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RESEARCH ARTICLE

Mechanisms of stable lipid loss in a social insect

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SUMMARY

Worker honey bees undergo a socially regulated, highly stable lipid loss as part of their behavioral maturation. We used large-scale transcriptomic and proteomic experiments, physiological experiments and RNA interference to explore the mechanistic basis for this lipid loss. Lipid loss was associated with thousands of gene expression changes in abdominal fat bodies. Many of these genes were also regulated in young bees by nutrition during an initial period of lipid gain. Surprisingly, in older bees, which is when maximum lipid loss occurs, diet played less of a role in regulating fat body gene expression for components of evolutionarily conserved nutrition-related endocrine systems involving insulin and juvenile hormone signaling. By contrast, fat body gene expression in older bees was regulated more strongly by evolutionarily novel regulatory factors, queen mandibular pheromone (a honey bee-specific social signal) and vitellogenin (a conserved yolk protein that has evolved novel, maturation-related functions in the bee), independent of nutrition. These results demonstrate that conserved molecular pathways can be manipulated to achieve stable lipid loss through evolutionarily novel regulatory processes.

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INTRODUCTION

Certain animal species have evolved the ability to adaptively change their body mass and composition in response to environmental conditions, including during hibernation in mammals (Rousseau et al., 2003), diapause in many insect species (Hahn and Denlinger, 2007) and behavioral maturation in honey bees (Toth and Robinson, 2005). While many studies have examined the homeostatic mechanisms by which animals maintain constant body mass (Morton et al., 2006), relatively little is known about the molecular mechanisms by which animals achieve changes in their body mass.

Studies with human subjects and with animal models such as mice and fruit flies (in which changes in body mass are usually maladaptive) have shown that nutritionally responsive hormones such as insulin and leptin adjust nutrient intake and utilization to achieve energy balance and stable body mass (Morton et al., 2006). Genetic and genomic studies suggest that this process involves hundreds to thousands of genes (Emilsson et al., 2008). However, these mechanisms appear ill-equipped to cope with changes in body mass: one cause of the current obesity epidemic in developed nations is that homeostatic mechanisms in humans quickly adjust to elevated adiposity as a norm and respond to subsequent decreases in adiposity as though to starvation (Morton et al., 2006).

Presumably, species that undergo adaptive changes in body mass are able to do so because of evolved mechanistic differences in regulatory pathways. It is known, for example, that day-length-mediated changes in adiposity in seasonal mammals can involve changes in the titer of the adipocyte hormone leptin and in leptin sensitivity, but these and other mechanisms of stable lipid gain or loss are not well understood in any organism (Rousseau et al., 2003).

Honey bees (*Apis mellifera* L.) show stable lipid loss (reviewed in Ament et al., 2010). Worker bees begin their adult life lean but develop larger 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 (Toth and Robinson, 2005). 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 (Winston, 1987). 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 (Crailsheim, 1992). Stable lipid loss occurs prior to a lifestyle transition from in-hive tasks to foraging outside the hive for nectar and pollen. Decreased body mass may improve a forager's capacity for long flights. It also is to the benefit of the entire colony if lipids are used up prior to the onset of foraging, because foragers are at high risk of dying outside the hive (Blanchard et al., 2000).

Changes in body mass result from an imbalance between the rate at which an animal ingests nutrients and the rate at which these nutrients are utilized for energy (Haslam and James, 2005). How an animal achieves such an energy imbalance can relate to a myriad of underlying processes including diverse changes in behavior, physiology, hormones, and molecular signaling and metabolic pathways. 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 (Crailsheim, 1992; Crailsheim et al., 1992). 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 (Harrison, 1986). Therefore, changes in energy balance during stable lipid loss in the bee likely arise from differences both in the intake and in the utilization of nutrients.

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 in the abdominal fat bodies (Schulz et al., 1998; Toth et al., 2005), suggesting that nutrition itself could be an important regulator of lipid loss. Insects store fat in the fat bodies that line the body walls, most thickly in the abdomen (Snodgrass, 1956). Insect fat bodies have analogous functions to both liver and adipose tissue because they are crucial for both carbohydrate metabolism and lipid storage.

Several conserved, nutritionally and metabolically related signaling pathways have been implicated in honey bee behavioral maturation (Ament et al., 2010), including insulin and target of rapamycin (TOR) (Ament et al., 2008), cGMP-dependent protein kinase (Ben-Shahar et al., 2002) and juvenile hormone (JH) (Jaycox et al., 1974; Robinson, 1987; Sullivan et al., 2000). These pathways might therefore also regulate stable lipid loss.

However, as discussed above, homeostatic regulation of these pathways does not allow for stable lipid loss in humans and standard model systems. This suggests that other molecular mechanisms for stable lipid loss remain to be discovered. We used honey bees to address this issue.

We hypothesized that stable lipid loss involves two kinds of regulatory differences between honey bees 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 (Ament et al., 2008). 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 (Amdam et al., 2003; Guidugli et al., 2005; Nelson et al., 2007). In addition, bees have evolved intricate mechanisms by which social signals influence physiology; queen mandibular pheromone (QMP) primarily functions to inhibit worker reproduction (Hoover et al., 2003), but also delays the transition of worker bees from hive work to foraging (Pankiw et al., 1998a) and causes an increase in lipid stores (Fischer and Grozinger, 2008), both of which may increase the colony's capacity to rear brood at times when the queen is actively laying eggs. We explored these hypotheses through a combination of transcriptomic and proteomic experiments, physiological experiments and RNA interference.

MATERIALS AND METHODS

Bees

Bees were maintained according to standard practices at the University of Illinois Bee Research 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 exclusively used colonies headed by 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 (Ament et al., 2008). Nurse bees used for nutritional manipulations were collected based on their age (8–9 days old) and proximity to the brood; this is also a reliable assay for nursing (Seeley, 1982). 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 h period. Bees were collected immediately (1 day old) or aged in a host colony to the desired age (5 or 9 days old). 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 a rich diet (pollen/honey) or a poor diet (sugar syrup) as previously described (Ament et al., 2008). The alternative royal jelly/honey diet was also as described (Hoover et al., 2006). We modified conditions slightly to improve the survival of older bees. One-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 1 day olds and nurses, and 20–30% for foragers. We verified that all groups consumed the diets by weighing and replacing feeders daily. After 4 days, bees were collected by brief CO_2 anesthetization and flash frozen in liquid nitrogen.

