatory states in nurses and foragers. An important goal for future research is to elucidate how signaling pathways generate these state transitions at the molecular level, in both fat cells and in the brain.

Toward a systems biology of honey bee division of labor

Division of labor, like other examples of phenotypic plasticity, involves massive and stable changes in gene expression and regulatory roles for hormones and signal transduction mechanisms [65]. While signal transduction mechanisms are likely the switches by which environmental cues regulate phenotypes, the broader changes in gene expression have important roles as the effectors of each behavioral or physiological state. According to this line of reasoning, various forms of phenotypic plasticity such as division of labor are regulated through hormonally-related transcriptional networks that link environmental changes to alternative gene

expression states, ultimately generating differences in phenotype. Systems biology provides the framework to test the generality of this idea.

The regulation of cellular regulatory states is best understood in the context of development, in which different regulatory states specify the different body parts that make up an animal. During development, the progression of states is controlled by hierarchical gene regulatory networks (GRNs) (reviewed in [66]). In these networks, signaling pathways specify when and where transcription factors activate batteries of target genes that determine each cell's phenotype. We hypothesize that in the context of honey bee division of labor, the hormonal signaling pathways that influence behavioral and physiological maturation do so by affecting the activity of GRNs in much the same way (Fig. 4.1). The difference is that these changes occur in cells that have already differentiated so that the regulatory networks specify the physiological, rather than developmental, states of cells.

A challenge in modeling the GRNs underlying division of labor will be to characterize the network properties that both enable and constrain phenotypic change. Specifically, how do nutritionally-related hormones determine network regulatory states, and how do these regulatory states in turn determine the behavioral and physiological characteristics of nurses and foragers? These questions can be addressed by characterizing the GRNs that underlie each behavioral state, focusing on the parts of the network that are regulated by hormones. A specific hypothesis, derived from studies of transcriptional networks in other contexts [67], is that the increase in insulin and JH during maturation induces forager-like traits by changing the locations that hormonally-responsive transcription factors bind in the genome, leading to activation of

foraging-related genes. Alternatively, as has been revealed in other contexts for some nuclear hormone receptors [68], hormonally-related transcription factors may bind at the same locations throughout maturation but have different effects on transcription depending on the presence or absence of ligands and co-factors. These changes in the activity of sequence-specific transcription factors are likely to occur both through changes in the expression of the transcription factors themselves and through changes in the open or closed states of chromatin structures at target genes. Although understanding the dynamic properties of transcriptional networks is an active area of research [68], it is known that network connections can be turned on or off rapidly in some contexts. For instance, the response of single-celled organisms to DNA damage involves changes in active network connections that occur within minutes of chemical perturbations [67], so it is reasonable to think that changes in transcription factor activity within GRNs occur in real time during plasticity. In honey bees, relevant GRNs are located in the fat cells and brain circuits, which are among the tissues that mediate physiological and behavioral differences between nurses and foragers.

The GRN framework suggests an established methodology through which to elucidate the mechanisms that specify the states of fat and brain cells in nurses and foragers. Currently available technologies in honey bees now allow elucidation of the active GRNs in nurse and forager tissues. Functional interactions between genes can be determined from large-scale microarray experiments by generating gene co-expression networks. Causal relationships within these correlational networks can then be established in a variety of ways. For instance, direct targets of transcription factors (TFs) can be identified by characterizing the genomic binding sites for TFs using chromatin immunoprecipitation coupled to genomic tiling microarrays (ChIP-

chip) or high-throughput sequencing (ChIP-seq). We have begun to use ChIP-chip to identify genomewide binding sites for *ultraspiracle* (USP), a transcription factor associated with JH signaling [70, 71] (Wang, Mizzen and Robinson, unpublished data). Targets of TFs can also be elucidated by gene expression profiling after genetic manipulations of TFs; genetic perturbation using RNA interference is now routine in the bee, especially when targeting peripheral tissues [72-74]. Finally, the functional relationships among genes discovered using any of these techniques can be inputted into computational models to construct a quantitative model of a GRN (e.g., [67], reviewed in [68]).

Between brain circuits and fat cells, it seems more prudent to begin by characterizing the GRNs in fat cells. This is because it is straightforward to harvest large numbers of relatively homogeneous cells from the fat bodies and because the roles of insulin and JH are well known in this tissue [29]. In order to identify dynamic changes in the network, GRNs will need to be deduced in both nurses and foragers. Since key hormones are already known, characterizing the targets of only a small handful of TFs will likely be sufficient to gain significant insight into this system. In time, this approach could be expanded to elucidate GRNs in brain circuits and for additional transcription factors. In addition to advancing our understanding of plasticity, this approach also has the potential to identify genes that regulate the stable weight loss that occurs during the maturation process.

Conclusion

In the future, it will be interesting to compare the structures of GRNs underlying development to GRNs underlying behavioral plasticity. One might speculate that GRNs underlying plasticity are

different in structure, owing to the need for greater flexibility. Body parts (such as an eye or a heart) are constructed once during development and must form in the same way regardless of conditions; perhaps for this reason, the GRNs that specify these body parts are extremely dense -- i.e., containing many interconnected transcription factors – a wiring pattern that is thought to be very stable [66]. Perhaps there are design principles for behavioral GRNs that allow animals to rapidly transition back and forth between regulatory states during processes of phenotypic plasticity. Comparing behaviorally-related GRNs in the honey bee and in solitary species could also shed light on how the social environment regulates the behaviors of individuals. The answers will require many experiments over many years, but now is the time to begin framing the questions.

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Box 4.1. Honey bee primer

Honey bees are social insects, living together in colonies containing tens of thousands of individuals (for an overview of honey bee biology, see [10]). Colony life is organized by a complex and sophisticated division of labor. Each colony contains a single queen, who is specialized for reproduction and spends most of her time laying eggs. Males, called drones, are relatively rare, and their sole role is to mate. The vast majority of the individuals in the hive are sterile worker bees that are responsible for all of the other tasks performed by the colony. The tasks performed by worker bees are further divided up among individuals via the process of behavioral maturation that is the subject of this review. For the first 2-3 weeks of adult life, worker bees specialize on broodcare ("nursing"). They then switch for a few days to any of a number of more specialized tasks such as building honeycomb cells, storing food in honeycomb cells, or guarding the hive entrance against intruders. Finally, for the remaining 1-2 weeks of their life, worker bees forage outside the hive for nectar and pollen, the colony's sole sources of food.

Honey bees have been the subjects of scientific study for hundreds of years (reviewed in [75]). Bees are noted models for behavioral plasticity, communication [76], and learning and memory [77], among other subjects. Many of these studies have relied on three key attributes of the honey bee system. First, the millennia-old tradition of beekeeping allows for high-throughput experiments under natural and semi-natural colony conditions in the field, and large numbers of bees of all life stages are readily available for experiments [10]. Second, causal experiments are possible using a variety of environmental, pharmacological, and molecular manipulations of whole colonies or of individuals within colonies (e.g., [16, 19, 22, 25, 43, 50, 59]). For studies of

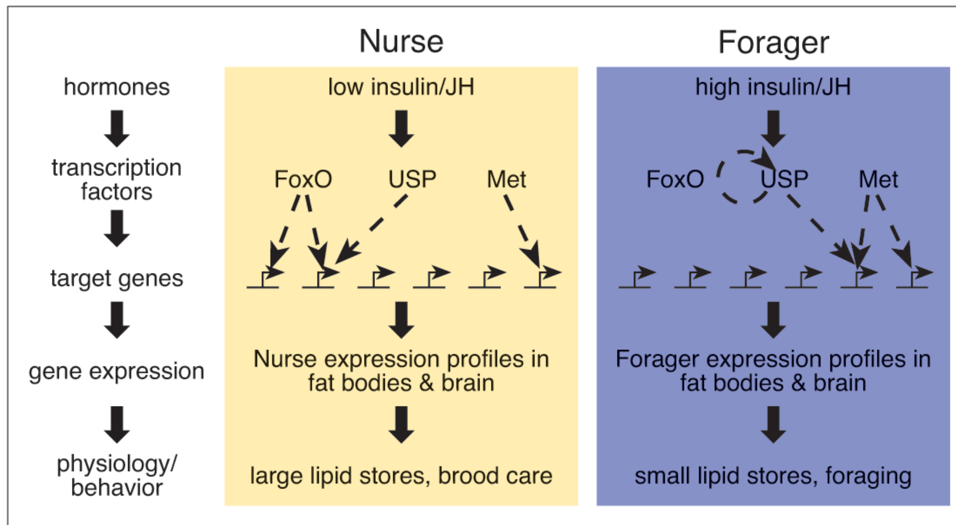
division of labor, these experiments typically involve marking bees of known age early in life, manipulating them, and tracking the age at which they first begin to forage. Often these studies are done using "single-cohort" colonies that are made from a single cohort of 1000-2000 one-day-old bees at the start of the experiment; because bees begin foraging at a much younger age in the absence of a pre-existing foraging force, the length of the experiment is reduced and certain treatment designs become more feasible. Finally, the sequencing of the honey bee genome [31] has enabled efficient discovery of candidate genes using microarrays (e.g., [7, 12, 78]), informatics [79], and other functional genomics technologies.

An important attribute of honey bee societies is that the colony is an integrated level of biological organization, not merely an aggregation of individuals [75]. Colonies have specific attributes or traits, but they can be traced to the traits expressed in individuals. A prime example of this comes from artificial selection of colonies for high and low pollen hoarding. In these experiments, selection for a colony-level trait, the amount of stored pollen in the hive, revealed genetic variation for a variety of nutritionally-related traits in individuals, including the age at first foraging, foraging preference for nectar or pollen, and gustatory sensitivity [80, 81]. Both the proximate and ultimate mechanisms by which social phenotypes arise from individual phenotypes are active areas of research [65].

Figure for Chapter 4

Figure 4.1 . Theoretical model for the role of gene regulatory networks (GRNs) in the regulation of worker division of labor. Signaling through insulin-like peptides and juvenile hormone (JH) is low in nurses and higher in foragers [25, 83]. These hormones regulate gene expression through interactions with transcription factors, some of which have already been identified in other insect species. Known transcriptional regulators include *FoxO*, which is involved in insulin action [84], as well as *ultraspiracle* (*usp*) and *methoprene-tolerant* (*met*), both of which are associated with JH [70, 85, 86]. Increased insulin signaling in foragers is likely to repress *FoxO* target genes by preventing FoxO protein from binding to their promoters [84]. Increased JH signaling causes increased *usp* expression in honey bees [87], as well as other hypothetical changes in target gene activation by usp and met. Specific target genes are not shown, but *vitellogenin* [88] and *for* (Wang, Ament, and Robinson, unpublished data) are candidate targets of *usp*. According to this framework, interactions among these and other transcription factors lead to the distinct gene expression profiles of nurses and foragers in brain [7, 12] and fat bodies (Ament and Robinson, unpublished data). These hormonally-controlled gene regulatory networks are hypothesized to be causal for behavioral maturation and stable lipid loss.

Figure 4.1



Chapter 5

A hormonally regulated transcriptional switch underlies phenotypic plasticity in the honeybee

Co-authored with Ying Wang, Feng Hong, Chieh-Chun Chen, Craig A. Mizzen, Saurabh Sinha, Sheng Zhong, and Gene E. Robinson

Abstract

Phenotypic plasticity – one genotype producing alternative phenotypes – is an important driving force in evolution. However, little is known about how the alternative molecular states underlying plasticity are specified and maintained, especially for alternative phenotypes that are stable for a long period of time. We studied this question in adult worker honeybees, which show striking age-related changes in behavioural phenotype that are regulated by endocrine and nutritional factors. We show that this behavioural maturation is influenced by a juvenile hormone (JH)-driven transcriptional regulatory network (TRN) in a peripheral nutrient-sensing tissue, the fat bodies. The network acts through the nuclear hormone receptor *Ultraspiracle (USP)*, which we show mediates the actions of JH. Inhibition of *USP* by RNAi delayed behavioural maturation and reduced transcriptional responses to JH while JH enhanced responses to *USP*, suggesting that JH and *USP* work together in a positive feedback loop. Identification of genome-wide binding sites for *USP* in the fat bodies by ChIP-chip and integration of these results with transcriptional targets of JH, *USP*, and behavioural maturation revealed a hierarchical TRN containing 773 direct *USP* targets and hundreds of potential indirect targets. This network was

enriched for other transcription factors and contained genes associated with macronutrient metabolism and plasticity-related signaling pathways. The TRN contained behaviourally-related sub-networks that were associated with binding sites for additional transcription factors that may act as *USP* co-factors. These results suggest that the highly stable alternative behavioural phenotypes that characterize honeybee behavioural maturation arise and are maintained by a JH-*USP* transcriptional switch mechanism. This study shows how hormonal signaling can be transcriptionally canalized to regulate long-term phenotypic plasticity.

Introduction

In the previous chapters of this thesis I demonstrated that the regulation of honeybee behavioural maturation involves novel regulation of conserved, nutritionally-related hormonal signaling pathways (Chapter 1) and massive changes in gene expression in the abdominal fat bodies, a peripheral nutrient-sensing tissue with causal influences on maturation (Chapter 3). These results suggest that although many regulatory steps separate transcription – particularly transcription in a peripheral organ – from behaviour, changes in transcription are a significant factor underlying the phenotypic differences between nurses and foragers. Based on these findings, I proposed that the regulation of behavioural maturation and other forms of phenotypic plasticity involves hormonally-driven transcriptional regulatory networks that take on alternative states (Chapter 4). Here I test this hypothesis for a key hormone underlying behavioural maturation, juvenile hormone (JH), and a JH-related transcription factor, *Ultraspiracle (USP)*.

JH is a highly pleiotropic hormone in insects [1]. It has a role in regulating metamorphosis across all holometabolous insects and is involved in diverse forms of plasticity including gregarious vs.

solitary forms in locusts [2], long vs. short wing morphs in crickets [3], and anautogeny in mosquitoes [4]. A prominent role for JH in worker honeybee behavioural maturation is supported by the following findings. i) JH titers rise prior to the onset of foraging and remain high throughout a bee's foraging career (reviewed in [5]). ii) Manipulation of bees with JH or JH analog treatments accelerates the pace of maturation, leading to an early onset of foraging [6, 7]. iii) Removal of the endocrine glands that produce JH (corpora allata) delays the onset of foraging [8]. JH affects behavioral maturation in part by regulating levels of key neuromodulators in the brain [9]. Furthermore, JH interacts with other environmental and hormonal factors that influence behavioral maturation – e.g., nutrition ([3, 4], Chapters 3 & 4), social pheromones [10], insulin signaling [11], and the yolk protein *vitellogenin* [11-14] – suggesting that it may have an integrating function linking these factors to maturation.

The transcription factor *Ultraspiracle (USP)* – the insect homolog of the Retinoid X Receptor (*RXR*) – mediates the actions of multiple insect hormones, including ecdysteroids (Ecd) [15] and JH [16, 17]. The molecular mechanisms of JH action are poorly understood, but *USP* is physically associated with a complex of proteins that mediate the actions of JH and Ecd [18]. In the bee, *USP* influences the timing of pupal development and the expression of a few genes related to Ecd and JH signaling [19], and JH analog treatments upregulate *usp* expression [20]. We hypothesized that *USP* mediates the effects of JH on behavioural maturation.

Results

***USP* influences honeybee behavioural maturation.** Despite the previously noted connections between *USP* and JH, a direct effect of *USP* on honeybee behaviour had not been shown. We

tested effects of *USP* RNAi on honeybee behavioural maturation in the field. Because JH accelerates maturation, we expected inhibition of *USP* to delay the onset of foraging. Abdominal injections with *USP* dsRNA caused a transient, ca. 35% drop in *USP* mRNA levels in the fat bodies after 3 days and a somewhat stronger knockdown of *USP* protein, but transcripts in the head were not affected (Fig. D.1). *USP* RNAi caused a significant delay in the onset of foraging; across 9 independent trials ca. 15% fewer *USP* RNAi-manipulated bees initiated foraging relative bees treated with a control dsRNA (Cox Proportional Hazards, P = 0.03; Fig. 5.1). We consider it unlikely that this delay in foraging ontogeny was due to toxic effects of RNAi. First, the effect was relatively mild – more than 50% of bees foraged despite *USP* RNAi. Second, stressors (e.g., parasite infection [21], social isolation [22]) typically accelerate rather than delay the onset of foraging in honeybees. In addition, we found replicable differences in the strength of this response between bees from different genetic sources (Fig. D.2), suggesting that there is naturally occurring genetic variation for sensitivity to *USP;* similar genetic variation has been reported for the effects of JH analog treatments [23]. These results demonstrate that *USP* has a causal effect on honeybee behavioural maturation, making it one of a small but growing number of transcription factors shown to influence behaviour [24].

***USP* mediates transcriptional responses to JH.** If *USP* influences behavioural maturation by mediating the actions of JH, then *USP* RNAi and JH treatment should influence similar genes, and *USP* RNAi should repress transcriptional responses to JH. We focused on the relationship between JH and *USP* in the fat bodies because of the causal effects of peripheral *USP* RNAi on behavioural maturation and because JH is known to influence behavioural and physiological plasticity in bees [25] and other insects [3] via its actions in this tissue. We studied the effects of

the JH analog methoprene (JHA) on fat body gene expression in bees treated with *USP* RNAi using deep mRNA sequencing. We treated bees orally with JHA for 24 hours, starting 2 days after the induction of RNAi; we confirmed that this dose of JHA caused precocious foraging (Fig. D.3).