Vitellogenin RNAi

Previously described *dsVg* probes (Amdam et al., 2006) were synthesized by *in vitro* transcription with T7 RNA polymerase. We diluted double-stranded (ds)RNA to a concentration of 10 $\mu g\,\mu l^{-1}$ in buffered saline solution and injected 1 µl intra-abdominally using a microinjection system (World Precision Instruments, Sarasota, FL, USA) equipped with a 34 gauge beveled needle. Control bees were injected with 1 µl 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 4 days then collected by flash freezing in liquid nitrogen. Mortality was 10–20% over 4 days. We validated knockdown by quantitative (q)PCR and selected 5 (out of 10) individuals per 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 (Pherotech, Trail, BC, Canada; 0.1 queen equivalents synthetic QMP in 90% isopropanol/10% water dried onto a microscope coverslip) was administered as previously described (Grozinger et al., 2003) to groups of 35 caged bees fed a rich or poor diet. Bees in control cages were administered solvent alone. We measured total daily food consumption for each cage every 24 h 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 Pankiw et al., 1994). Nurse bees were captured from each colony and caged overnight (15 bees per cage) without QMP, and 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 min. 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 three 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 µl of 50 mmol l^{-1} NH_4HCO_3, 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 (Ament et al., 2008; Toth and Robinson, 2005).

Sample preparation for microarrays and qPCR

Abdomens of frozen bees were soaked overnight in 0.6 ml RNAlater-ICE (Ambion, Austin, TX, USA). We then removed the gut and extracted total RNA from the remaining fat body and annealing cuticle using RNeasy kits (Qiagen, Valencia, CA, USA).

Microarray procedures

The microarray has been characterized previously (Alaux et al., 2009a) and contains 28,800 spotted oligos, including 13,440 experimental probes spotted in duplicate, 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. The effects of maturation, diet quality and QMP were measured in one integrated study with N=16–17 samples per group (see supplementary material Fig. S1), and the effects of *vg* RNAi were measured separately with N=10 samples per group (see supplementary material Fig. S2). Microarray procedures were slightly modified from Alaux et al. (Alaux et al., 2009a). RNA from the fat bodies of individual bees was amplified according to the manufacturer's instructions with the MessageAmp II aRNA Amplifcation kit (Ambion) starting with 500 ng RNA. Dye coupling and labeled amplified (a)RNA cleanup were performed with the Kreatech Universal Labeling System (Open Biosystems, Huntsville, AL, USA), using 2 µg aRNA in each reaction. Paired aRNA samples having incorporated 60 pmol Cy3/Cy5 dye were hybridized to microarrays overnight. Slides were scanned with an Axon 4000B scanner (Axon Instruments, Union City, CA, USA), and images were analyzed using GENEPIX software (Agilent Technologies, Santa Clara, CA, USA).

Microarray data analysis

Analysis was performed as described previously (Alaux et al., 2009a). 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 $\log_2$-transformed gene intensity 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 false discovery rate (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 (differentially expressed) 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 were based on the main effects of diet and QMP, respectively, in the two-factor model, excluding probes that showed a significant diet × QMP interaction (FDR<0.05). The *vg* RNAi microarray results were evaluated using a one-factor model with three levels (dsRNA injected, saline injected, uninjected); the *vg* RNAi gene list consists of probes that were significant in the *post hoc* dsRNA-injected *vs* saline-injected contrast.

Functional enrichment analysis

Gene ontology (GO) directional bias analysis was performed with *Drosophila melanogaster* orthologs to bee genes (Alaux et al., 2009b). Statistical bias was determined using the DAVID Bioinformatics Resources 2008 functional annotation tool (Dennis et al., 2003), based on the ratio of up- *vs* down-regulated genes in each GO category, compared with the ratio among all genes with annotated *Drosophila* orthologs. Differentially expressed genes were mapped to pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Goto, 2000), primarily for visual purposes, using the Color Objects in Pathways tool on the KEGG website (www.genome.jp/kegg). 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 (Alaux et al., 2009b). This factor is observed divided by the expected number of overlapping genes. The denominator is calculated as the product of the number of genes differentially expressed in each experiment divided by the total number of genes analyzed. We tested statistical significance by using an exact hypergeometric test (1-tailed) for the overlap between two gene sets.

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

Quantitative PCR (qPCR)

qPCR was performed as described previously (Ament et al., 2011). cDNA was synthesized from 200 ng total RNA. qPCR was performed with an ABI Prism 7900 (Foster City, CA, USA) sequence detector and specific primers (supplementary material Table S1). The 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, USA). Data were combined from three independent trials using different colonies.

Proteomics: quantitative liquid chromatography/mass spectrometry (LC/MS)

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. Abdomens were

Table 1. Functional categories of genes showing directionally biased responses during stable lipid loss, and their responses to diet quality, *vg* RNAi and queen mandibular pheromone (QMP)

Biological process	Maturation Nurse ↑/forager ↑	Diet quality Rich ↑/poor ↑	*vg*RNAi Control ↑/*vg* RNAi ↑	QMP QMP ↑/control ↑
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 macromolecular metabolic process	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)	–	–
Regulation of developmental process	2/22 (*P*=2.35e–03)	12/26 (*P*=4.61e–03)	–	–
Cellular component organization & 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 process	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 chemical 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 biological quality	7/30 (*P*=9.50e–03)	–	–	–
Cell structure morphogenesis	14/44 (*P*=9.82e–03)	25/68 (*P*=9.09e–09)	–	–

We identified gene ontology (GO) biological process categories for which genes showed a consistent directional bias in expression. For 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 melanogaster* orthologs ('All genes'), based on a modified version of Fisher's exact test (Dennis et al., 2003). Data for these same 'behaviourally 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). Not shown are results for several additional GO terms with lower significance and represented by >70% of the same genes as one of the terms in the table. 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. Supplementary material Tables S2, S3, S5, S6 and S8–S11 contain complete lists of GO terms showing directional bias for all experiments.

washed three times in PBS, then 100 µl lysis buffer (1% NP-40, 150 mmol l^{-1} NaCl, 20 mmol l^{-1} Tris-HCl pH 7.5) including a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA; at 8 times the suggested concentration) was added and homogenized by 10 strokes through a syringe tipped with a 25 gauge needle. The sample was clarified for 10 min at 16,100 ***g*** at 4°C and the pelleted debris was discarded, while the supernatant proteins were precipitated by adding ethanol and sodium acetate as described elsewhere (Foster et al., 2003). Hemolymph was processed as described previously (Chan et al., 2006) and hemolymph and fat body proteins were prepared for mass spectrometry, isotopically labeled and analyzed on an LTQ-OrbitrapXL (ThermoFisher, Loughborough, Leics, UK) exactly as described before (Chan et al., 2009). 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.