JHA influenced a total of 213 genes in the fat bodies either in *USP* RNAi-treated bees or control bees or in both groups (False Discovery Rate [FDR] < 0.3, corresponding to $P < 0.002$). JHA-responsive genes identified in this study included well-known targets of JH such as the yolk protein *vitellogenin* and genes related to the synthesis and degradation of insect hormones (*JH esterase*, *Cyp305a1, JH acid methyltransferase*), as well as genes associated with metabolic processes known to differ between nurses and foragers such as lipid metabolism and transport (*ceramidase*, several odorant-binding proteins) and carbohydrate metabolism (*alpha-glucosidase, alpha-amylase*).

Comparing these results to the effects of maturation on fat body gene expression (using microarray gene expression profiles from nurses and foragers described in Chapter 3) revealed that nearly half the JHA-responsive genes also differed significantly between nurses and foragers (106 genes; 2.1-fold more than expected by chance, $P = 6.51e-23$). Moreover, JHA generally caused 'forager-like' changes in gene expression (i.e., genes upregulated by JHA were usually also higher in foragers than nurses; 76% concordant; $P = 3.6e-9$), and the effects of JHA and maturation on genes influenced by both were positively correlated ($r = 0.382$, $P = 1.3e-5$; Fig. 5.2A). These results demonstrate that the transcriptional responses to JH in the fat bodies are strongly associated with behavioural maturation, consistent with its well-established regulatory

role. A similar relationship was found in a previously published report on the effects of JH and behavioural maturation on gene expression in the brain [26].

We also identified 136 genes that responded to *USP* RNAi (in JHA-treated bees or controls or in both conditions; FDR < 0.3). *USP*-responsive genes were enriched for genes that were differentially expressed in nurses and foragers (62 genes; 1.94-fold expected by chance, P = 6.43e-13). However, there was no directional relationship between the effects of *USP* and maturation on these genes, regardless of stimulation by JHA (fold-change response to maturation vs. *USP* RNAi without JHA: r = 0.06, P = 0.60; with JHA: r = -0.11, P = 0.34; Fig. 5.2B). These results suggest that the effects of *USP* on behavioural maturation are unlikely due to direct effects of *USP* on behaviourally-related gene expression.

Instead, we found evidence that *USP* mediates responses to JH. 77% of *USP*-responsive genes also responded to JHA, 49 times more than expected by chance (105 genes; P = 5.86e-161), and the responses of these genes to *USP* and JH were highly correlated (r = 0.68, P = 1.33e-15; Fig. 5.2C). Moreover, *USP* RNAi inhibited the responses of many genes to JH. Of the 105 genes that responded to both JH and *USP*, 65 responded to JH at least 1.5-fold less strongly when we treated bees with *USP* RNAi (compared to only 5 genes that responded that much more strongly to JHA after RNAi; binomial test, P = 2.2e-14), leading to a strongly attenuated transcriptional response to JH overall (t-test on fold-change responses to JHA in control vs. *USP* RNAi condition for 105 genes regulated by both, P = 3.29e-16; Fig. 5.2D). It is mathematically equivalent to state that these same 65 genes responded more strongly to *USP* RNAi in the high JH condition, and indeed we found that twice as many genes responded significantly to *USP* only

in the high JH condition than only in the low JH condition (61 genes vs. 29 genes, binomial test, P = 9.7e-4; comparison of responses to *USP* RNAi in control vs. JHA conditions: t-test, P = 6.08e-10; Fig. 5.2E). These results suggest both that *USP* mediates the actions of JH, and JH mediates the actions of *USP* in the fat bodies. This should generate a positive feedback loop so that a change in either JH titre or *USP* expression would be stabilized and strengthened by increases in the other component. Therefore, our results suggest that JH and *USP* act together as a bistable switch. These insights are based on the broad patterns of response to JH and *USP* across all shared targets. In order to extend this inference to individual genes, it will be necessary to characterize genes for which there are statistical interactions between *USP* and JH, and the statistical methods required for this analysis have not yet been broadly implemented for RNA-seq data (see Methods). However, the plausibility of the idea that JH and USP act together as a bistable switch is supported by the fact that the shared targets of JH and *USP* include transcription factors (e.g., *SoxNeuro*, Fig. 5.2G) and other regulatory genes (e.g., *Ceramidase*, Fig. 5.2H) that may propagate JH and *USP* signals to downstream transcriptional and non-transcriptional targets.

Both JH- and *USP*-driven changes may be relevant to phenotypic plasticity in the bee. As previously mentioned, JH titres rise during maturation. In addition, *usp* transcription fluctuates naturally in adult bees by at least as much as the 35% knockdown we achieved by RNAi [27], and JH itself activated *USP* expression in our study by approximately this much (Fig. 5.2h), which has been shown previously in the bee in other contexts [20]. We propose that the transcriptional stability imposed by the JH-*USP* switch may contribute to the behavioural stability of the nursing and foraging states. Interestingly, this mechanism may be at least partially

distinct from a previously described mutually-inhibitory relationship between JH and the lipoprotein *vitellogenin* since *USP* RNAi had no effect of *vg* transcription either in our study (Fig. 5.2) or in a previously published report [18]. Unlike JH and Vg, JH and *USP* are thought to interact more or less directly at the promoters of target genes [17]. As a consequence, it should be possible to elucidate molecular mechanisms of the JH-*USP* switch by exploring the relationship between *USP* and its direct targets in the genome.

Direct targets of *USP* in the fat bodies of nurses and foragers. We hypothesized that phenotypic plasticity involves JH-related changes in how *USP* interacts with its direct targets in the genome. We used chromatin immunoprecipitation—genomic tiling microarray analysis to characterize the direct targets of *USP* in the fat bodies of nurses and foragers, and we integrated this information with gene expression data to test hypotheses about the *USP* TRN. Peak-finding algorithms identified a total of 1360 putative genomic binding sites for *USP* across 6 biological replicates of the ChIP-chip experiment (3 nurse samples and 3 forager samples). We characterized 773 putative direct target genes that were located within 10kb of a binding site; *USP* binding sites were equally likely to be up- or downstream from transcriptional start sites (Fig. D.3), as has previously been reported for *RXR* in mammals [28]. *USP* target genes were highly enriched for other transcription factors (GO: "regulation of transcription", 62 genes, 2.1-fold enriched, $P < 3.02e\text{-}9$), including 8 of the 19 nuclear hormone receptors in the honeybee genome involved in hormonally-related signaling cascades (e.g., *Hr46, E75, Svp,* and *USP* itself), and other transcription factors with well established roles in the regulation of behavior [23] such as *fruitless* and the Egr homolog *stripe*. In addition, the targets of *USP* included genes related to conserved nutritionally-related signaling pathways that are known to be involved in the

regulation of behavioural maturation (reviewed in [29) including the *insulin-related receptor 1* and the cGMP-dependent protein kinase *foraging*. These results suggest that *USP* influences a large, hierarchical transcriptional regulatory network, involving several genes already known to function in phenotypic plasticity but that had not been known to work together.

Our results also suggest a high level of evolutionary conservation for the direct targets of *USP*. 243 genes identified as *USP* targets in the bee (47% of those with clear orthologs; more than expected by chance, P = 1.3e-5) had previously been identified as *USP* targets in the fruit fly, *Drosophila melanogaster* [30], despite ca. 250 million years of divergence. Moreover, by scanning the DNA around *USP* binding sites for conserved *cis*-regulatory sequences we found that 716 binding sites (53%) contained at least one copy of the *CF1 cis*-regulatory motif bound by *USP* in *Drosophila*, the second-most strongly enriched motif out of more than 600 that we examined ($P < 10e-6$).

We found that only a fraction of the direct targets of *USP* were transcriptionally responsive to *USP* RNAi; using a lenient statistical cutoff ($P < 0.05$) for differential expression, 17 of 334 *USP* RNAi-responsive genes were direct targets. This result presumably reflects both the subtlety of the RNAi knockdown and the complicated relationship between genomic binding and transcriptional regulation and is consistent with findings for other transcription factors [31]. We also found 21 direct targets of *USP* that were responsive to JHA (out of 538 differentially expressed at $P < 0.05$) and 107 that were differentially expressed between nurses and foragers (out of 2641 differentially expressed at $FDR < 0.05$). These genes represent a behaviourally-

related subnetwork among the direct targets of *USP* that are likely to play a role in phenotypic plasticity.

Behaviourally-related subnetworks in the *USP* transcriptional regulatory network. That behaviourally-related targets represent only a fraction of the *USP* TRN is not surprising since *USP* interacts with multiple endocrine factors in addition to JH. However, we were curious how these subsets of *USP* targets were specified because this might hold clues to the limits on phenotypic plasticity. Manipulations of JH speed up or slow down behavioural maturation, but they do not change the order of these maturational stages, cause bees to take on novel phenotypes, or cause a breakdown in the organization of the colony. Thus, the specificity of JH action to the timing of maturation suggests constraints on the kinds of phenotypes that can be produced in adult bees. To begin to address the mechanisms underlying behavioural canalization, we explored mechanisms that might explain why JH and *USP* target specific sets of genes during maturation and not others.

One mechanism by which JH could influence phenotypic plasticity at the molecular level is by inducing changes in where (or how strongly) subsets of *USP* targets bind in the genome; alternatively, JH could change how *USP* targets influence gene expression without changes in binding. Both mechanisms have previously been described for *RXR* in developmental or physiological contexts [15, 32].

To test the first hypothesis, we searched for differential binding of *USP* in the genomes of nurses and foragers in the ChIP-chip results. *USP* binding intensity was highly correlated between nurse

and forager samples across all 1360 specific binding sites in the genome ($r = 0.96$, $P << 2.2e-16$; Fig. 5.3; Fig. D.4) with no binding sites (measured by the mean intensity of probes within the bounds of a statistically-characterized peak region) differing between nurses and foragers by even 1.5-fold. Moreover, statistical analyses did not identify a single probe or binding site that was differentially bound between the two conditions (ANOVA; False Discovery Rate [FDR] > 0.17 for all probes; FDR > 0.999 for all peaks). Even a very small fold-difference in binding could potentially influence gene expression; we identified 116 *USP* binding sites that showed small (1.25-1.46-fold) differences in binding intensity between nurses and foragers, but there was no relationship between the intensity of *USP* binding at these sites and microarray-based gene expression estimates for adjacent target genes in nurse and forager fat bodies (Pearson correlation, $r = -0.1$, $P = 0.34$). These results suggest that differential binding of *USP* to its binding sites is not an important mechanism underlying its effects on maturation, and that the JH-*USP* switch more likely functions through hormonally-induced changes in the activation of *USP* targets that do not involve changes in binding.

The second hypothesis to explain how JH could influence phenotypic plasticity at the molecular level is that JH could change how *USP* targets influence gene expression without changes in binding. Behaviourally-specific responses to *USP* might be determined by other transcription factors that co-localize with *USP* at its binding sites. These co-factors could include other nuclear hormone receptors with which *USP* forms heterodimers or members of other families of transcription factors (TFs) that interact with *USP* as part of larger protein complexes at promoters [18]. To explore this hypothesis, we searched for signatures of potential co-factors by scanning *USP* binding sites for conserved *cis*-regulatory motifs and looking for motifs that i)

were enriched at all *USP* binding sites (compared to the rest of the genome), ii) predicted that adjacent target genes would be differentially expressed between nurses and foragers; or iii) predicted the direction of response between nurses and foragers. These analyses revealed coherent, non-overlapping sets of enriched motifs for each test (Fig. 5.4a-d), supporting the idea that behaviourally-related responses are a feature of a distinct sub-network of *USP* targets that are specified by combinatorial actions of *USP* and other transcription factors.

Our results suggest specific hypotheses about classes of transcription factors responsible for different roles in the specification of behaviourally-related sub-networks. 4 of the 5 motifs that predicted a "general" behavioural response (i.e., enriched in peak regions near diferentially expressed genes) were highly similar "E-box" motifs associated with bHLH transcription factors. No motifs specifically predicted that a gene would be upregulated in foragers, but a cluster of motifs related to Homeobox genes predicted that a behaviourally-related *USP* target would be upregulated in nurses. From these results, we speculate that the specification of behaviourally-related sub-network involves the following combinatorial *cis*-regulatory code (Fig. 5.4e): *USP* and a bHLH transcription factor together cause *USP* targets to be upregulated in foragers; however, if in addition to these factors there is a Homeobox transcription factor present the gene is upregulated in nurses. A formal meta-analysis of motif co-localization could clarify how well these or similar combinations of motifs predict behavioural responses within the *USP* network. Given the similarity between the various motifs from each of these classes, it is impossible to discern which individual bHLH or Homeobox TFs are responsible for these functions based on motif presence alone. Rigorous testing of this hypothesis would be possible with additional

ChIP-chip (or ChIP-seq) experiments to confirm binding of transcription factors at these locations.

In hopes of identifying specific genes that could act as cofactors to *USP*, we looked for differentially expressed TFs among the *USP* targets that might be responsible for propagation of JH/*USP* signaling to indirect targets and participate in feedback regulation of other direct *USP* targets. We identified three TFs among *USP* targets -- *Chd64, E75,* and *Stat* -- that were upregulated in foragers. *Chd64* has been shown to bind JH response elements present in the honeybee genome and to physically interact with *USP* as part of a JH signaling protein complex [18]. *E75* is a nuclear hormone receptor critical for responses to JH and ecdysone during development [33]. *Stat* is predicted to bind one of the motifs that was enriched among *USP* targets (*dStat*). Together, these results suggest that the regulation of plasticity by the JH-*USP* transcriptional switch involves the upregulation of various components of a protein complex involved in JH signaling, providing a potential mechanism for positive feedback. These hypotheses should be tested in future experiments, perhaps using RNAi to knock down potential JH and *USP* cofactors.

Discussion

We have shown that the hormonally-related transcription factor *USP* influences phenotypic plasticity in adult worker honeybees and reduces transcriptional responses to juvenile hormone, suggesting that phenotypic plasticity involves a JH/*USP* transcriptional switch-like mechanism. We characterized a transcriptional regulatory network of direct and indirect *USP* targets to understand how this switch might function. We showed that the specification of behaviourally-

related sub-networks among *USP* targets does not involve differential binding of *USP* in the genomes of nurses and foragers. But *cis*-regulatory motif analysis predicted behaviourally-related transcriptional cofactors that may act combinatorially with JH and *USP* to affect downstream processes in the brain, fat bodies and elsewhere that influence phenotypic plasticity.

JH has many important functions in insect development and behaviour, yet understanding its mode of action has been a long-standing frustration for insect physiologists [34]. While our results suggest a critical role for *USP* in mediating the actions of JH in adult worker honeybees, a second TF, *Methoprene-tolerant (Met)*, has been shown to mediate responses to JH during beetle and fly development [34]. *USP* and *Met* each previously have been proposed to function on their own as JH receptors or together as part of a larger molecular complex [16, 18, 36]. Our results confirm a functional role for *USP* in JH signaling in adult honeybees, and the involvement of cofactors in this response support the existence of larger protein complexes mediating JH action. Unraveling the combinatorial effects of different cofactors within such a JH signaling complex may hold the key to understanding how JH is involved with such diverse processes both within a species and across arthropod evolution.

Our results contribute to a growing appreciation of the importance of transcriptional regulation to phenotypic plasticity [24] and suggest a specific mechanism that may contribute to the canalization of individuals into distinct and stable alternative states. Our results suggest that *USP* and JH together form a positive feedback loop in which each strengthens responses to the other. This feedback loop could form a bistable transcriptional switch to drive the JH/*USP* transcriptional regulatory network into distinct states. In the context of honeybee maturation, this

switch is likely flipped by a rise in JH titer prior to the onset of foraging. However, changes to either component over physiological or developmental timescales could be responsible for driving diverse forms of endocrine-mediated plasticity in insects, providing a mechanism for hormonal pleiotropy. In principal, genetic differences in how these changes occur could contribute to the evolution of plasticity itself, an important force underlying the evolution of phenotypic differences between species.

Methods

Effects of *USP* RNAi and JH analog treatment on foraging ontogeny and fat body gene expression. Bees were maintained at the University of Illinois Beekeeping Facility according to standard beekeeping practices. We used exclusively bees from source colonies headed by single-drone inseminated queens to reduce within-trial genetic variation; all experiments were replicated in at least two independent trials using queens from distinct European genotypes.

We used previously described dsUSP probes to knock down *USP* expression [19]; *dsUSP* was synthesized for us as a gift from Beeologics Inc. (Rehovot, Israel). Control dsRNA was *dsGFP* (also a gift from Beelogics) or (in a few behavioural trials) *ds-pUC* synthesized with standard *in vitro* transcription methods. We observed no differences between the two control probes in their effects on *USP* transcription or on age at onset of foraging. One-day-old bees were injected intra-abdominally with 20 ug *dsUSP* or control *dsRNA* dissolved in 1 ul deionized water. They were then painted with an identifying mark on the thorax (Testor's enamel) and placed into Plexiglas cages containing ca. 25 bees with equal numbers from each group, fed pollen paste (45% pollen / 45% honey / 10% water) and sugar syrup (50% sucrose w/v in water), replaced daily (as in [37]).

For behavioural observation, dsRNA-treated bees were placed into single-cohort colonies after 3 days of caging, and we monitored bees that initiated foraging from 5-9-days-old as described previously [37]. Bees used for gene expression measurements were from the two source colonies showing the strongest behavioural responses to *USP* RNAi. In addition to RNAi treatments, we added the JH analog methoprene into food during the third day of caging at a concentration of 20mg/g food. Bees were killed by flash freezing at the end of the third day of caging.

We soaked frozen abdomens in RNAlater-ICE, removed the gut from the abdomen and extracted total RNA from the fat bodies and annealing cuticle using RNeasy kits (Qiagen, Valencia, CA; as described in Chapter 3). We confirmed knockdown of *USP* by RT-qPCR using standard protocols, and we selected individuals showing typical knockdown for mRNA sequencing. mRNA libraries were constructed from 4 biological replicates per group using the Illumina (San Diego, CA) mRNA-seq protocol with multiplex adapters; each replicate contained pooled RNA from 4 individual bees. We synthesized 75nt mRNA sequences using an Illumina Genome Analyzer IIx, with 4 libraries in each lane, to a read depth of ca. 4-9 million reads / library. Library construction and mRNA sequencing were performed at the University of Illinois W.M. Keck Center for Comparative and Functional Genomics. Reads were mapped to the *A. mellifera* Official Gene Set 2 using the Bowtie rapid alignment tool, and unmapped reads (ca. 50%) were discarded. We found sequences mapping to 10406 OGS gene models, 9323 of which had >5 reads in at least one library; this is similar to the transcript diversity quantified in this tissue using microarrays. There are currently no publically available tools for the statistical analysis of

mRNA-seq data that can perform tests other than pairwise comparisons. We therefore analyzed the effects of *USP* RNAi and of JHA separately in each condition using the R package *DESeq*.

ChIP-chip. We collected age-matched 8-10-day-old nurses observed placing their heads into honeycomb cells containing larvae, and 21-23-day-old foragers returning to the hive carrying nectar or pollen [36]. We dissected fat body tissue from freshly collected bees and immediately performed cross-linking reactions and isolated nuclei from fat cells pooled from 8 individual bees. Chromatin immunoprecipitation was then performed on fresh material or nuclei stored at -80°C for up to 1-2 months using the EZ-ChIP kit (Millipore, Billerica, MA) according to standard protocols and a custom antibody specific to honeybee *USP* (Fig. D.6). We used custom genomic tiling microarrays (Nimblegen, Madison, WI) with 50bp probes and 100bp resolution designed from the *A. mellifera* genome sequence Assembly 4.0. Each two-color array was hybridized with genomic DNA pulled down using the *USP* antibody and with input genomic DNA, and the binding intensity of *USP* was calculated as their ratio. Hybridization and data extraction were performed according to standard operating procedures by NimbleGen. We used the Mpeak and Tamalpais algorithms to identify specific peak regions bound by *USP* and described the union of regions identified by these programs as putative *USP* binding sites. We validated a few binding sites in foragers using a different *USP* antibody (Fig D.7).

***cis*-Regulatory analysis.** We scanned the genome sequences around *USP* binding sites using the SWAN algorithm, for 602 cis-regulatory motifs compiled from FlyREG (*D. melanogaster*), TRANSFAC (*D. melanogaster, Homo sapiens*), and Jaspar (*H. sapiens*) [38, 39]. We then looked for statistical enrichment of motifs in the following comparisons: i) in peak regions

compared to the rest of the genome (empirical P-value calculated by searching for the number of motif instances among peak regions compared to an equivalent number of random genomic regions); ii) in peaks adjacent to behaviourally-related genes compared to peaks adjacent to non-behaviourally-related gene (hypergeometric test); iii) in peaks adjacent to genes upregulated in foragers compared to peaks adjacent to genes upregulated in nurses (Fisher's Exact test).

Acknowledgments

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Figures for Chapter 5

Figure 5.1. The transcription factor *USP* influences worker honeybee behavioural maturation. The age at onset of foraging was observed for bees injected with *USP* RNAi and for controls injected with a control dsRNA. RNAi significantly delayed the age at onset of foraging (Cox Proportional Hazards; P = 0.03). Pooled data from 9 trials.

Figure 5.2. *USP* RNAi inhibits behaviourally-related transcriptional responses to juvenile hormone. a. Correspondence between effects of juvenile hormone analog (JHA) and behavioural maturation on fat body gene expression for 106 genes influenced significantly by both (log2 fold change responses). **b**. Correspondence between effects of *USP* RNAi and behavioural maturation on fat body gene expression for 61 genes influenced significantly by both. **c.** Correspondence between effects of *USP* RNAi and JHA on fat body gene expression for 105 genes influenced significantly by both. **d.** Distribution of fold change responses to JHA in fat bodies of *USP* RNAi-treated bees and dsGFP-treated controls for 105 genes that responded to both JHA and *USP* RNAi. **e.** Distribution of fold change responses to *USP* RNAi in fat bodies of JHA-treated bees and untreated controls for 105 genes that responded to both JHA and *USP* RNAi. **f,g.** Examples of genes regulated in fat bodies by JHA and *USP* RNAi (mRNA-seq). **h.** Effect of JHA on *usp* mRNA levels in fat bodies (RT-qPCR, t-test, P = 9.9e-9, n=22-23 bees). **i.** Effects of JH but not *USP* RNAi on *vitellogenin* gene expression (mRNA-seq).

Figure 5.3. Binding sites of *USP* do not differ in the genomes of nurses and foragers. Correspondence between the intensity of *USP* binding in the genomes of nurses and foragers.

The mean intensities from three nurse and three forager ChIP-chip replicates are shown for probes located within statistical peak regions (n=9944).

Figure 5.4. *cis*-regulatory sequences predict potential *USP* cofactors. A total of 602 DNA-binding sequences for transcription factors – derived from studies in other species and compiled from public databases – were tested for enrichment at *USP* binding sites. **a.** *cis*-regulatory motifs enriched (FDR < 0.1) at *USP* binding sites compared to the rest of the genome. **b.** *cis*-regulatory motifs enriched at *USP* binding sites adjacent to genes differentially expressed between nurses and foragers, compared to other *USP* binding sites. **c.** *cis*-regulatory motifs enriched at *USP* binding sites adjacent to genes upregulated in nurses, compared to binding sites near genes upregulated in foragers. **d.** There were no motifs enriched at binding sites near genes upregulated in foragers, compared to binding sites near genes upregulated in nurses. **e.** Verbal model for the specification of behaviourally-related sub-networks of *USP* targets by *USP* and cofactors.

Figure 5.1

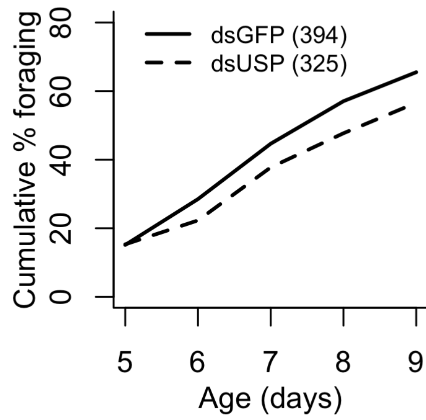


Figure 5.2

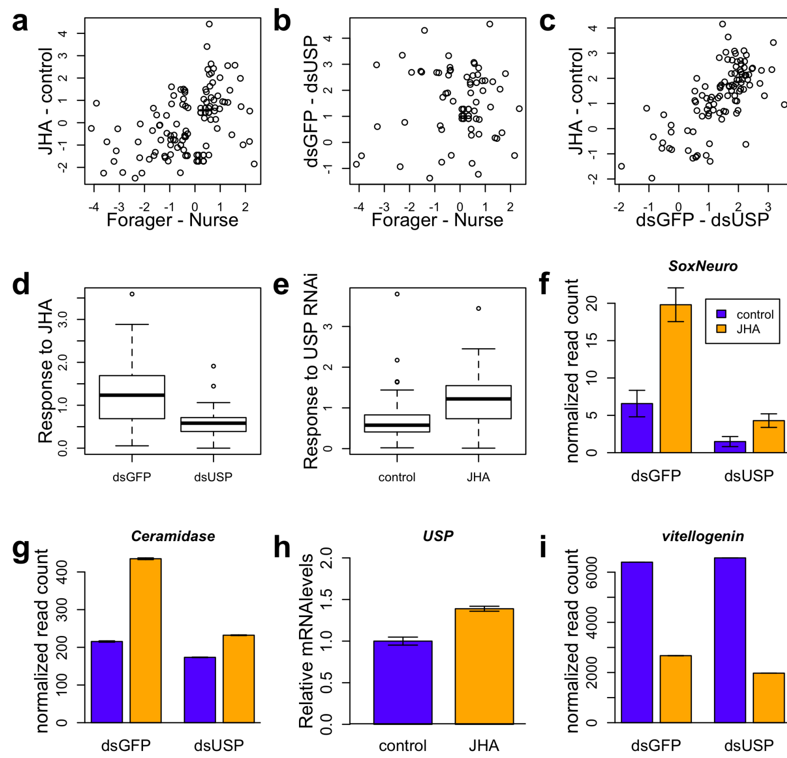


Figure 5.3

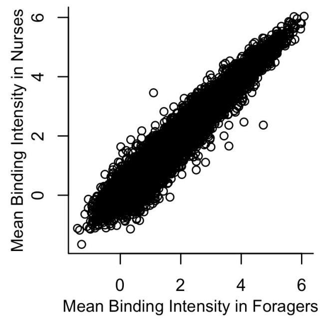


Figure 5.4

a

General *USP* Target
I_CF1_Q1, Adf1, Dstat, Ftz-f1, SUH, V_PPARA_Q6

b

Any Behavioural Response
V_DEC_Q1, V_SREBP_Q1, V_MAX_Q1, V_USF2_Q6, HLHm5

c

Nursing > Foraging
prd, gsb, ftz, I_EN_Q6, V_HOXA4_Q2, BEAF_32B, MafB, kni, bicoid, V_POU1F1_Q6

d

Foraging > Nursing
none enriched, FDR < 0.1

e

***USP* + *bHLH* + *NOT Hox* = ↑ forager**

***USP* + *bHLH* + *Hox* = ↑ nurse**

Chapter 6

Conclusions

Understanding how genes influence behavior is a daunting challenge but is essential both to address human behavioral disorders and to understand the remarkable behavioral diversity of other species. New technologies, particularly in genomics, are now making it possible to study the relationships between genes and behavior in an increasingly wide range of species. These studies have the potential to identify both shared and unique mechanisms shaping animal and human behavior, as well as providing insight into the processes shaping behavioral evolution. In the work described in this thesis, I studied mechanisms underlying the sophisticated and highly derived social behaviors of honey bees, taking an integrative approach that combines behavioral studies in the field and laboratory, functional genomics and systems biology, physiology, and molecular, pharmacological, and environmental manipulations.

A prominent theory in evolutionary biology proposes that novel traits arise through changes in the regulation of conserved genes. It has been well established that a conserved set of genes regulates the development of body plans across taxa, but this idea has only recently begun to be tested in the context of behavior. I chose to test this idea in honey bees because of their intensively studied and highly derived social behaviors and the availability of an increasing number of molecular tools, including a sequenced genome. I focused on the division of labor between workers, one of the bee's well-understood and evolutionarily important behavioral innovations. Worker division of labor in honey bee colonies is accomplished through a process of behavioral maturation. Worker bees perform brood care ("nursing")

and other tasks inside hive for the first few weeks of adult life; they then transition to foraging outside the hive for nectar and pollen for the final 1-2 weeks of life. The age at which this transition occurs is flexible and depends on colony needs. Based on previous results showing a relationship between nutritional status and foraging ontogeny, I hypothesized that division of labor involves conserved hormonal signaling pathways that regulate feeding-related behaviors in other species.

I tested this hypothesis for insulin/insulin-like growth factor signaling (Chapter 1), a conserved pathway that regulates organismal responses to nutritional status in species ranging from nematode worms to man. Insulin-related transcripts were upregulated in the brains and abdomens of foragers compared to nurses. Moreover, pharmacological inhibition of the insulin-related TOR pathway delayed the age at which bees living in experimental colonies in the field initiated foraging, suggesting a causal role for canonical nutritionally-related signaling pathways in behavioral maturation. Interestingly, insulin-related genes responded to nutritional cues in the opposite direction in honey bee workers compared to other species, and the strength of these responses to nutrition depended on a bee's behavioral state[i]. Therefore, my results support the idea that the evolution of honey bee social behavior involved novel regulation of these conserved genes.

My studies of a second nutritionally-related signaling pathway, Neuropeptide Y (NPY)-like signaling, suggest that evolution has selectively modulated different signaling pathways in distinct ways. NPY-related transcripts showed foraging-specific responsiveness to nutrition but were less consistently associated with maturation than were insulin-related transcripts, suggesting that these pathways are involved in different aspects of honey bee social behavior.

My research on insulin and NPY supported the hypothesis that the evolution of honey bee social behavior involved changes in the regulation of conserved nutritionally-related pathways. Yet previous studies have also identified a number of evolutionarily novel factors in honey bees, such as novel hormonally-related

functions for the yolk protein *vitellogenin* and novel roles for social pheromones in the regulation of physiology and behavior. In order to understand the relationship between these two mechanisms for the evolution of novel traits in the bee, I focused on the genomic responses to maturation, nutrition, *vg*, and Queen Mandibular Pheromone that underlie an endophenotype of worker division of labor – stable lipid loss occurring prior to the onset of foraging (Chapter 3). I discovered that stable lipid loss involves age-related changes in how (conserved) metabolic pathways and nutritionally-related signaling pathways respond to nutritional cues, as well as robust maturationally-related gene expression responses to *vg* and QMP. Intriguingly, these results suggest that conserved and evolutionarily novel regulators of stable lipid loss and division of labor converge on a single hormonal signaling pathway, juvenile hormone signaling.

I developed a strategy to elucidate JH-related transcriptional regulatory networks underlying division of labor in the bee using high-throughput molecular systems biology techniques (Chapter 4), which I then implemented (Chapter 5). I discovered that a JH-related transcription factor, *Ultraspiracle (USP),* functions in the fat bodies to influence division of labor. I then used chromatin immunoprecipitation to identify several hundred putative direct target genes of *USP* and the combination of *USP* RNAi and deep mRNA sequencing to characterize 105 functional genes regulated by JH analog treatments and *USP* in the fat bodies. My results suggest that JH and *USP* work together as a transcriptional switch. These results suggest that a JH-driven transcriptional regulatory network in fat cells is involved in worker honey bee division of labor

Together, the discoveries arising from my doctoral work elucidate a framework for understanding the relationship between genes and honey bee social behavior. I discovered that worker division of labor involves novel regulation of conserved signaling pathways, including JH and insulin signaling. These changes in regulation occur both over evolutionary time (linking nutritional cues to social behavior) and within the lifetime of an individual bee (causing behaviorally-related differences in responsiveness to

nutrition). In the JH-*USP* transcriptional switch, I have elucidated a mechanism that could underlie both kinds of regulatory change.

The studies I have described in this thesis represent a significant step forward in our understanding of how genes influence behavior in social insects. However, a limitation of these results is that we still know little about the brain circuits that underlie division of labor and other forms of phenotypic plasticity. Presumably, changes similar to those that I have described in fat cells occur in transcriptional regulatory networks within neurons, and it the combination of transcriptional networks acting within neuronal circuits that somehow produces the behavioral states of organisms. I intend to tackle this challenging problem in my postdoctoral work at the University of California at Berkeley, where I will focus on characterizing transcriptional and neuronal networks underlying the effects of hunger on feeding-related behaviors in the fruit fly. Ultimately, my goal is to attain a faculty position and to lead my own laboratory to investigate the mechanisms and evolution of complex behaviors in insects.

Appendix A: Supplemental figures for Chapter 1.

Figure A.1. Unrooted neighbor-joining tree showing the relationships of representative vertebrate and invertebrate members of the insulin/insulin-like peptide family. Signal and C peptide sequences were removed from analysis. Numbers above the branches represent bootstrap support values higher than 50%. The statistical significance of branch order was estimated by the generation of 10,000 replications of bootstrap re-sampling of the original aligned amino acid sequences.