RESULTS

Gene expression changes in fat body during naturally occurring lipid loss

Fat body tissue from nurses and foragers differed in the expression of 2641 transcripts (ANOVA, FDR<0.05), which amounted to 21% of the quantified transcripts in this tissue. Mapping differentially expressed transcripts to GO (Ashburner et al., 2000) (Table 1; see supplementary material Tables S2 and S3) and to pathways from the KEGG (Fig. 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) (Klowden, 2002) (Fig. 1), as well as energy metabolism pathways (e.g. oxidative phosphorylation, cofactor metabolic process). It is not possible to discern from this experiment whether these changes represent a cause or consequence of the differences in adiposity between nurses and foragers.

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* (Nilsen et al., 2011). 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 in nurses. It is not known whether any of these 'developmental' genes are causal for stable lipid loss, but their reuse during maturation suggests commonalities between development and adult plasticity (Sinha et al., 2006).

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, $P<0.05$; supplementary material Table S4), RNA and protein measurements for 104 genes were strongly correlated (Pearson correlation, $r=0.71$, $P \ll 2.2e{-}16$; Fig. 2), suggesting that transcriptional differences are

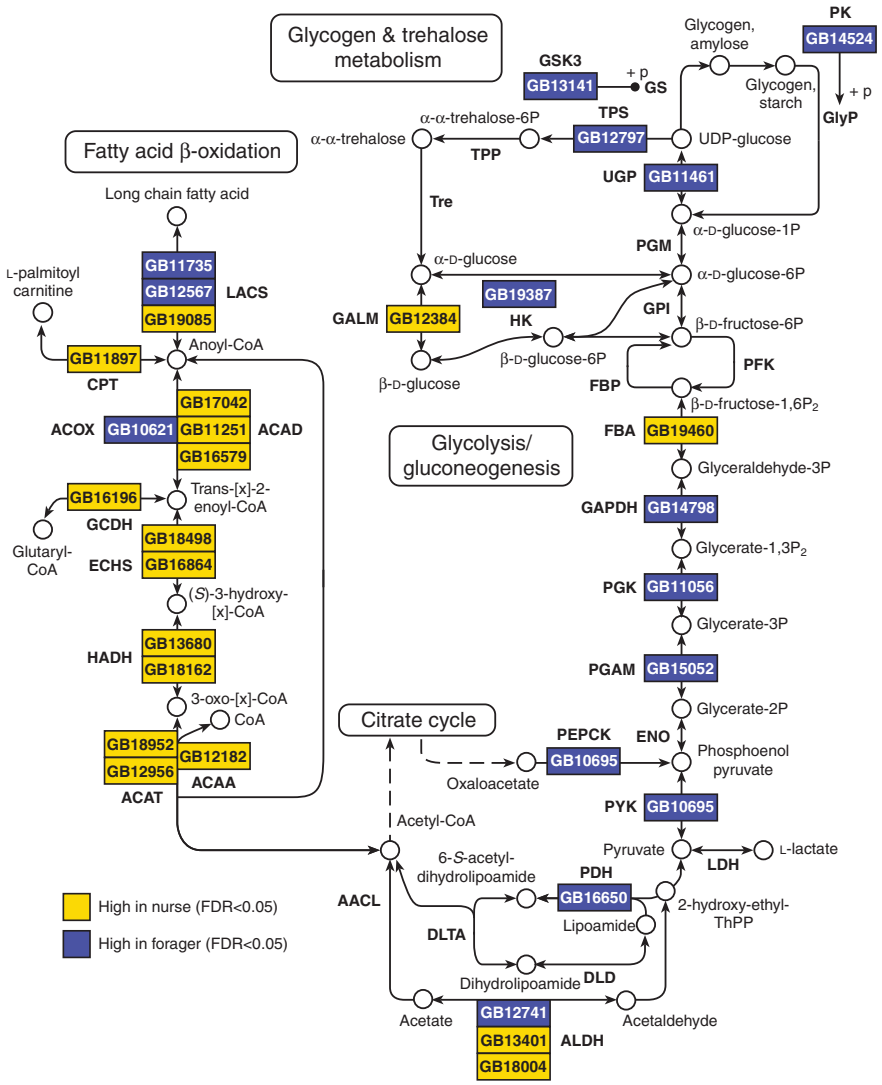

Fig. 1. Maturational changes in fatty acid and carbohydrate metabolism gene expression. Transcripts differentially expressed between nurse and forager fat bodies (false discovery rate, FDR<0.05) were mapped to metabolic pathways based on the union of direct annotations from the Kyoto Encyclopedia of Genes and Genomes (KEGG) 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).

often 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. We did this bioinformatically, by comparing the transcriptional changes during maturation to transcriptional responses induced by factors that are known to influence nutrient stores in the bee.

Effects of diet on fat body gene expression

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 4 days after adult emergence) or a nutrient-poor diet (sugar syrup only, *ad libitum*). This experimental protocol mimics the lipid gain during early life (Ament et al., 2008) and allowed us to characterize the transcriptional changes induced by nutrition in isolation from social stimuli in the hive. We predicted that the 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 the expression of genes related to 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 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). This surprising result held when we applied more and less stringent statistical cutoffs in defining gene lists, supporting the conclusion that the fat body transcriptomic response to maturation and diet includes a mix of concordant and discordant changes (supplementary material Fig. S3). These results suggest that although naturally occurring, stable lipid loss involves changes in the expression of diet-regulated genes, these changes are unlikely to be due to diet *per se*.

GO functional analysis further supported this conclusion. A total of 14 out of 20 GO categories with biased representation between nurses and foragers also had biased representation between rich and

Table 2. Overlap of differentially expressed genes between experiments

	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

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

poor diet (Table 1; supplementary material Tables S5 and S6). 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. 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.

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, indicating that maturational differences in energy metabolism are unlikely to be caused by dietary changes.

The conclusion that stable lipid loss likely involves a mix of nutritionally dependent and independent changes was also supported by quantitative proteomic analysis (supplementary material Table S7). 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. 2).

Diet quality also influenced the expression of a number of genes and processes that were not differentially expressed during maturation (supplementary material Tables S3 and S4). For instance, genes related to amino acid metabolism (58 genes) were upregulated with the 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* (Accili and Arden, 2004), the cholesterol sensor *Hr96* (Bujold et al., 2010) and the cGMP-dependent protein kinase *foraging* (Kaun and Sokolowski, 2009), 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.