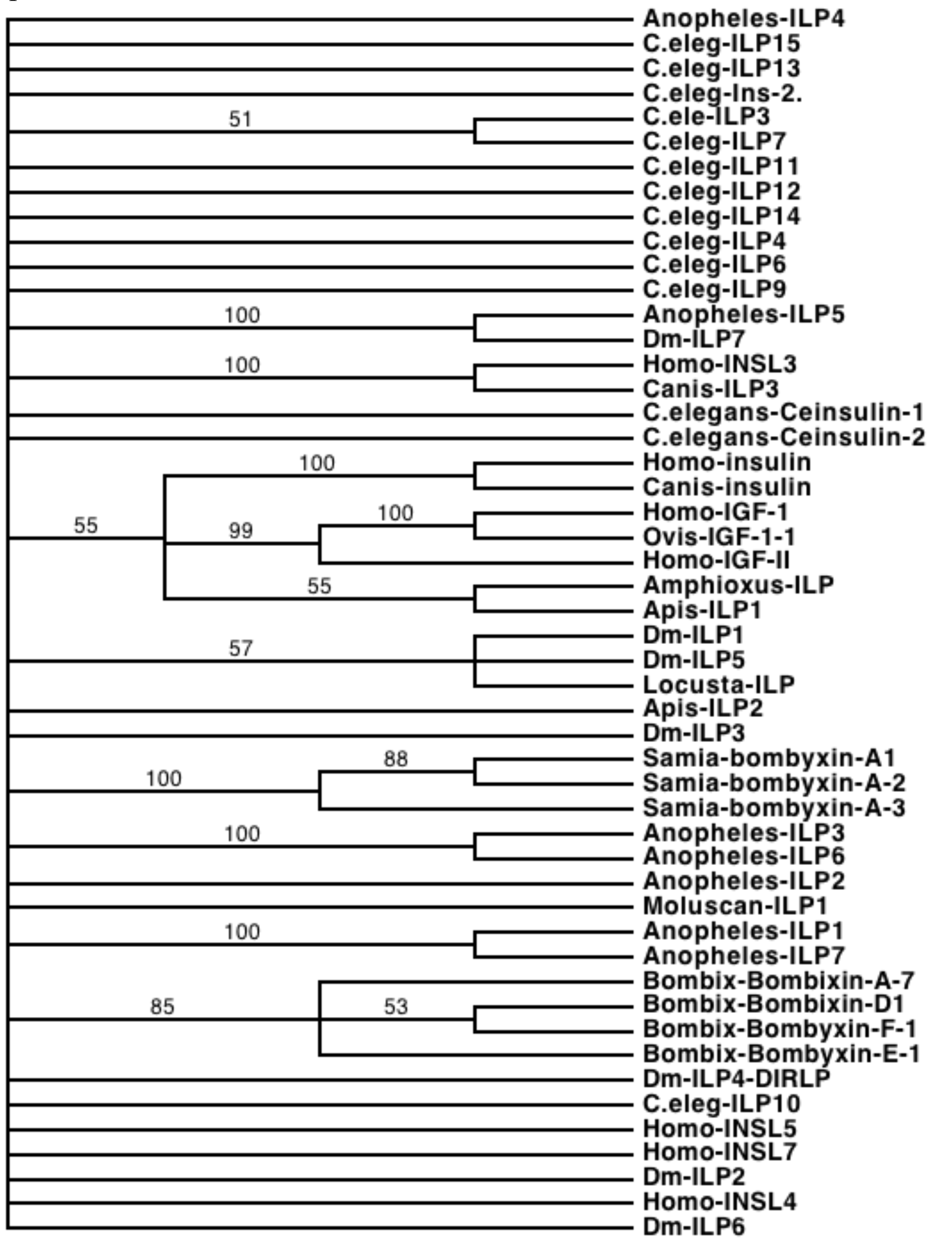


Figure A.2. Expression of genes related to insulin signaling and AKH signaling in brains and abdomens of nurses and foragers. Data from these same four trials are pooled in Figure 1.1A. Student's t-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

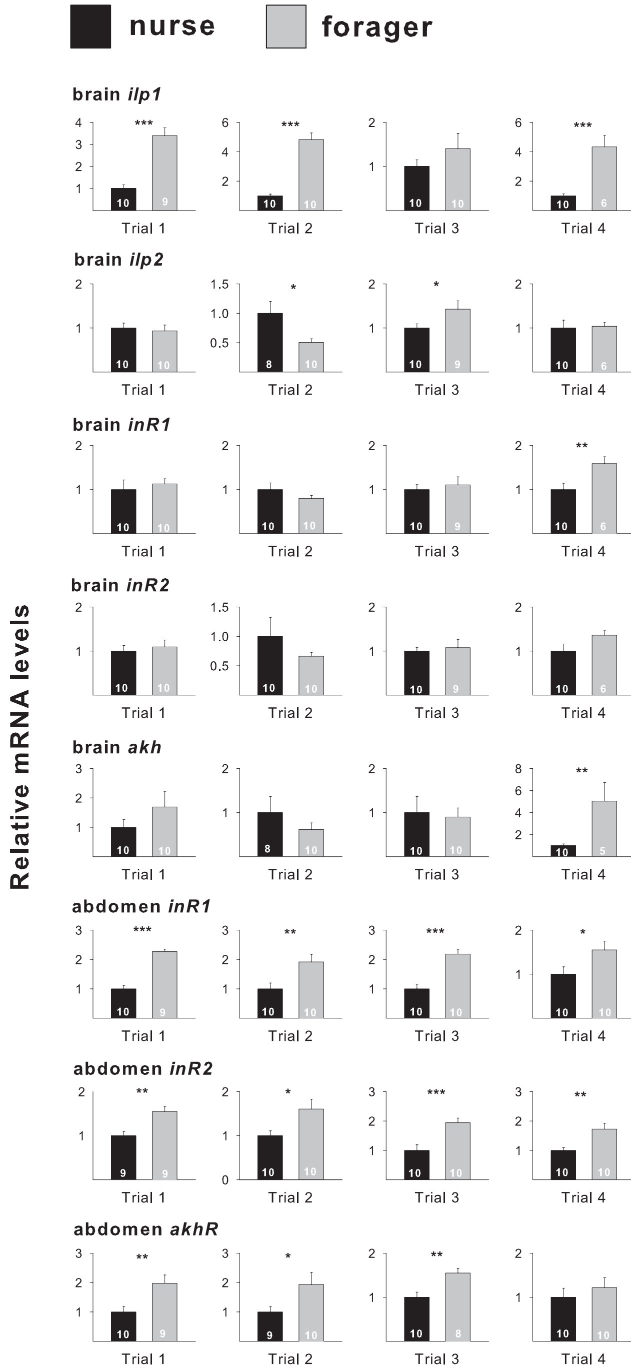
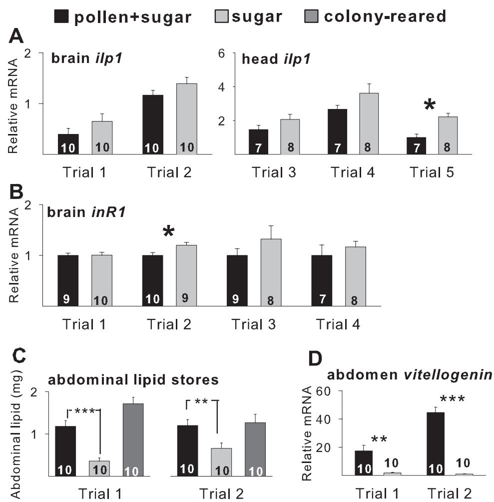


Figure A.3. Effects of diet on insulin signaling gene expression and nutrient stores. **A,B)** Data from the individual trials shown in A and B are pooled in Figure 1.1B. **A)** Expression of *ilp1* in brains of 4-day-old bees and heads of 6-day-old bees fed pollen and sugar or sugar-only diet (individual trials) and of age-matched bees raised in a colony and of foragers (F). **B)** Expression of *inR1* in brains of 4- or 5-day-old bees fed pollen+sugar or sugar-only diet. **C**) Abdominal lipid mass of 5-day-old caged bees fed pollen+sugar or sugar-only and of age-matched bees raised in a colony. **D)** Effect of diet on abdominal *vitellogenin (vg)* expression in 4-day-old bees fed pollen+sugar or sugar-only diet.



Appendix B: Supplemental tables for Chapter 3

The following tables show Gene Ontology terms that had biased representation between the two groups in each experiment. The p-value shown is for a modified Fisher's Exact Test.

Table B.1. Gene Ontology terms with a directional bias toward foragers (F) > nurses (N).

Term	PValue	Category F > N	Category N < F	Total F > N	Total N < F
GO:0065007~biological regulation	7.91E-05	147	60	591	410
GO:0051179~localization	2.51E-04	182	84	591	410
GO:0048749~compound eye development	5.98E-04	28	3	591	410
GO:0007243~protein kinase cascade	9.20E-04	18	0	591	410
GO:0001745~compound eye morphogenesis	1.00E-03	24	2	591	410
GO:0051234~establishment of localization	1.08E-03	152	70	591	410
GO:0050896~response to stimulus	1.11E-03	99	39	591	410
GO:0007560~imaginal disc morphogenesis	1.26E-03	36	7	591	410
GO:0006810~transport	1.60E-03	150	70	591	410
GO:0002164~larval development	1.68E-03	44	11	591	410
GO:0002117~larval development (sensu Amphibia)	1.96E-03	37	8	591	410
GO:0007552~metamorphosis	1.96E-03	37	8	591	410
GO:0050789~regulation of biological process	1.99E-03	124	55	591	410
GO:0001654~eye development	2.02E-03	30	5	591	410
GO:0050793~regulation of developmental process	2.35E-03	22	2	591	410
GO:0048592~eye morphogenesis	2.50E-03	27	4	591	410
GO:0016043~cellular component organization and biogenesis	2.71E-03	165	81	591	410
GO:0032502~developmental process	2.89E-03	155	75	591	410
GO:0007444~imaginal disc development	3.03E-03	40	10	591	410
GO:0051188~cofactor biosynthetic process	3.03E-03	24	3	591	410
GO:0006796~phosphate metabolic process	3.22E-03	71	26	591	410
GO:0006793~phosphorus metabolic process	3.22E-03	71	26	591	410
GO:0042221~response to chemical stimulus	3.56E-03	33	7	591	410
GO:0006119~oxidative phosphorylation	4.61E-03	30	6	591	410
GO:0007423~sensory organ development	4.97E-03	32	7	591	410
GO:0000165~MAPKKK cascade	6.29E-03	14	0	591	410
GO:0009887~organ morphogenesis	7.59E-03	43	13	591	410
GO:0051641~cellular localization	8.21E-03	67	26	591	410
GO:0065008~regulation of biological quality	9.50E-03	30	7	591	410
GO:0000902~cell morphogenesis	9.82E-03	44	14	591	410
GO:0032989~cellular structure morphogenesis	9.82E-03	44	14	591	410
GO:0009790~embryonic development	1.01E-02	38	11	591	410

GO:0009653~anatomical structure morphogenesis	1.09E-02	78	33	591	410
GO:0007275~multicellular organismal development	1.09E-02	128	63	591	410
GO:0035214~eye-antennal disc development	1.18E-02	25	5	591	410
GO:0007455~eye-antennal disc morphogenesis	1.18E-02	25	5	591	410
GO:0001751~compound eye photoreceptor cell differentiation	1.19E-02	18	2	591	410
GO:0030154~cell differentiation	1.32E-02	89	40	591	410
GO:0007610~behavior	1.35E-02	33	9	591	410
GO:0009108~coenzyme biosynthetic process	1.39E-02	20	3	591	410
GO:0048869~cellular developmental process	1.50E-02	90	41	591	410
GO:0007254~JNK cascade	1.60E-02	12	0	591	410
GO:0031098~stress-activated protein kinase signaling pathway	1.60E-02	12	0	591	410
GO:0016192~vesicle-mediated transport	1.60E-02	44	15	591	410
GO:0042775~organelle ATP synthesis coupled electron transport	1.76E-02	17	2	591	410
GO:0042773~ATP synthesis coupled electron transport	1.76E-02	17	2	591	410
GO:0051649~establishment of cellular localization	1.95E-02	63	26	591	410
GO:0046530~photoreceptor cell differentiation	2.00E-02	19	3	591	410
GO:0032501~multicellular organismal process	2.04E-02	151	80	591	410
GO:0015672~monovalent inorganic cation transport	2.17E-02	21	4	591	410
GO:0048468~cell development	2.25E-02	76	34	591	410
GO:0006811~ion transport	2.34E-02	33	10	591	410
GO:0006457~protein folding	2.35E-02	25	6	591	410
GO:0045165~cell fate commitment	2.35E-02	25	6	591	410
GO:0006139~nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	2.50E-02	126	65	591	410
GO:0048856~anatomical structure development	2.50E-02	110	55	591	410
GO:0022604~regulation of cell morphogenesis	2.52E-02	11	0	591	410
GO:0022603~regulation of anatomical structure morphogenesis	2.52E-02	11	0	591	410
GO:0008360~regulation of cell shape	2.52E-02	11	0	591	410
GO:0009607~response to biotic stimulus	2.85E-02	18	3	591	410
GO:0001754~eye photoreceptor cell differentiation	2.85E-02	18	3	591	410
GO:0006952~defense response	2.98E-02	34	11	591	410
GO:0035220~wing disc development	3.03E-02	20	4	591	410
GO:0050794~regulation of cellular process	3.14E-02	94	46	591	410
GO:0006812~cation transport	3.17E-02	28	8	591	410
GO:0043412~biopolymer modification	3.24E-02	79	37	591	410
GO:0019953~sexual reproduction	3.25E-02	50	20	591	410
GO:0007276~gamete generation	3.25E-02	50	20	591	410
GO:0003006~reproductive developmental process	3.26E-02	13	1	591	410
GO:0046034~ATP metabolic process	3.26E-02	13	1	591	410
GO:0046907~intracellular transport	3.95E-02	56	24	591	410
GO:0016311~dephosphorylation	4.03E-02	17	3	591	410

GO:0048731~system development	4.05E-02	94	47	591	410
GO:0016310~phosphorylation	4.17E-02	54	23	591	410
GO:0030707~ovarian follicle cell development	4.20E-02	19	4	591	410
GO:0006464~protein modification process	4.54E-02	77	37	591	410
GO:0043283~biopolymer metabolic process	4.58E-02	162	91	591	410
GO:0006754~ATP biosynthetic process	4.86E-02	12	1	591	410
GO:0006753~nucleoside phosphate metabolic process	4.86E-02	12	1	591	410
GO:0015992~proton transport	4.86E-02	12	1	591	410
GO:0042048~olfactory behavior	4.86E-02	12	1	591	410
GO:0006399~tRNA metabolic process	4.86E-02	12	1	591	410
GO:0007635~chemosensory behavior	4.86E-02	12	1	591	410
GO:0006818~hydrogen transport	4.86E-02	12	1	591	410
GO:0048513~organ development	5.11E-02	73	35	591	410
GO:0016477~cell migration	5.44E-02	26	8	591	410
GO:0048522~positive regulation of cellular process	5.75E-02	18	4	591	410
GO:0007476~imaginal disc-derived wing morphogenesis	5.75E-02	18	4	591	410
GO:0007472~wing disc morphogenesis	5.75E-02	18	4	591	410
GO:0000003~reproduction	5.76E-02	54	24	591	410
GO:0048737~imaginal disc-derived appendage development	5.76E-02	20	5	591	410
GO:0048736~appendage development	5.76E-02	20	5	591	410
GO:0035114~imaginal disc-derived appendage morphogenesis	5.76E-02	20	5	591	410
GO:0035107~appendage morphogenesis	5.76E-02	20	5	591	410
GO:0043687~post-translational protein modification	5.80E-02	59	27	591	410
GO:0002165~instar larval or pupal development	6.09E-02	52	23	591	410
GO:0009791~post-embryonic development	6.09E-02	52	23	591	410
GO:0006928~cell motility	6.22E-02	31	11	591	410
GO:0051674~localization of cell	6.22E-02	31	11	591	410
GO:0007242~intracellular signaling cascade	6.67E-02	43	18	591	410
GO:0010324~membrane invagination	6.77E-02	27	9	591	410
GO:0006897~endocytosis	6.77E-02	27	9	591	410
GO:0015986~ATP synthesis coupled proton transport	7.16E-02	11	1	591	410
GO:0006120~mitochondrial electron transport, NADH to ubiquinone	7.16E-02	11	1	591	410
GO:0007154~cell communication	7.34E-02	101	54	591	410
GO:0016044~membrane organization and biogenesis	7.45E-02	32	12	591	410
GO:0051707~response to other organism	7.80E-02	15	3	591	410
GO:0051704~multi-organism process	7.80E-02	15	3	591	410
GO:0045045~secretory pathway	8.61E-02	26	9	591	410
GO:0046903~secretion	8.61E-02	26	9	591	410
GO:0032940~secretion by cell	8.61E-02	26	9	591	410
GO:0002376~immune system process	9.41E-02	22	7	591	410
GO:0009617~response to bacterium	9.48E-02	8	0	591	410

GO:0042742~defense response to bacterium	9.48E-02	8	0	591	410

Table B.2. Gene Ontology terms with a directional bias toward nurses (N) > foragers (F).