Maturational changes in fat body transcriptomic 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 a subset of metabolic and signaling-related genes under a broader set of conditions using 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 4 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 (Ament et al., 2008; Ament et al., 2011)] that were involved in protein storage (*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 included two additional genes related to well-known, nutritionally related peptide signaling pathways – the *adipokinetic hormone receptor* (*akhR*) (Kim and Rulifson, 2004) and the *short neuropeptide F receptor* (*snpfR*) (Root et al., 2011) – 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). Expression of 8 of 10 genes (all except *akhR* and *snpfR*) differed between pre-nursing bees fed the nutrient-poor sugar syrup diet and those fed the nutrient-rich pollen/honey diet (supplementary material Fig. S4). Expression of 8 out of 10 genes (all except *lsd2* and *snpfR*) differed between bees fed the sugar syrup diet and those fed a different nutrient-rich diet made from a mixture of royal jelly and honey (Fig. 3; we used two rich diets here to facilitate comparisons with foragers below – foragers cannot digest pollen and are fed jelly from workers) (Crailsheim et al., 1992). These results provide validation for maturational and

Table 3. Directional relationships of differentially expressed genes between experiments

	Maturation (2641)			Diet (3372)			*vg* RNAi (2136)		
	F>N	N>F	Significance	P>R	R>P	Significance	V>C	C>V	Significance
Poor>rich diet	346	281	χ^2=20.55						
Rich>poor diet	288	390	P=5.88e-6						
vg RNAi>control	370	105	χ^2=170.12	207	175	χ^2=2.56			
Control>*vg* RNAi	208	350	P<2.2e–16	271	286	n.s.			
Control>QMP	60	49	χ^2=3.45	34	104	χ^2=5.16	19	80	χ^2=0.09
QMP>control	26	41	P=0.05	28	40	P=0.02	11	37	n.s.

We compared the distribution of up- and down-regulated genes between each pair of experiments. Chi-square tests were used to determine whether there was significant directional bias in these relationships. Numbers represent the intersection between the conditions indicated.
P, poor diet; R, rich diet; F, forager; N, nurse; V, vitellogenin; C, control.

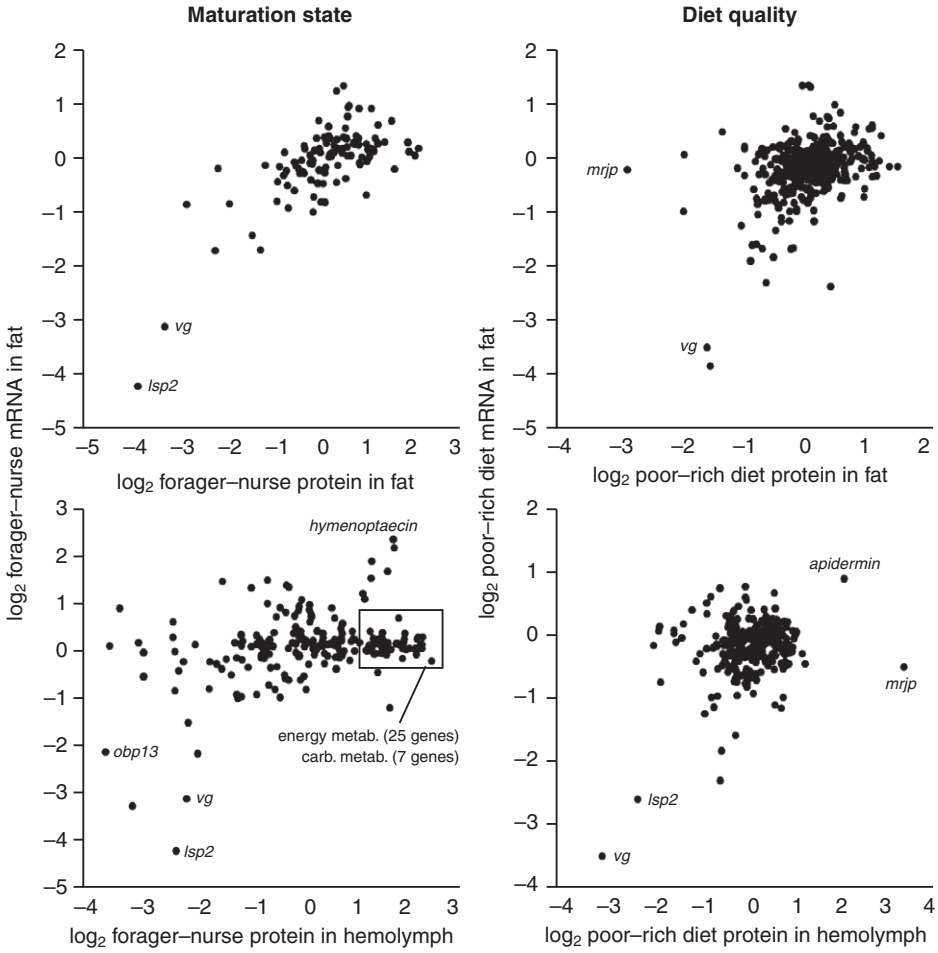

Fig. 2. Correlations of transcriptional responses in fat bodies to maturation and diet with protein differences in the fat bodies and hemolymph. $\log_2$ expression differences are shown for mRNA (microarrays) and protein (liquid chromatography/mass spectrometry, LC/MS) for all genes quantified in both platforms. Labels are shown for selected genes (*lsp2*, *larval serum protein 2*; *vg*, *vitellogenin*; *obp13*, *odorant binding protein 13*) and categories [energy metabolism: gene ontology (GO) biological processes tricarboxylic acid cycle, ATP synthesis, or oxidative phosphorylation]; carbohydrate metabolism (GO: carbohydrate metabolic process).

nutritional expression patterns discovered in our microarray studies. In addition, the similar responses to the two rich diet regimes suggest that the observed maturational differences relate to diet quality in general rather than being a specific response to pollen.

Nurses and foragers responded very differently from pre-nursing bees to the same nutritional manipulations (Fig. 3). Nurse bees were not responsive to rich *vs* poor diet; there was no differential expression of any of the 10 genes. Instead, most genes retained expression levels similar to those of 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 less strongly or not at all to diet, and generally were fixed at low levels similar to those of foragers in the hive. However, the three peptide signaling receptors – *inR1*, *akhR* and *snpfR* – had equal or greater sensitivity to nutrition compared with pre-nursing bees. The differing expression patterns of JH- and neuropeptide-related genes indicate that maturational changes in responsiveness to diet reflect the actions of different signaling pathways, regulated independently.