Term	*PValue*	*Category F > N*	*Category N > F*	*Total F > N*	*Total N > F*
GO:0006412~translation	9.83E-11	33	79	588	413
GO:0009059~macromolecule biosynthetic process	1.13E-08	46	85	588	413
GO:0044249~cellular biosynthetic process	1.42E-07	84	116	588	413
GO:0009058~biosynthetic process	1.23E-06	99	124	588	413
GO:0010467~gene expression	1.32E-04	101	113	588	413
GO:0006631~fatty acid metabolic process	1.44E-04	4	20	588	413
GO:0044260~cellular macromolecule metabolic process	1.47E-03	168	157	588	413
GO:0044238~primary metabolic process	3.82E-03	354	284	588	413
GO:0044267~cellular protein metabolic process	6.51E-03	167	150	588	413
GO:0006629~lipid metabolic process	7.47E-03	31	41	588	413
GO:0032787~monocarboxylic acid metabolic process	1.11E-02	12	22	588	413
GO:0008152~metabolic process	1.14E-02	416	320	588	413
GO:0044255~cellular lipid metabolic process	1.31E-02	25	34	588	413
GO:0019538~protein metabolic process	2.38E-02	177	151	588	413
GO:0009124~nucleoside monophosphate biosynthetic process	2.91E-02	1	8	588	413
GO:0009123~nucleoside monophosphate metabolic process	2.91E-02	1	8	588	413
GO:0006635~fatty acid beta-oxidation	3.43E-02	2	9	588	413
GO:0019395~fatty acid oxidation	3.43E-02	2	9	588	413
GO:0006044~N-acetylglucosamine metabolic process	3.80E-02	3	10	588	413
GO:0006041~glucosamine metabolic process	3.80E-02	3	10	588	413
GO:0006040~amino sugar metabolic process	3.80E-02	3	10	588	413
GO:0006637~acyl-CoA metabolic process	4.61E-02	0	6	588	413
GO:0043170~macromolecule metabolic process	4.82E-02	278	219	588	413
GO:0009161~ribonucleoside monophosphate metabolic process	5.66E-02	1	7	588	413
GO:0009156~ribonucleoside monophosphate biosynthetic process	5.66E-02	1	7	588	413
GO:0006030~chitin metabolic process	5.66E-02	1	7	588	413
GO:0044237~cellular metabolic process	9.45E-02	373	280	588	413

Table B.3. Gene Ontology with a directional bias toward poor diet (P) > rich diet (R).

Term	*PValue*	*Category P > R*	*Category R > P*	*Total P > R*	*Total R > P*
GO:0050794~regulation of cellular process	3.82E-12	126	61	511	653
GO:0009653~anatomical structure morphogenesis	3.87E-12	112	49	511	653
GO:0050789~regulation of biological process	5.08E-11	150	88	511	653
GO:0032502~developmental process	7.05E-11	182	121	511	653
GO:0048856~anatomical structure development	7.53E-11	144	83	511	653

GO:0048731~system development	5.41E-10	124	68	511	653
GO:0007275~multicellular organismal development	1.30E-09	158	103	511	653
GO:0048869~cellular developmental process	2.76E-09	113	61	511	653
GO:0032501~multicellular organismal process	4.58E-09	178	127	511	653
GO:0065007~biological regulation	6.17E-09	165	114	511	653
GO:0030154~cell differentiation	7.11E-09	111	61	511	653
GO:0000902~cell morphogenesis	9.09E-09	68	25	511	653
GO:0032989~cellular structure morphogenesis	9.09E-09	68	25	511	653
GO:0048513~organ development	9.80E-09	97	49	511	653
GO:0031323~regulation of cellular metabolic process	2.68E-08	81	37	511	653
GO:0019222~regulation of metabolic process	3.85E-08	85	41	511	653
GO:0006350~transcription	4.03E-08	79	36	511	653
GO:0019219~regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	5.45E-08	76	34	511	653
GO:0007444~imaginal disc development	5.92E-08	51	15	511	653
GO:0007154~cell communication	6.26E-08	125	79	511	653
GO:0007165~signal transduction	1.20E-07	109	65	511	653
GO:0010468~regulation of gene expression	1.50E-07	74	34	511	653
GO:0010467~gene expression	1.53E-07	156	113	511	653
GO:0045449~regulation of transcription	3.56E-07	65	28	511	653
GO:0043283~biopolymer metabolic process	8.39E-07	186	151	511	653
GO:0007560~imaginal disc morphogenesis	9.47E-07	42	12	511	653
GO:0009887~organ morphogenesis	9.51E-07	49	17	511	653
GO:0006351~transcription, DNA-dependent	2.32E-06	66	32	511	653
GO:0032774~RNA biosynthetic process	2.32E-06	66	32	511	653
GO:0002117~larval development (sensu Amphibia)	2.76E-06	43	14	511	653
GO:0007552~metamorphosis	2.76E-06	43	14	511	653
GO:0006355~regulation of transcription, DNA-dependent	2.80E-06	56	24	511	653
GO:0048468~cell development	5.85E-06	85	51	511	653
GO:0016070~RNA metabolic process	7.20E-06	100	66	511	653
GO:0007242~intracellular signaling cascade	1.36E-05	54	25	511	653
GO:0007399~nervous system development	3.56E-05	60	32	511	653
GO:0016043~cellular component organization and biogenesis	6.58E-05	173	152	511	653
GO:0007423~sensory organ development	7.21E-05	37	14	511	653
GO:0048523~negative regulation of cellular process	1.07E-04	40	17	511	653
GO:0051301~cell division	1.62E-04	26	7	511	653
GO:0009790~embryonic development	1.85E-04	46	23	511	653
GO:0048519~negative regulation of biological process	1.94E-04	40	18	511	653
GO:0001654~eye development	2.15E-04	31	11	511	653
GO:0000003~reproduction	2.46E-04	51	28	511	653
GO:0007010~cytoskeleton organization and biogenesis	2.57E-04	52	29	511	653
GO:0048736~appendage development	2.89E-04	25	7	511	653
GO:0006357~regulation of transcription from RNA polymerase II promoter	2.90E-04	33	13	511	653
GO:0048749~compound eye development	3.08E-04	29	10	511	653
GO:0007276~gamete generation	3.23E-04	47	25	511	653
GO:0019953~sexual reproduction	3.23E-04	47	25	511	653

GO:0022008~neurogenesis	3.37E-04	40	19	511	653
GO:0035107~appendage morphogenesis	5.10E-04	24	7	511	653
GO:0007292~female gamete generation	5.30E-04	39	19	511	653
GO:0048477~oogenesis	5.30E-04	39	19	511	653
GO:0048699~generation of neurons	5.30E-04	39	19	511	653
GO:0002165~instar larval or pupal development	5.95E-04	51	30	511	653
GO:0006366~transcription from RNA polymerase II promoter	6.42E-04	42	22	511	653
GO:0002164~larval development	6.77E-04	43	23	511	653
GO:0007476~imaginal disc-derived wing morphogenesis	7.15E-04	22	6	511	653
GO:0007472~wing disc morphogenesis	7.15E-04	22	6	511	653
GO:0035214~eye-antennal disc development	7.50E-04	26	9	511	653
GO:0000904~cellular morphogenesis during differentiation	8.25E-04	38	19	511	653
GO:0048737~imaginal disc-derived appendage development	8.91E-04	23	7	511	653
GO:0030030~cell projection organization and biogenesis	1.20E-03	36	18	511	653
GO:0032990~cell part morphogenesis	1.20E-03	36	18	511	653
GO:0048858~cell projection morphogenesis	1.20E-03	36	18	511	653
GO:0006996~organelle organization and biogenesis	1.21E-03	84	65	511	653
GO:0048592~eye morphogenesis	1.26E-03	25	9	511	653
GO:0007455~eye-antennal disc morphogenesis	1.26E-03	25	9	511	653
GO:0043170~macromolecule metabolic process	1.45E-03	276	293	511	653
GO:0035220~wing disc development	1.54E-03	22	7	511	653
GO:0035114~imaginal disc-derived appendage morphogenesis	1.54E-03	22	7	511	653
GO:0048812~neurite morphogenesis	1.62E-03	33	16	511	653
GO:0048667~neuron morphogenesis during differentiation	1.62E-03	33	16	511	653
GO:0031175~neurite development	1.62E-03	33	16	511	653
GO:0045165~cell fate commitment	1.64E-03	27	11	511	653
GO:0009791~post-embryonic development	1.94E-03	51	33	511	653
GO:0007166~cell surface receptor linked signal transduction	1.96E-03	52	34	511	653
GO:0007049~cell cycle	2.13E-03	38	21	511	653
GO:0030036~actin cytoskeleton organization and biogenesis	2.39E-03	25	10	511	653
GO:0030029~actin filament-based process	2.39E-03	25	10	511	653
GO:0008283~cell proliferation	2.39E-03	25	10	511	653
GO:0048518~positive regulation of biological process	2.63E-03	21	7	511	653
GO:0048666~neuron development	2.66E-03	33	17	511	653
GO:0030182~neuron differentiation	2.80E-03	34	18	511	653
GO:0022402~cell cycle process	2.94E-03	35	19	511	653
GO:0001745~compound eye morphogenesis	3.05E-03	22	8	511	653
GO:0007169~transmembrane receptor protein tyrosine kinase signaling pathway	3.06E-03	12	1	511	653
GO:0006928~cell motility	3.64E-03	30	15	511	653
GO:0006259~DNA metabolic process	4.39E-03	34	19	511	653
GO:0050793~regulation of developmental process	4.61E-03	26	12	511	653
GO:0016477~cell migration	4.95E-03	27	13	511	653

GO:0007409~axonogenesis	5.04E-03	21	8	511	653
GO:0007369~gastrulation	6.03E-03	11	1	511	653
GO:0007350~blastoderm segmentation	6.03E-03	11	1	511	653
GO:0051674~localization of cell	6.08E-03	31	17	511	653
GO:0009888~tissue development	7.19E-03	25	12	511	653
GO:0003006~reproductive developmental process	7.28E-03	9	0	511	653
GO:0007163~establishment and/or maintenance of cell polarity	7.81E-03	15	4	511	653
GO:0043687~post-translational protein modification	8.97E-03	60	47	511	653
GO:0007243~protein kinase cascade	9.32E-03	16	5	511	653
GO:0022403~cell cycle phase	9.77E-03	22	10	511	653
GO:0000278~mitotic cell cycle	9.77E-03	22	10	511	653
GO:0051276~chromosome organization and biogenesis	1.04E-02	23	11	511	653
GO:0003008~system process	1.05E-02	41	28	511	653
GO:0007411~axon guidance	1.07E-02	17	6	511	653
GO:0008356~asymmetric cell division	1.17E-02	10	1	511	653
GO:0051248~negative regulation of protein metabolic process	1.17E-02	10	1	511	653
GO:0051246~regulation of protein metabolic process	1.20E-02	18	7	511	653
GO:0006139~nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	1.27E-02	139	138	511	653
GO:0040007~growth	1.42E-02	20	9	511	653
GO:0001703~gastrulation with mouth forming first	1.50E-02	8	0	511	653
GO:0010004~gastrulation involving germ band extension	1.50E-02	8	0	511	653
GO:0048754~branching morphogenesis of a tube	1.50E-02	8	0	511	653
GO:0001763~morphogenesis of a branching structure	1.50E-02	8	0	511	653
GO:0045935~positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	1.59E-02	11	2	511	653
GO:0016071~mRNA metabolic process	1.88E-02	27	16	511	653
GO:0048522~positive regulation of cellular process	1.93E-02	17	7	511	653
GO:0007067~mitosis	1.93E-02	17	7	511	653
GO:0000087~M phase of mitotic cell cycle	1.93E-02	17	7	511	653
GO:0009408~response to heat	1.97E-02	12	3	511	653
GO:0035282~segmentation	1.97E-02	12	3	511	653
GO:0031325~positive regulation of cellular metabolic process	1.97E-02	12	3	511	653
GO:0009893~positive regulation of metabolic process	1.97E-02	12	3	511	653
GO:0001738~morphogenesis of a polarized epithelium	1.97E-02	12	3	511	653
GO:0046530~photoreceptor cell differentiation	2.08E-02	18	8	511	653
GO:0043412~biopolymer modification	2.11E-02	75	67	511	653
GO:0009966~regulation of signal transduction	2.21E-02	19	9	511	653
GO:0003012~muscle system process	2.24E-02	9	1	511	653
GO:0006936~muscle contraction	2.24E-02	9	1	511	653
GO:0009266~response to temperature stimulus	2.31E-02	13	4	511	653
GO:0000226~microtubule cytoskeleton organization and biogenesis	2.31E-02	13	4	511	653
GO:0048646~anatomical structure formation	2.42E-02	21	11	511	653
GO:0045941~positive regulation of transcription	2.88E-02	10	2	511	653

GO:0048589~developmental growth	2.88E-02	10	2	511	653
GO:0016055~Wnt receptor signaling pathway	2.88E-02	10	2	511	653
GO:0040008~regulation of growth	2.88E-02	10	2	511	653
GO:0007446~imaginal disc growth	3.05E-02	7	0	511	653
GO:0016570~histone modification	3.05E-02	7	0	511	653
GO:0016569~covalent chromatin modification	3.05E-02	7	0	511	653
GO:0001754~eye photoreceptor cell differentiation	3.06E-02	16	7	511	653
GO:0001751~compound eye photoreceptor cell differentiation	3.06E-02	16	7	511	653
GO:0006325~establishment and/or maintenance of chromatin architecture	3.23E-02	17	8	511	653
GO:0006323~DNA packaging	3.23E-02	17	8	511	653
GO:0007264~small GTPase mediated signal transduction	3.23E-02	17	8	511	653
GO:0022414~reproductive process	3.42E-02	11	3	511	653
GO:0007164~establishment of tissue polarity	3.42E-02	11	3	511	653
GO:0001736~establishment of planar polarity	3.42E-02	11	3	511	653
GO:0048598~embryonic morphogenesis	3.51E-02	19	10	511	653
GO:0009607~response to biotic stimulus	3.51E-02	19	10	511	653
GO:0000279~M phase	3.51E-02	19	10	511	653
GO:0009628~response to abiotic stimulus	3.61E-02	20	11	511	653
GO:0006397~mRNA processing	3.83E-02	24	15	511	653
GO:0000165~MAPKKK cascade	4.22E-02	13	5	511	653
GO:0051726~regulation of cell cycle	4.51E-02	14	6	511	653
GO:0000074~regulation of progression through cell cycle	4.51E-02	14	6	511	653
GO:0009987~cellular process	4.61E-02	452	554	511	653
GO:0007167~enzyme linked receptor protein signaling pathway	4.74E-02	15	7	511	653
GO:0007517~muscle development	5.08E-02	17	9	511	653
GO:0051049~regulation of transport	5.10E-02	9	2	511	653
GO:0006396~RNA processing	5.26E-02	28	20	511	653
GO:0001700~embryonic development via the syncytial blastoderm	5.37E-02	21	13	511	653
GO:0007610~behavior	5.37E-02	21	13	511	653
GO:0050896~response to stimulus	5.61E-02	73	70	511	653
GO:0008361~regulation of cell size	5.78E-02	10	3	511	653
GO:0000910~cytokinesis	5.78E-02	10	3	511	653
GO:0051252~regulation of RNA metabolic process	5.78E-02	10	3	511	653
GO:0006464~protein modification process	6.05E-02	70	67	511	653
GO:0050877~neurological system process	6.23E-02	35	28	511	653
GO:0016568~chromatin modification	6.30E-02	11	4	511	653
GO:0007398~ectoderm development	6.99E-02	13	6	511	653
GO:0042221~response to chemical stimulus	7.19E-02	26	19	511	653
GO:0035239~tube morphogenesis	7.21E-02	14	7	511	653
GO:0007498~mesoderm development	7.21E-02	14	7	511	653
GO:0006468~protein amino acid phosphorylation	7.27E-02	25	18	511	653
GO:0030707~ovarian follicle cell development	7.37E-02	15	8	511	653
GO:0009792~embryonic development ending in birth or egg hatching	7.54E-02	21	14	511	653

GO:0042127~regulation of cell proliferation	7.71E-02	7	1	511	653
GO:0009968~negative regulation of signal transduction	8.79E-02	8	2	511	653
GO:0007267~cell-cell signaling	9.26E-02	27	21	511	653
GO:0003002~regionalization	9.43E-02	26	20	511	653
GO:0007389~pattern specification process	9.43E-02	26	20	511	653
GO:0007265~Ras protein signal transduction	9.53E-02	9	3	511	653
GO:0019226~transmission of nerve impulse	9.59E-02	25	19	511	653

Table B.4. Gene Ontology terms with a directional bias toward rich diet (R) > poor diet (P).

Term	*PValue*	*Category P > R*	*Category R > P*	*Total P > R*	*Total R > P*
GO:0019752~carboxylic acid metabolic process	5.12E-11	14	89	506	658
GO:0006082~organic acid metabolic process	5.12E-11	14	89	506	658
GO:0055086~nucleobase, nucleoside and nucleotide metabolic process	3.41E-09	2	47	506	658
GO:0009117~nucleotide metabolic process	1.42E-07	2	40	506	658
GO:0009058~biosynthetic process	2.79E-07	67	167	506	658
GO:0006725~aromatic compound metabolic process	3.33E-07	1	35	506	658
GO:0006807~nitrogen compound metabolic process	6.28E-07	16	72	506	658
GO:0006520~amino acid metabolic process	7.92E-07	10	58	506	658
GO:0009165~nucleotide biosynthetic process	9.74E-07	1	33	506	658
GO:0006119~oxidative phosphorylation	9.74E-07	1	33	506	658
GO:0051186~cofactor metabolic process	9.82E-07	5	45	506	658
GO:0009259~ribonucleotide metabolic process	1.16E-06	0	29	506	658
GO:0006519~amino acid and derivative metabolic process	1.56E-06	12	61	506	658
GO:0009260~ribonucleotide biosynthetic process	2.02E-06	0	28	506	658
GO:0009308~amine metabolic process	2.05E-06	16	69	506	658
GO:0006732~coenzyme metabolic process	2.52E-06	5	43	506	658
GO:0006163~purine nucleotide metabolic process	3.52E-06	0	27	506	658
GO:0006164~purine nucleotide biosynthetic process	6.11E-06	0	26	506	658
GO:0009150~purine ribonucleotide metabolic process	6.11E-06	0	26	506	658
GO:0006091~generation of precursor metabolites and energy	9.72E-06	19	71	506	658
GO:0009152~purine ribonucleotide biosynthetic process	1.06E-05	0	25	506	658
GO:0008152~metabolic process	1.46E-05	334	509	506	658
GO:0044249~cellular biosynthetic process	2.51E-05	62	143	506	658
GO:0006629~lipid metabolic process	4.10E-05	15	59	506	658
GO:0032787~monocarboxylic acid metabolic process	5.72E-05	3	31	506	658
GO:0006631~fatty acid metabolic process	1.04E-04	2	27	506	658
GO:0046483~heterocycle metabolic process	1.78E-04	1	23	506	658
GO:0009112~nucleobase metabolic process	2.73E-04	0	19	506	658
GO:0006118~electron transport	3.44E-04	14	51	506	658
GO:0051188~cofactor biosynthetic process	4.84E-04	1	21	506	658
GO:0009108~coenzyme biosynthetic process	4.84E-04	1	21	506	658
GO:0009205~purine ribonucleoside triphosphate metabolic process	7.90E-04	0	17	506	658

GO:0009199~ribonucleoside triphosphate metabolic process	7.90E-04	0	17	506	658
GO:0009144~purine nucleoside triphosphate metabolic process	7.90E-04	0	17	506	658
GO:0009141~nucleoside triphosphate metabolic process	7.90E-04	0	17	506	658
GO:0044255~cellular lipid metabolic process	1.26E-03	15	49	506	658
GO:0009206~purine ribonucleoside triphosphate biosynthetic process	1.34E-03	0	16	506	658
GO:0009201~ribonucleoside triphosphate biosynthetic process	1.34E-03	0	16	506	658
GO:0009145~purine nucleoside triphosphate biosynthetic process	1.34E-03	0	16	506	658
GO:0009142~nucleoside triphosphate biosynthetic process	1.34E-03	0	16	506	658
GO:0044271~nitrogen compound biosynthetic process	1.75E-03	2	21	506	658
GO:0009309~amine biosynthetic process	1.75E-03	2	21	506	658
GO:0044237~cellular metabolic process	3.12E-03	303	447	506	658
GO:0015992~proton transport	3.77E-03	0	14	506	658
GO:0006144~purine base metabolic process	3.77E-03	0	14	506	658
GO:0046034~ATP metabolic process	3.77E-03	0	14	506	658
GO:0006818~hydrogen transport	3.77E-03	0	14	506	658
GO:0008652~amino acid biosynthetic process	4.29E-03	2	19	506	658
GO:0044270~nitrogen compound catabolic process	5.42E-03	1	16	506	658
GO:0009310~amine catabolic process	5.42E-03	1	16	506	658
GO:0009063~amino acid catabolic process	5.42E-03	1	16	506	658
GO:0006754~ATP biosynthetic process	6.28E-03	0	13	506	658
GO:0006753~nucleoside phosphate metabolic process	6.28E-03	0	13	506	658
GO:0015986~ATP synthesis coupled proton transport	1.04E-02	0	12	506	658
GO:0009064~glutamine family amino acid metabolic process	1.04E-02	0	12	506	658
GO:0044248~cellular catabolic process	1.14E-02	26	60	506	658
GO:0045454~cell redox homeostasis	1.36E-02	1	14	506	658
GO:0042775~organelle ATP synthesis coupled electron transport	1.36E-02	1	14	506	658
GO:0042773~ATP synthesis coupled electron transport	1.36E-02	1	14	506	658
GO:0006635~fatty acid beta-oxidation	1.72E-02	0	11	506	658
GO:0019395~fatty acid oxidation	1.72E-02	0	11	506	658
GO:0009056~catabolic process	1.87E-02	28	61	506	658
GO:0022618~protein-RNA complex assembly	2.14E-02	1	13	506	658
GO:0022613~ribonucleoprotein complex biogenesis and assembly	2.47E-02	4	19	506	658
GO:0042440~pigment metabolic process	2.81E-02	0	10	506	658
GO:0006626~protein targeting to mitochondrion	2.81E-02	0	10	506	658
GO:0019748~secondary metabolic process	3.32E-02	1	12	506	658
GO:0007005~mitochondrion organization and biogenesis	3.54E-02	2	14	506	658
GO:0019725~cellular homeostasis	4.47E-02	6	21	506	658
GO:0042592~homeostatic process	4.47E-02	6	21	506	658
GO:0046148~pigment biosynthetic process	4.56E-02	0	9	506	658

GO:0009161~ribonucleoside monophosphate metabolic process	4.56E-02	0	9	506	658
GO:0009156~ribonucleoside monophosphate biosynthetic process	4.56E-02	0	9	506	658
GO:0009124~nucleoside monophosphate biosynthetic process	4.56E-02	0	9	506	658
GO:0009123~nucleoside monophosphate metabolic process	4.56E-02	0	9	506	658
GO:0006413~translational initiation	5.09E-02	1	11	506	658
GO:0006839~mitochondrial transport	7.65E-02	2	12	506	658
GO:0051187~cofactor catabolic process	7.73E-02	1	10	506	658
GO:0045333~cellular respiration	7.73E-02	1	10	506	658
GO:0006099~tricarboxylic acid cycle	7.73E-02	1	10	506	658
GO:0009109~coenzyme catabolic process	7.73E-02	1	10	506	658
GO:0009060~aerobic respiration	7.73E-02	1	10	506	658
GO:0046356~acetyl-CoA catabolic process	7.73E-02	1	10	506	658
GO:0044238~primary metabolic process	8.73E-02	308	428	506	658
GO:0006811~ion transport	9.59E-02	17	36	506	658

Table B.5. Gene Ontology terms with a directional bias toward *vg* RNAi (V) > control (C).

Term	*PValue*	*Category V > C*	*Category C > V*	*Total V > C*	*Total C > V*
GO:0019538~protein metabolic process	4.16E-06	125	91	336	418
GO:0044267~cellular protein metabolic process	3.05E-05	116	87	336	418
GO:0043170~macromolecule metabolic process	3.35E-05	185	167	336	418
GO:0044260~cellular macromolecule metabolic process	7.65E-05	118	92	336	418
GO:0016043~cellular component organization and biogenesis	9.20E-04	112	94	336	418
GO:0033036~macromolecule localization	1.98E-03	43	25	336	418
GO:0022607~cellular component assembly	2.07E-03	34	17	336	418
GO:0043283~biopolymer metabolic process	2.17E-03	100	84	336	418
GO:0010467~gene expression	2.29E-03	87	70	336	418
GO:0008104~protein localization	2.74E-03	40	23	336	418
GO:0006911~phagocytosis, engulfment	3.55E-03	22	8	336	418
GO:0065003~macromolecular complex assembly	5.81E-03	27	13	336	418
GO:0045184~establishment of protein localization	8.13E-03	35	21	336	418
GO:0009987~cellular process	8.61E-03	297	342	336	418
GO:0016070~RNA metabolic process	8.95E-03	51	37	336	418
GO:0006909~phagocytosis	1.13E-02	22	10	336	418
GO:0015031~protein transport	1.17E-02	34	21	336	418
GO:0046907~intracellular transport	1.23E-02	38	25	336	418
GO:0006996~organelle organization and biogenesis	1.60E-02	51	39	336	418
GO:0016311~dephosphorylation	1.63E-02	8	0	336	418
GO:0006508~proteolysis	1.64E-02	32	20	336	418
GO:0009059~macromolecule biosynthetic process	1.68E-02	46	34	336	418
GO:0022618~protein-RNA complex assembly	1.76E-02	11	2	336	418
GO:0006413~translational initiation	1.76E-02	11	2	336	418

GO:0007292~female gamete generation	1.93E-02	23	12	336	418
GO:0048477~oogenesis	1.93E-02	23	12	336	418
GO:0006886~intracellular protein transport	2.30E-02	30	19	336	418
GO:0006605~protein targeting	2.65E-02	20	10	336	418
GO:0051641~cellular localization	3.02E-02	43	33	336	418
GO:0006412~translation	3.05E-02	42	32	336	418
GO:0044237~cellular metabolic process	3.09E-02	227	253	336	418
GO:0065007~biological regulation	3.21E-02	89	84	336	418
GO:0019953~sexual reproduction	3.23E-02	29	19	336	418
GO:0010324~membrane invagination	3.23E-02	29	19	336	418
GO:0006897~endocytosis	3.23E-02	29	19	336	418
GO:0007276~gamete generation	3.23E-02	29	19	336	418
GO:0000003~reproduction	3.25E-02	33	23	336	418
GO:0016044~membrane organization and biogenesis	3.25E-02	32	22	336	418
GO:0051704~multi-organism process	4.24E-02	12	4	336	418
GO:0022403~cell cycle phase	4.65E-02	13	5	336	418
GO:0022613~ribonucleoprotein complex biogenesis and assembly	4.99E-02	14	6	336	418
GO:0051649~establishment of cellular localization	5.18E-02	41	33	336	418
GO:0048592~eye morphogenesis	5.27E-02	15	7	336	418
GO:0016192~vesicle-mediated transport	5.42E-02	38	30	336	418
GO:0006366~transcription from RNA polymerase II promoter	5.68E-02	17	9	336	418
GO:0007389~pattern specification process	6.02E-02	20	12	336	418
GO:0006470~protein amino acid dephosphorylation	6.46E-02	6	0	336	418
GO:0050789~regulation of biological process	6.77E-02	80	78	336	418
GO:0000279~M phase	7.32E-02	12	5	336	418
GO:0006457~protein folding	7.94E-02	14	7	336	418
GO:0001745~compound eye morphogenesis	7.94E-02	14	7	336	418
GO:0048468~cell development	8.01E-02	42	36	336	418
GO:0051276~chromosome organization and biogenesis	8.29E-02	16	9	336	418
GO:0006139~nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	8.97E-02	75	74	336	418
GO:0044238~primary metabolic process	9.27E-02	216	247	336	418

Table B.6. Gene Ontology terms with directional bias toward control (C) > *vg* RNAi (V)

Term	*PValue*	*Category V > C*	*Category C > V*	*Total V > C*	*Total C > V*
GO:0006732~coenzyme metabolic process	1.71E-04	4	30	335	419
GO:0006629~lipid metabolic process	4.83E-04	13	46	335	419
GO:0051186~cofactor metabolic process	7.85E-04	6	31	335	419
GO:0044255~cellular lipid metabolic process	1.19E-03	9	36	335	419
GO:0044262~cellular carbohydrate metabolic process	2.62E-03	5	26	335	419
GO:0005975~carbohydrate metabolic process	3.56E-03	14	42	335	419
GO:0009056~catabolic process	8.68E-03	20	49	335	419
GO:0044248~cellular catabolic process	1.41E-02	18	44	335	419

GO:0051188~cofactor biosynthetic process	2.91E-02	2	14	335	419
GO:0009108~coenzyme biosynthetic process	2.91E-02	2	14	335	419
GO:0044275~cellular carbohydrate catabolic process	2.91E-02	2	14	335	419
GO:0008610~lipid biosynthetic process	2.91E-02	2	14	335	419
GO:0006091~generation of precursor metabolites and energy	3.93E-02	18	40	335	419
GO:0009064~glutamine family amino acid metabolic process	3.98E-02	0	9	335	419
GO:0032787~monocarboxylic acid metabolic process	4.03E-02	4	17	335	419
GO:0016052~carbohydrate catabolic process	4.25E-02	3	15	335	419
GO:0006096~glycolysis	4.37E-02	1	11	335	419
GO:0006066~alcohol metabolic process	4.40E-02	7	22	335	419
GO:0046164~alcohol catabolic process	4.40E-02	2	13	335	419
GO:0046365~monosaccharide catabolic process	4.40E-02	2	13	335	419
GO:0019320~hexose catabolic process	4.40E-02	2	13	335	419
GO:0006007~glucose catabolic process	4.40E-02	2	13	335	419
GO:0006006~glucose metabolic process	4.40E-02	2	13	335	419
GO:0044265~cellular macromolecule catabolic process	5.39E-02	8	23	335	419
GO:0003008~system process	5.58E-02	17	37	335	419
GO:0016310~phosphorylation	7.02E-02	17	36	335	419
GO:0005996~monosaccharide metabolic process	8.07E-02	4	15	335	419
GO:0019318~hexose metabolic process	8.07E-02	4	15	335	419
GO:0009057~macromolecule catabolic process	8.69E-02	11	26	335	419
GO:0006936~muscle contraction	9.63E-02	2	11	335	419
GO:0030534~adult behavior	9.63E-02	2	11	335	419
GO:0003012~muscle system process	9.63E-02	2	11	335	419

Table B.7. Gene Ontology terms with directional bias toward control (C) > QMP (Q).

Term	***PValue***	***Category C > Q***	***Category Q > C***	***Total C > Q***	***Total Q > C***
GO:0006520~amino acid metabolic process	3.27E-03	12	1	50	56
GO:0006519~amino acid and derivative metabolic process	3.27E-03	12	1	50	56
GO:0006807~nitrogen compound metabolic process	1.23E-02	13	3	50	56
GO:0009309~amine biosynthetic process	1.77E-02	8	0	50	56
GO:0044271~nitrogen compound biosynthetic process	1.77E-02	8	0	50	56
GO:0008652~amino acid biosynthetic process	1.77E-02	8	0	50	56
GO:0009308~amine metabolic process	2.26E-02	12	3	50	56
GO:0006082~organic acid metabolic process	2.63E-02	13	4	50	56
GO:0019752~carboxylic acid metabolic process	2.63E-02	13	4	50	56
GO:0009064~glutamine family amino acid metabolic process	7.19E-02	6	0	50	56

Table B.8. Gene Ontology terms with directional bias toward QMP (Q) > control (C)

Term	*PValue*	*Category C > Q*	*Category Q > C*	*Total C > Q*	*Total Q > C*
GO:0044260~cellular macromolecule metabolic process	9.52E-06	3	26	50	56
GO:0044267~cellular protein metabolic process	9.52E-06	3	26	50	56
GO:0019538~protein metabolic process	9.52E-06	3	26	50	56
GO:0009057~macromolecule catabolic process	2.97E-03	0	12	50	56
GO:0044265~cellular macromolecule catabolic process	5.80E-03	0	11	50	56
GO:0006508~proteolysis	9.87E-03	2	14	50	56
GO:0009987~cellular process	1.03E-02	33	49	50	56
GO:0043285~biopolymer catabolic process	1.11E-02	0	10	50	56
GO:0030163~protein catabolic process	1.11E-02	0	10	50	56
GO:0043170~macromolecule metabolic process	1.23E-02	14	30	50	56
GO:0044257~cellular protein catabolic process	2.08E-02	0	9	50	56
GO:0019941~modification-dependent protein catabolic process	2.08E-02	0	9	50	56
GO:0006511~ubiquitin-dependent protein catabolic process	2.08E-02	0	9	50	56
GO:0051603~proteolysis involved in cellular protein catabolic process	2.08E-02	0	9	50	56
GO:0043632~modification-dependent macromolecule catabolic process	2.08E-02	0	9	50	56
GO:0043283~biopolymer metabolic process	2.91E-02	6	18	50	56
GO:0043412~biopolymer modification	5.98E-02	1	9	50	56
GO:0006464~protein modification process	5.98E-02	1	9	50	56

Appendix C: Supplemental figures for Chapter 3

Figure C.1. Number and proportion of differentially expressed genes (DEGs) concordantly and discordantly regulated by maturation and diet as a function of p-value cutoff. y-axis on right and solid bars indicate the number of genes differentially expressed in response to both maturation and diet quality and regulated concordantly (i.e., in the direction predicted by lipid stores; solid gray) or discordantly (solid red). y-axis on left and dotted black line indicate the proportion of concordant changes out of all genes differentially expressed in both experiments.