The results above suggest that dietary changes on their own are insufficient to explain how nurses lose lipid stores prior to the onset of foraging, so naturally occurring stable lipid loss in honey bees would need to involve 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. 4). 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 *snpfR* (r=–0.00 to –0.21). JH, Vg and insulin signaling have coordinated actions in the bee (Corona et al., 2007; Nilsen et al., 2011), and JH has well-known effects on lipid metabolism in other insects (e.g. Zhao and Zera, 2002) and energy metabolism in the bee (Sullivan et al., 2003). Based on these results, we hypothesized that non-dietary processes involving JH, Vg and insulin signaling mediate stable lipid loss.

Effects of *vg* 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 honey bees (Guidugli et al., 2005; Nelson et al., 2007), influences maturation and nutrient storage, we measured fat body gene expression following RNAi knockdown 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 (supplementary material Tables S8 and S9). Of these transcripts, 1030 (48%) were also differentially expressed between nurses and foragers, more than twice as many as expected by chance (Table 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*

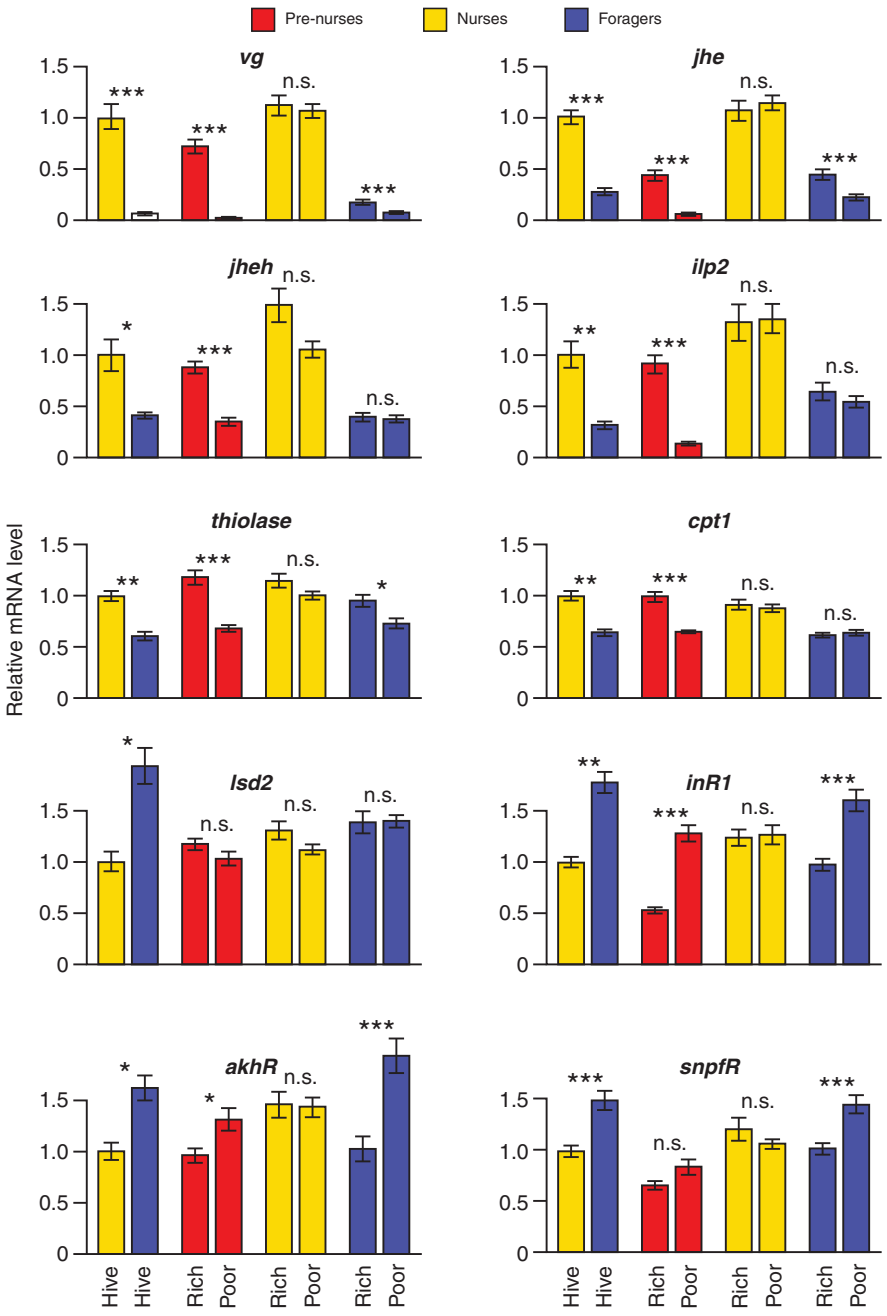

Fig. 3. Age-related changes in responsiveness of metabolic and hormonal signaling pathways in fat bodies to nutritional stimuli. Fat body gene expression for nurses and foragers collected directly from the hive and for pre-nurses, nurses and foragers that were caged and fed either rich or poor diet. 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 1* (*cpt1*), *lipid storage droplet 2* (*lsd2*), *insulin-related receptor 1* (*inR1*), *adipokinetic hormone receptor* (*akhR*) and *small neuropeptide F receptor* (*snpfr*). *N*=19–30 bees/group.

diet 56% concordant; Fisher's exact test, $P<4.5e{-}11$; Table 3), suggesting a tighter fat body transcriptional relationship with maturational state for *vg* than for diet.

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 1). Consistent with its effects causing precocious foraging (Nelson et al., 2007), *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 1; supplementary material Tables S5 and S6). 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 the Vg hemolymph titer acts independent of nutrient availability *per se* as a signal of nutritional status.

vg RNAi also influenced a number of pathways that were not altered during maturation. Many aspects of the translational machinery were upregulated after *vg* knockdown (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 *vg* led to increased protein turnover in the fat bodies.

Effects of QMP on lipid stores, food intake and fat body gene expression

Nutrition and social factors are known to have independent effects on maturation (Toth et al., 2005). We investigated a role for one

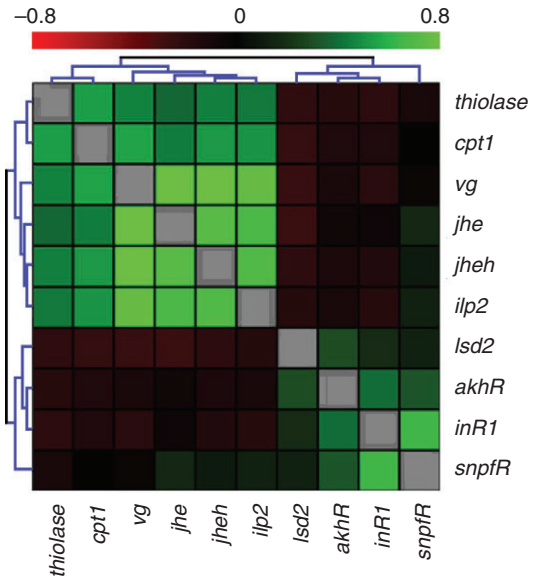

Fig. 4. Co-expression patterns of genes related to metabolic and hormonal signaling pathways. Pearson correlation matrix for co-expression between genes shown in Fig. 3 (qPCR, $N \approx 250$) with average-linkage hierarchical clustering based on Euclidean distance.

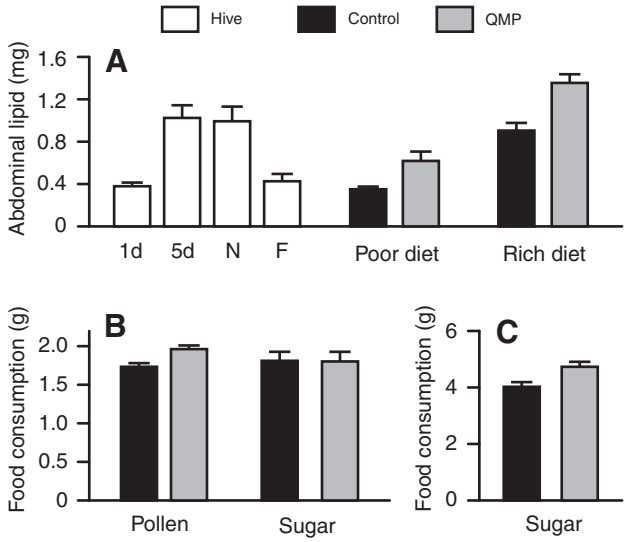

Fig. 5. Effects of queen mandibular pheromone (QMP) on abdominal lipid stores and food consumption. (A) Abdominal lipid stores of hive-reared 1 day old bees (1d), 5 day old bees (5d), nurses (N) and foragers (F), and of cage-reared 5 day old bees fed either rich or poor diet in combination with exposure to QMP or a solvent control. Means + s.e.m. N=30 bees. ANOVA for diet × QMP factorial: P_{diet}=1.0e–8, P_{QMP}=0.006, $P_{diet \times QMP}$=0.20. (B) Effects of QMP on food consumption (total consumption over 4 days for cages containing 35 bees) by bees fed a rich diet of 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 a poor diet of 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. $^*P_{QMP}<0.05$.

such factor, QMP, in stable lipid loss and explored interactions between QMP and dietary factors. We first confirmed (Fischer and Grozinger, 2008) that QMP exposure caused young bees fed a rich diet to have larger lipid stores (Fig. 5A). 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 both fed 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. We were thus able, 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. 5B) and bees fed a poor diet to consume more sugar syrup (Fig. 5C). 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 (supplementary material Fig. S5). Similar genetic variability has been seen previously in laboratory assays using QMP (Pankiw et al., 1994). 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* RNAi, disproportionately influenced genes that were also influenced by maturation (Table 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 1). And although QMP increased pollen consumption, it appeared to cause bees to degrade proteins (supplementary material Tables S10 and S11). 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 these findings reflect a subtle balancing of physiological processes by QMP to promote efficient colony functioning.

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

Our results support the idea that diet, *vg* and QMP all contribute to stable lipid loss during maturation. We performed additional bioinformatic analyses to explore relationships between the responses to diet, *vg* and QMP in order to determine whether they influence nutrient storage *via* shared or independent genes and mechanisms.

The sets of genes regulated by maturation, diet, *vg* and QMP all overlapped significantly more than expected by chance (Table 2). This result suggests that these factors influence shared mechanisms. In addition, as described above, the directional responses to

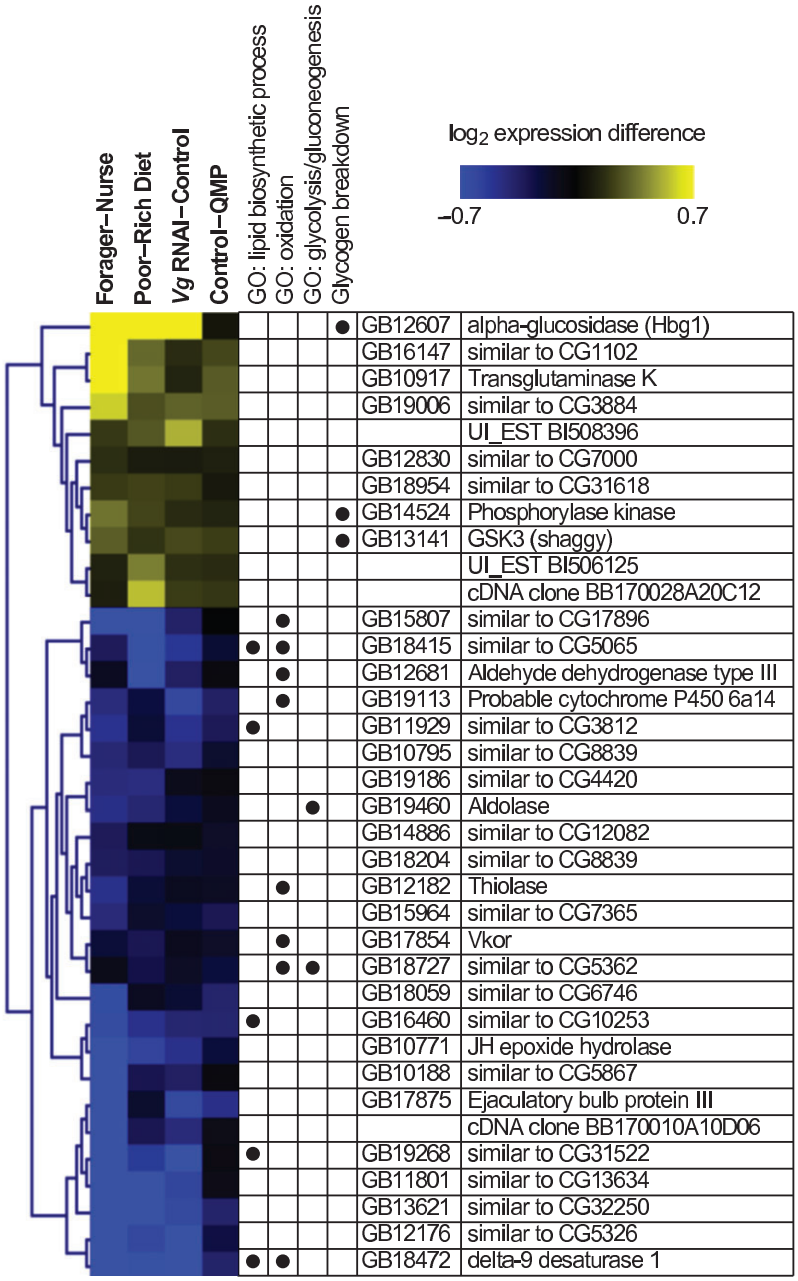

Fig. 6. Genes with concordant responses to maturation, diet, *vg* RNAi and QMP. Genes are shown that responded significantly (FDR<0.2) in all four experiments in concordant directions relative to the effects of each factor on a bee's lipid stores. Heatmap shows the $\log_2$ transformed difference estimate in each experiment. Dots indicate annotation of a gene to the GO biological processes listed or manually annotated to glycogen breakdown. Gene names are listed according to the *A. mellifera* Official Gene Set 2 (Honeybee Genome Sequencing Consortium, 2006) 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.