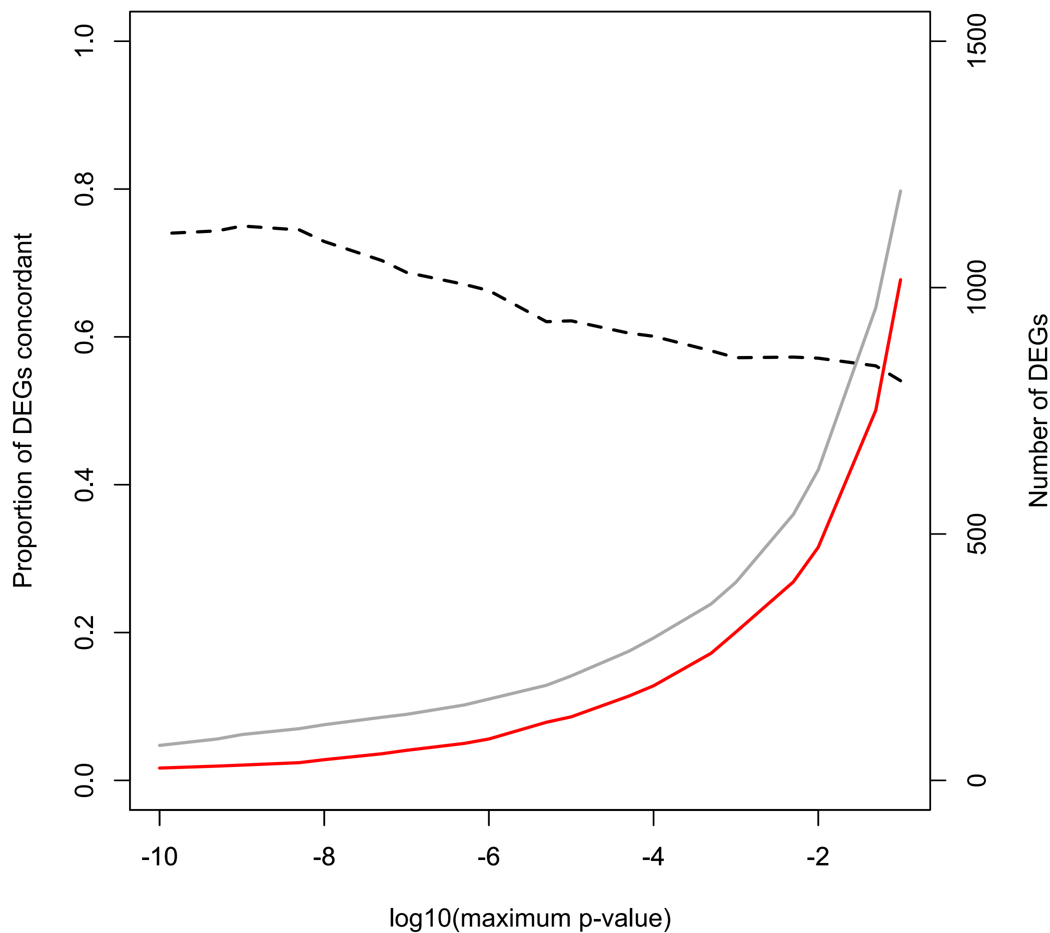
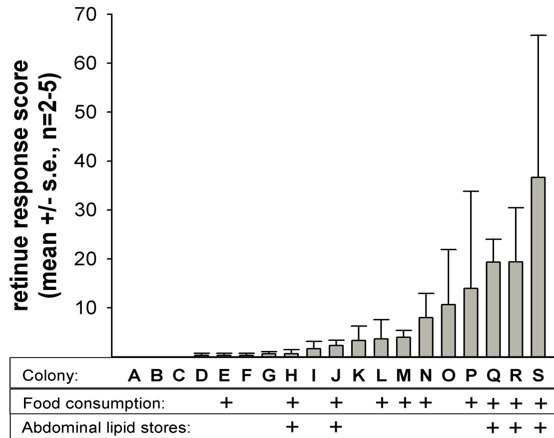
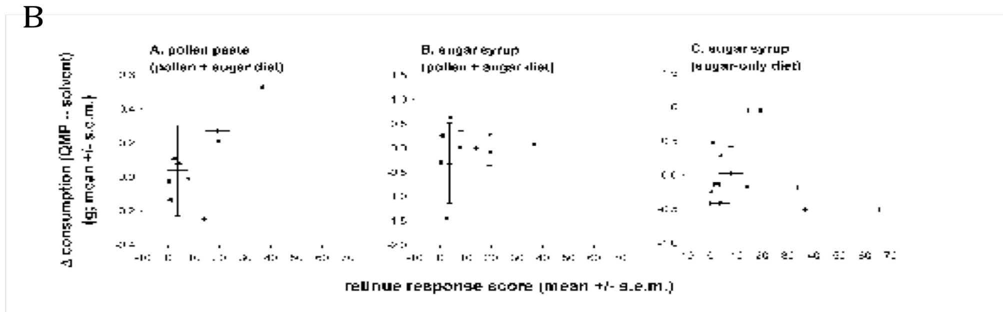


Figure C.2. Relationship between responsiveness to QMP and its effects of lipid stores and food consumption. A. Retinue response scores for bees from 19 colonies (A-S), and the colonies selected for analyses of food consumption, lipid stores, and fat body gene expression. Mean and S.E.M. are shown for 2-5 replicate retinue response assays with each colony. B. Consumption of pollen and of sugar syrup in cages fed both, and of sugar syrup in cages fed that only, plotted as a function of retinue response score. C, D. Effects of QMP and diet on lipid stores of bees from colonies with very high (C) and low to moderate (D) responsiveness to QMP in the retinue assay. Results in Fig. 3 are pooled from the three trials shown in C.





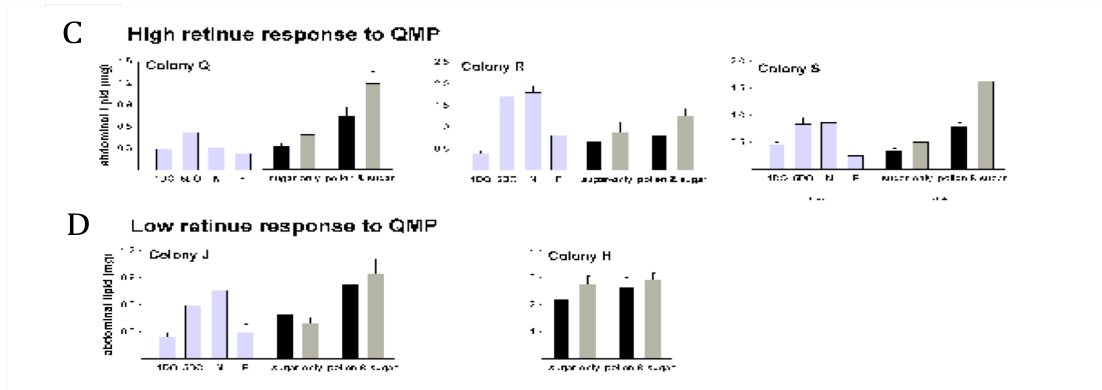


Figure C.3. Microarray hybridization scheme for the Maturation, Diet, and QMP experiments. Each arrow represents a microarray with the sample at the tail labeled with Cy3 and the sample at the head labeled with Cy5. Samples are labeled as follows: SS11 = group SS, trial 1, sample 1. Group abbreviations: N, nurse; F, forager; SS, solvent (no QMP) / sugar-only diet; QS, QMP / sugar-only diet; SPS, solvent / pollen&sugar diet; QPS, QMP / pollen&sugar diet. This design uses 128 microarrays to measure 96 samples (2 trials * 8 samples * 6 groups).

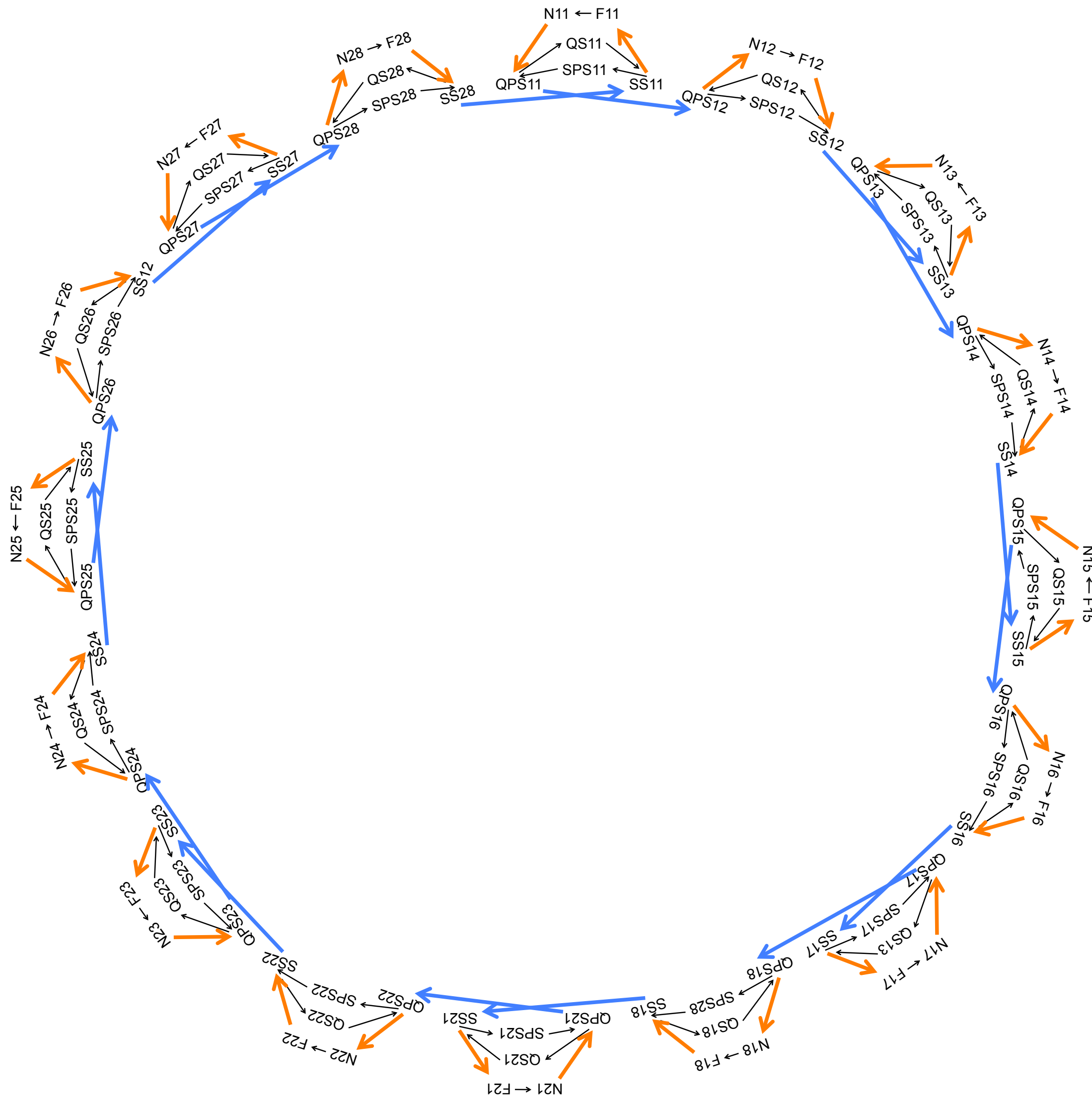


Figure C.4. Microarray hybridization scheme for the Vg RNAi experiment. Each arrow represents a microarray with the sample at the tail labeled with Cy3 and the sample at the head labeled with Cy5. Groups: Vg, dsVg; EB, saline buffer injection control; Noi, no injection control. This study used 33 microarrays to measure 30 samples (2 trials * 3 groups * 5 samples/group/trial).

Colony A

Colony C

Appendix D. Supplemental Figures for Chapter 5.

Figure D.1. *USP* RNAi causes transcriptional and translational knockdown of *USP* in the fat bodies but not in head.

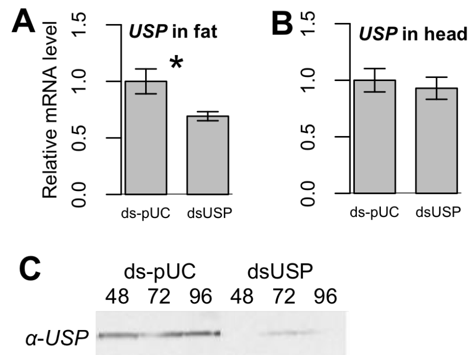


Figure D.2. Replicable differences between bees from genetically distinct sources in the effect of *USP* RNAi on the age at onset of foraging. Bees from each source colony were tested in separate colonies during trial 1 and together in a shared host single-cohort colony in Trial 2. Genotypes A and B were used for mRNA sequencing. Results shown in Fig. 5.1 are pooled from these trials and from 5 additional trials using bees from three additional genetic sources.

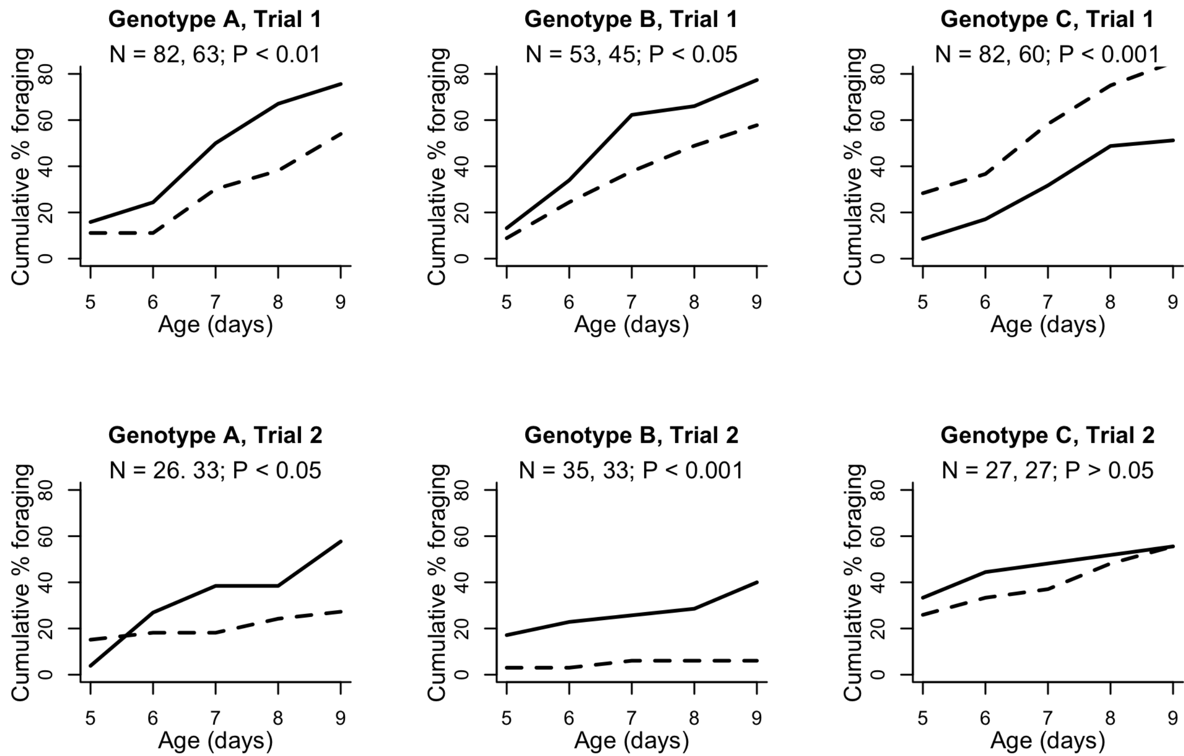


Figure D.3. Juvenile hormone analog methoprene administered orally to 3-day-old bees influences foraging ontogeny.

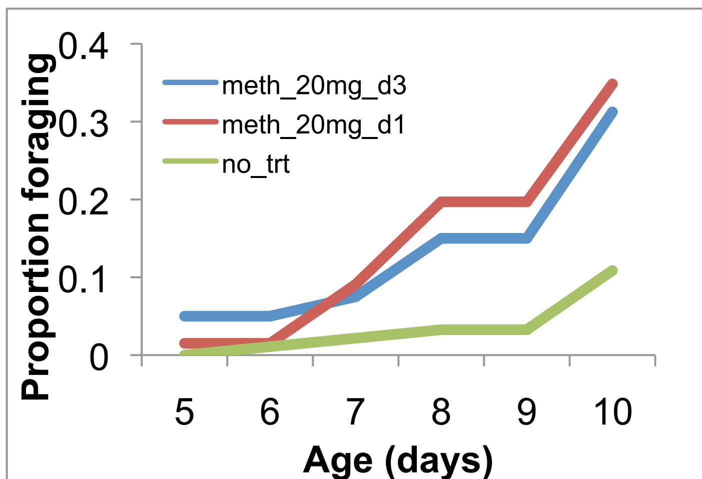


Figure D.4. Distribution of *USP* binding sites relative to predicted start sites of genes within 10kb.

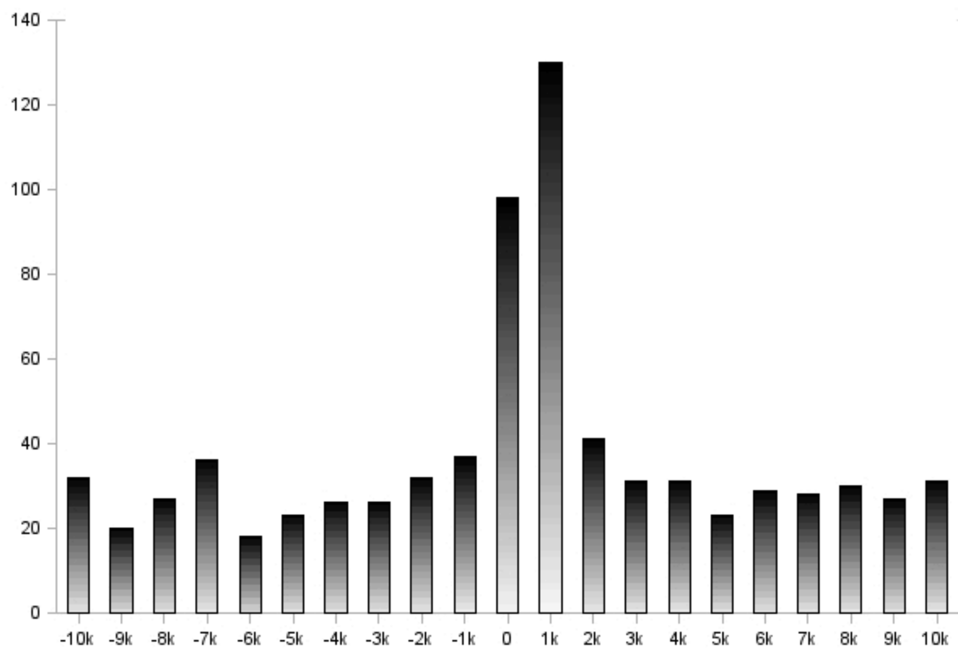


Figure D.5. Binding intensity at probes within *USP* binding sites is highly replicable across 6 biological replicates, both within and between behavioural groups.