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) were either non-significant (diet *vs vg*, *vg vs* QMP) or biased in the direction opposite to predictions (diet *vs* QMP). These results may reflect the differing roles of diet, *vg* and QMP outside the context of maturation (Hoover et al., 2003; Seehuus et al., 2006) or differences between physiological, genetic and social manipulations, respectively.

As 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 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. 6) (using FDR<0.2). Some of the genes associated with large lipid stores were related to lipid biosynthetic processes (five genes) and, more generally, oxidation/reduction (eight 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 (Jaycox et al., 1974; Robinson, 1987; Sullivan et al., 2000), as are its antagonistic relationships with Vg (Guidugli et al., 2005; Pinto et al., 2000; Rutz and Luscher, 1974) and QMP (Grozinger and Robinson, 2007; Kaatz et al., 1992). 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. Furthermore, these results suggest molecular mechanisms through which JH might exert these effects on metabolism.

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 (Klowden, 2002). Using mass spectrometry, we identified 47 proteins (out of 212 quantified) that were differentially abundant in hemolymph from nurses and foragers (supplementary material Table S12). 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. 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 (supplementary material Table S13), 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.

Hemolymph from bees fed a poor diet contained an increased abundance of proteins related to energy metabolism, carbohydrate metabolism and immune functions. The former category is somewhat surprising given that transcripts encoding 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 demonstrate concordance in mRNA and protein changes related to maturation and diet, especially those related to metabolism. Similarly Alaux and colleagues (Alaux et al., 2009b) reported similar concordance between changes in brain expression of genes related to metabolism and changes in metabolic enzyme activity in mitochondrial preparations made from brain tissue. Based on the concordance, we infer that many maturation- and diet-related proteins are secreted out of the fat bodies and thus 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 used large-scale, genomic analyses to learn about mechanisms that are associated with stable lipid loss in honey bees. We found that this lipid loss involves massive changes in gene expression in the abdominal fat bodies, including many changes in core metabolic and signaling pathways. By analyzing how these changes relate to those caused by a variety of intrinsic and extrinsic factors, we are able to make the following two conclusions about stable lipid loss in honey bees. First, it involves the regulation of nutritionally related pathways by both dietary and non-dietary factors, most likely including evolutionarily novel, hormonally related signals, i.e. Vg and QMP. Second, there are maturational changes in the responsiveness of nutritionally related signaling and metabolic pathways to the nutritional environment. We had 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, as our results would seem to indicate, Vg is a causal factor in the nutritionally independent regulation of nutritionally related genes, then our original two hypotheses converge. A causal role for Vg in mediating physiological changes during maturation is also supported by a recent report showing interactions between Vg titer, amino acids and the expression of insulin-signaling transcripts (Nilsen et al., 2011). The maturational changes were unexpected, but are consistent with the profound changes in behavior and physiology associated with behavioral maturation in honey bees and other social insects.

Our 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.

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 (Pankiw and Page, 1999). 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 with the in-hive feeding behaviors of nurses. However, it had been a mystery why these differences persist, given that foragers are not obviously food deprived and consume food inside the hive prior to their foraging flights (Winston, 1987). Our results suggest that nurses maintained large nutrient stores despite several days without dietary protein and lipid, while foragers remained lean despite 4 days consuming a rich diet and being prevented from flying. Diet-independent regulation

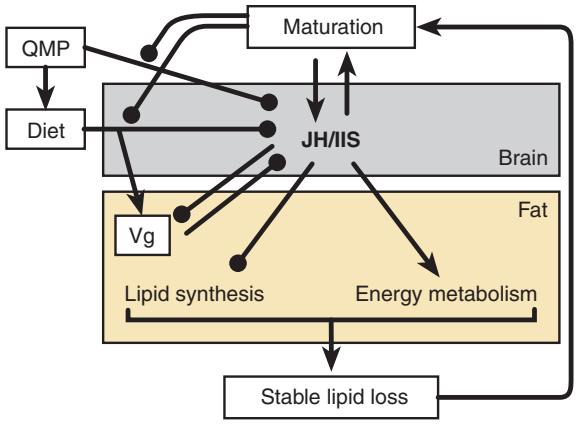

Fig. 7. Verbal model for the regulation of stable lipid loss and its coordination with behavioral maturation. We propose that stable lipid loss occurs through a shift from the utilization of nutrients for lipid biosynthesis to their utilization via energy metabolism pathways. These metabolic pathways are regulated by juvenile hormone (JH) and insulin/insulin-like growth factor (IIS), much as in other species, and by novel regulatory inputs to JH and IIS 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 (Grozinger and Robinson, 2007). In addition, there is a well-established mutually repressive relationship between JH and Vg that is thought to contribute to the timing of maturation (Amdam and Page, 2010; Guidugli et al., 2005; Rutz and Luscher, 1974). Communication between the brain and fat is 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.

of nutritionally related pathways provides a reasonable explanation for how these differences between nurses and foragers are established and maintained; i.e. nurses are buffered from fluctuations in colony nutrition and maintain high levels of lipid metabolism (necessary for brood feeding), whereas in foragers nutrient-sensing pathways are regulated by diet but carbohydrate metabolism is held consistently high (necessary for long flights). That foragers show a unique and specialized metabolic profile is illustrated by comparisons with *D. melanogaster*. The upregulation of carbohydrate metabolism in bees fed a sugar-only diet differs from the genomic responses to food deprivation reported in *Drosophila* (Li et al., 2010; Zinke et al., 2002), during which all major macronutrient metabolism pathways are tuned down to preserve nutrient stores. Humans also respond to food deprivation by conserving energy, one of the mechanisms making it difficult for obese humans to lose weight (Haslam and James, 2005); 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.