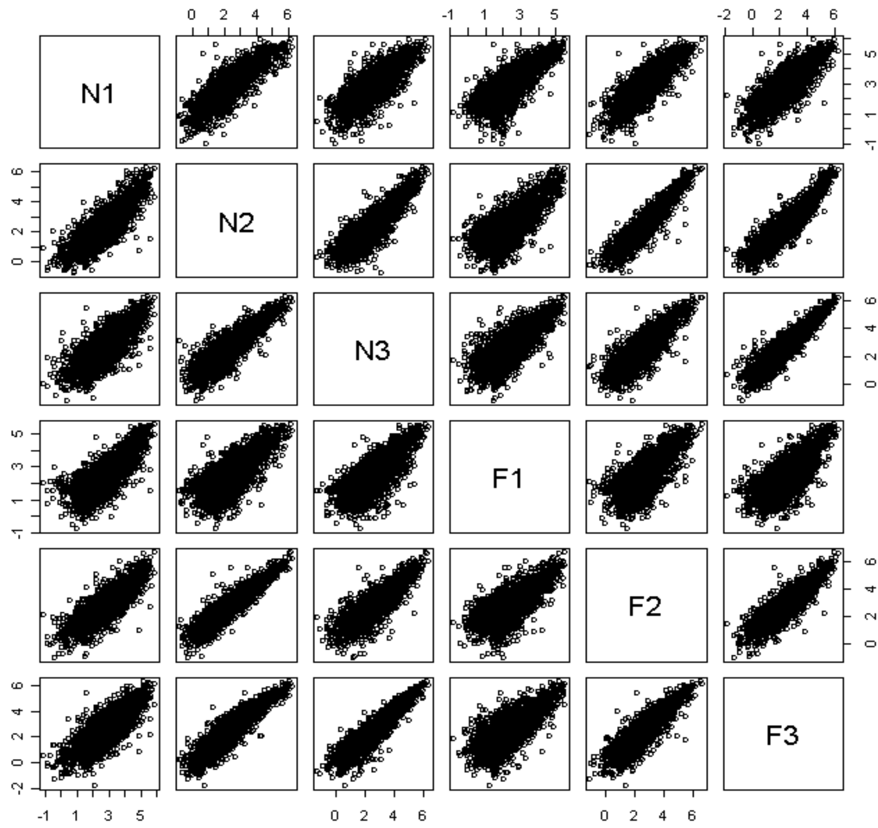


Figure D.6. Western blots using antibodies that recognize honey bee USP. Fat body protein extracts were used in both assays. Left lane: the antibody used in ChIP-chip. Right lane: a different USP antibody generated using a different peptide as antigen used for validation with ChIP-qPCR. Lanes were treated separately with the two primary antibodies but otherwise handled together.

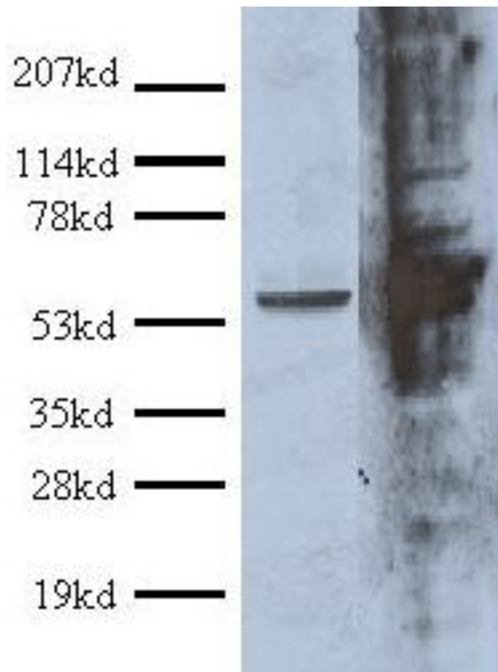
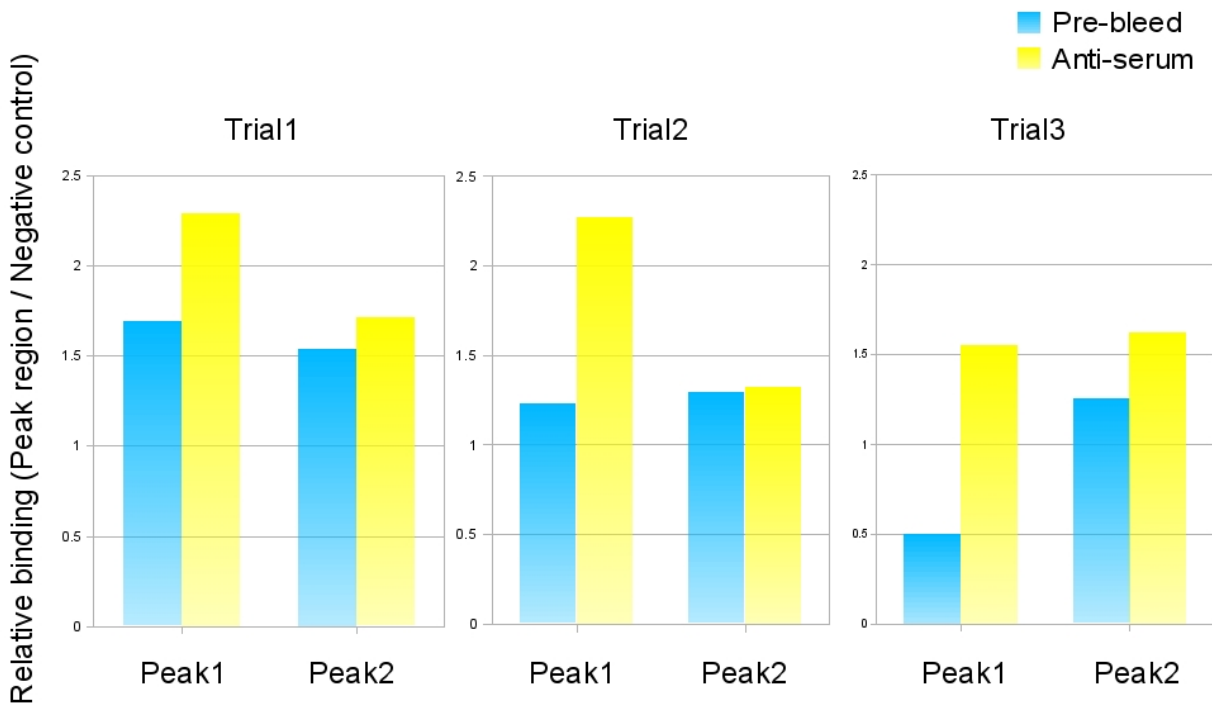


Figure D.7. Validation of *USP* binding sites by ChIP-qPCR. We used ChIP-qPCR and an antibody to honeybee *USP* different from the one used in ChIP-chip for validation of selected peak regions. We confirmed that there was a ca. 2-fold enrichment at peak region 1 in DNA pulled down with *USP* anti-serum compared to pre-bleed control (paired t-test, $P < 0.05$). There was a ca. 1.2-fold enrichment at peak region 2, but this was not significant (paired t-test, n=3, $P > 0.05$).



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Curriculum vitae

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Education

A.B.	Harvard University (*cum laude* in Biology), 2003
Ph.D.	University of Illinois, Neuroscience, 2010

Honors and Awards

Fellowships and Training Grants

2002	Center for Genomics Research Internship, Harvard University
2004-2006	Cell and Molecular Biology Training Grant, NIH / U. Illinois
2006-2009	NSF Graduate Research Fellowship ($90.000 / 3 yrs.)
2009-2010	Sensory Neuroscience Training Grant, NIH / U. Illinois

Honors

1999	Finalist, Intel Science Talent Search ($3000)
2000-2003	Harvard College Scholarship
2009	Ladd Prosser Memorial Award, Neuroscience Program, University of Illinois ($500)
2010	Neuroscience Program Award for Outstanding Research ($500)
2010	Co-winner, Procter & Gamble Company Doctoral Student Research Award ($1000)

Travel Awards

2004	Neuroscience Program Travel Award ($300)
2005	Neuroscience Program Travel Award ($550)
2006	University of Illinois Graduate College Travel Award ($250)
2006	Neuroscience Program Travel Award ($550)
2007	Neuroscience Program Travel Award ($550)
2008	Neuroscience Program Travel Award ($550)
2009	University of Illinois Graduate College Travel Award ($175)

2009 International Behavioral and Neural Genetics Society Travel Award ($775)

Research Experience

1998 Laboratory Assistant, Laboratory of Allen Mensinger, Marine Biological Laboratory, Woods Hole, MA: toadfish behavior.

1998-2000 Laboratory Assistant, Laboratory of Roger Hanlon, Marine Biological Laboratory, Woods Hole, MA: cephalopod behavior and physiology.

2000 Laboratory Assistant, Laboratory of Mitchell Sogin, Marine Biological Laboratory, Woods Hole, MA: *Giardia lamblia* genome sequencing project.

2001 Laboratory Assistant, Laboratory of Thomas Benjamin, Department of Pathology, Harvard Medical School: molecular biology of tumor suppressor genes.

2002-2003 Research for Honors Thesis, Laboratory of Hans Hofmann, Bauer Center for Genomics Research, Harvard University: cichlid fish behavior and genomics, mechanisms of social organization.

2003-2004 Research Technician, Laboratory of James Traniello, Department of Biology, Boston University: ant neuroanatomy, neurochemistry, and social behavior.

2004-2010 Ph.D. Candidate, Laboratory of Gene Robinson, Neuroscience Program, University of Illinois at Urbana-Champaign: honey bee sociogenomics.

Teaching and Mentoring Experience

2003-2004 Supervisor of research assistants, Boston University (supervised two undergraduate students)

2004-2009 Supervisor of research assistants, University of Illinois (supervised one entering PhD student, one Masters of Biology student, one post-bachelors student, six undergraduate students, and one high school student)

2007 Illinois Summer Neuroscience Institute, laboratory coordinator (designed and ran laboratory component of week-long institute for 20 undergraduate students).

2008 Seminar on "Honey Bee Nutrition," University of Illinois Short Course on Bees and Beekeeping

2008 Illinois Summer Neuroscience Institute, laboratory coordinator and panel discussant on "Genes and Behavior"

Spring 2009 Teaching Assistant for Merit Sections, MCB 252, "Cells, Tissues, and Development"

Research Talks

Invited Talks at Symposia

2005 "Student Data Blitz," Gordon Research Conference on Genes and Behavior, Ventura, CA

2006 "Graduate Student and Postdoctoral Symposium," W.M. Keck Center for Behavioral Biology, North Carolina State University, Raleigh, NC

2006	Woods Hole Behavior Symposium, Marine Biological Laboratory, Woods Hole, MA
2007	"Neurobiology and Behavior," Workshop on Honey Bee Genomics and Biology, Cold Spring Harbor Laboratories, NY
2008	"Student Data Blitz," Gordon Research Conference on Genes and Behavior, Barga-Gallicano, Italy
2008	"Future Challenges and Promises of Neuroethology," Gordon Research Conference on Neuroethology (Graduate Research Seminar), Magdalen College, Oxford, UK
2009	"Evo-Behavio: cross disciplinary studies in behavior and its evolution," International Behavioral and Neural Genetics Society, Dresden, Germany
2010	Gordon Research Conference on Genes & Behavior, Ventura, CA

<u>Invited Seminars</u>

2008	Department of Biology, Southeastern Missouri State University, Port Girardeau, MO
2009	Laboratory of Kristin Scott, Department of Molecular and Cellular Biology, University of California at Berkeley

<u>Intramural</u>

2006	Neuroscience Program First-Year Student Presentation
2006-2009	Neuroscience Student Data Blitz
2007	University of Illinois Ecolunch
2007	Cell and Molecular Biology Training Grant Seminar
2009	Advances in Sensory and Developmental Neuroscience Seminar
2009	Cell and Molecular Biology Training Grant Seminar

Contributed Poster Presentations

2005	Society for Neuroscience (with G.E. Robinson)
2006	Gordon Research Conference on Genes and Behavior (with G.E. Robinson)
2006	Society for Neuroscience (with R.A. Velarde and G.E. Robinson)
2006	Entomological Society of America (with R.A. Velarde and G.E. Robinson)
2007	Institute for Genomic Biology Symposium (with R.A. Velarde and G.E. Robinson)
2007	Society for Neuroscience (with M. Corona and G.E. Robinson)
2007	Cell and Molecular Biology Training Grant Symposium, UIUC (with M. Corona and G.E. Robinson)
2008	Institute for Genomic Biology Symposium (with M. Corona, H.S. Pollock, and G.E. Robinson)
2008	Gordon Research Conference on Genes and Behavior (with M. Corona, H.S. Pollock, and G.E. Robinson)
2008	Gordon Research Conference on Neuroethology (with M. Corona and G.E. Robinson)

2009 — Cold Spring Harbor Laboratory Meeting on Systems Biology: Networks (with Y. Wang, F. Hong, S. Zhong, C.A. Mizzen, and G.E. Robinson)

Seminars and Symposia Organized

Extramural

2010 — Chair, Gordon Research Seminar on Genes and Behavior, to be held in conjunction with the Gordon Research Conference on Genes and Behavior in Ventura, CA, March 13-14, 2010.

Intramural

2006 — Cell and Molecular Biology Training Grant Symposium (Posters Co-Chair)

2007, 2008 — Neuroscience Student Data Blitz

Publications

Primary research articles

1. Brockmann A. Annangudi S.P., Richmond T.A., Ament S.A., Xie F., Southey B.R., Rodriguez-Zas S.R., Sweedler J.V., and Robinson G.E. 2009. Quantitative peptidomics reveal brain peptide signatures of behavior. ***Proc Natl Acad Sci USA.*** 106:2383-8.
2. Ament S.A., Corona M., Pollock H.S., and Robinson G.E. (2008) Insulin signaling is involved in the regulation of worker division of labor in honey bee colonies. ***Proc Natl Acad Sci USA,*** 105:4226-31.
3. Honeybee Genome Sequencing Consortium (2006) Insights into social insects from the genome of the honeybee *Apis mellifera*. ***Nature*** 443:931-49.
4. Kunieda T.*, Fujiyuki T.*, Kucharski R.*, Foret S.*, Ament S.A.*, Toth A.L.*, Ohashi K., Takeuchi H., Kamikouchi A., Kage E., Morioka M., Beye M., Kubo T., Robinson G.E., and Maleszka R. (2006) Carbohydrate metabolism genes and pathways in insects: insights from the honey bee genome. ***Insect Mol Biol*** 15:563-576.
5. Shashar N., Borst D.T., Ament S.A**.**, Saidel W.M., Smolowitz R.M., and Hanlon R.T., (2001) Polarization reflecting iridophores in the arms of the squid *Loligo pealeii.* ***Biol Bull*** 201:267-268.
6. Hanlon R.T., Ament S.A., and Gabr H. (1999) Behavioral aspects of sperm competition in cuttlefish, *Sepia officinalis* (Sepioidea: Cephalopoda). ***Marine Biol*** 134:719-728.
7. Ament S.A., Bullis R., Hanlon R.T., and Mensinger A. (1997) Righting response and escape response in *Opsanus tau* are temperature dependent. ***Biol Bull*** 193:265-266.

Invited review

8. Ament S.A., Wang Y., and Robinson G.E. Nutritional regulation of worker division of labor in honey bee colonies: a systems perspective. *Wiley Interdiscipl Rev: Systems Biol Med.* 2(5): 566-576.

Reviewed for

PLoS Genetics

Scientific Outreach

2006-2009	Brain Awareness Day, UIUC. "What's the Buzz About?"
2007	Nontechnical research presentation to the Heart of Illinois Beekeeper's Association
2009	Nontechnical research presentation to the Cook-Dupage Beekeeping Association
2009	Data blitz talk for Illinois Summer Neuroscience Institute
2009	Nontechnical research presentation for the University of Illinois Pollinatarium (Pollinator Museum)

Service and Activities with the Neuroscience Program

2006	Coordinator, Open House Poster Session
2006-2007	Student Member to the Admissions Committee
2006-2007	Neuroscience Student Organization, *ad hoc* committee to draft an organizational Constitution
2008	Contributor to the "Illinois NeuroNews"; article on "The Future of Neuroethology" republished in the International Society for Neuroethology Newsletter, January, 2009
2007-2008	Co-Chair, Student-Invited Speaker Committee

Professional Affiliations

American Association for the Advancement of Science
Entomological Society of America
International Behavioral and Neural Genetics Society
International Society for Neuroethology
Society for Neuroscience

Interests

Cello performance: pursued Masters-level studies in cello performance at the Boston University School of Fine Arts (2003-2004).

Running: completed two marathons.