We found that there are maturational changes in responsiveness to nutrition, and previous work has shown maturational changes in responsiveness to social signals. For instance, as bees mature, they become decreasingly responsive to QMP (Pankiw et al., 1994). 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 (Le Conte et al., 2001; Pankiw et al., 1998b). 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.

We identified two evolutionarily novel factors – Vg and QMP – that likely contribute to diet-independent regulation of stable lipid loss. The strong, maturationally related, response to *vg* RNAi is consistent with the idea that Vg has taken on signaling-like roles in honey bees (Nelson et al., 2007; Amdam and Page, 2010) and could therefore have causal influences on lipid loss.

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 being the most likely target; 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 honey bees. Similarly, a recent study showed that a second pheromone, brood pheromone, also influences Vg titer (Smedal et al., 2009). 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 (Wilson, 1971).

Our results, together with previous work, allow us to propose a model for how stable lipid loss is achieved in honey bees (Fig. 7). We suggest that relationships between JH, insulin/insulin-like growth factor signaling (IIS) and metabolism are largely conserved between honey 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 (Amdam and Page, 2010; Grozinger and Robinson, 2007) 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 (Amdam and Omholt, 2003), 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 responsiveness to diet and QMP.

Our results suggest that stable lipid loss in honey bees arises from modification of conserved, nutritionally related metabolic and signaling pathways associated with body mass regulation. Studies of diapause in flies and other species (Hahn and Denlinger, 2007) and oogenesis in mosquitoes (Attardo et al., 2005) indicate that these traits, like stable lipid loss in honey bees, also involve novel regulation of conserved, nutritionally related metabolic and signaling pathways.

Might this provide any insights into the potential for stable lipid loss in humans? The situation is promising, because 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 IIS, neuropeptide Y-like signaling

and the TOR system (Rulifson et al., 2002; Wu and Brown, 2006; Wu et al., 2005). 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, AKH (Kim and Rulifson, 2004) [which also has homology with vertebrate gonadotropin-releasing hormone, GnRH (Lindemans et al., 2009)], and similarities between the functions and mode of action for vertebrate thyroid hormone and JH, a structurally related insect hormone (Flatt et al., 2006). The complement of macronutrient and energy metabolism enzymes is virtually unchanged between vertebrates and insects (Kanehisa and Goto, 2000). Anatomical features are less well conserved than the molecular components, but analogous functions are easy to discern and new studies are uncovering surprisingly high conservation between insect fat bodies and liver and adipose tissue (Leopold and Perrimon, 2007). Our studies are consistent with observations that stable lipid loss is unlikely to succeed based simply on nutritional interventions alone, perhaps because, as in honey bees, there are maturation-related factors to contend with. However, because stable lipid loss in honey bees largely results from novel regulation of pathways that also control body mass in humans, a promising approach might be to find new ways to regulate these pathways in humans to facilitate weight loss.

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REFERENCES

Accili, D. and Arden, K. C. (2004). FoxOs at the crossroads of cellular metabolism, differentiation, and transformation. *Cell* **117**, 421-426.

Alaux, C., Le Conte, Y., Adams, H. A., Rodriguez-Zas, S. L., Grozinger, C. M., Sinha, S. and Robinson, G. E. (2009a). Regulation of brain gene expression in honey bees by brood pheromone. *Genes Brain Behav.* **8**, 309-319.

Alaux, C., Sinha, S., Hasadsri, L., Hunt, G. J., Guzmn-Novoa, E., DeGrandi-Hoffman, G., Uribe-Rubio, J. L., Southey, B. R., Rodriguez-Zas, S. L. and Robinson, G. E. (2009b). Honey bee aggression supports a link between gene regulation and behavioral evolution. *Proc. Natl. Acad. Sci. USA* **106**, 15400-15405.

Amdam, G. V. and Omholt, S. W. (2003). The hive bee to forager transition in honeybee colonies: the double repressor hypothesis. *J. Theor. Biol.* **223**, 451-464.

Amdam, G. V. and Page, R. E. (2010). The developmental genetics and physiology of honeybee societies. *Anim. Behav.* **79**, 973-980.

Amdam, G. V., Norberg, K., Hagen, A. and Omholt, S. W. (2003). Social exploitation of vitellogenin. *Proc. Natl. Acad. Sci. USA* **100**, 1799-1802.

Amdam, G. V., Norberg, K., Page, R. E., Erber, J and Scheiner, R. (2006). Downregulation of vitellogenin gene activity increases the gustatory responsiveness of honey bee workers (*Apis mellifera*). *Behav. Brain Res.* **169**, 201-205.

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**, 4226-4231.

Ament, S. A., Wang, Y. and Robinson, G. E. (2010). Nutritional regulation of division of labor in honey bees: toward a systems biology perspective. *Wiley Interdiscip. Rev. Syst. Biol. Med.* **2**, 566-576.

Ament, S. A., Velarde, R. A., Kolodkin, M., Moyse, D. and Robinson, G. E. (2011). Neuropeptide Y-like signaling and nutritionally-mediated gene expression and behavior in the honey bee. *Insect Mol. Biol.* **20**, 335-345.

Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., Davis, A. P., Dolinski, K., Dwight, S. S., Eppig, J. T. et al. (2000). Gene ontology: tool for the unification of biology. *Nat. Genet.* **25**, 25-29.

Attardo, G., Hansen, I. and Raikhel, A. (2005). Nutritional regulation of vitellogenesis in mosquitoes: implications for anautogeny. *Insect Biochem. Mol. Biol.* **35**, 661-675.

Ben-Shahar, Y., Robichon, A., Sokolowski, M. B. and Robinson, G. E. (2002). Influence of gene action across different time scales on behavior. *Science* **296**, 741-744.

Blanchard, G. B., Orledge, G. M., Reynolds, S. E. and Franks, N. R. (2000). Division of labour and seasonality in the ant *Leptothorax albipennis*: worker corpulence and its influence on behaviour. *Anim. Behav.* **59**, 723-738.

Bujold, M., Gopalakrishnan, A., Nally, E. and King-Jones, K. (2010). Nuclear receptor DHR96 acts as a sentinel for low cholesterol concentrations in *Drosophila melanogaster*. *Mol. Cell. Biol.* **30**, 793-805.

Chan, Q. W., Howes, C. G. and Foster, L. J. (2006). Quantitative comparison of caste differences in honeybee hemolymph. *Mol. Cell. Proteomics* **5**, 2252-2262.

Chan, Q. W., Melathopoulos, A., Pernal, S. and Foster, L. J. (2009). The innate immune and systemic response in honey bees to a bacterial pathogen, *Paenibacillus larvae*. *BMC Genomics* **10**, 387-395.

Corona, M., Velarde, R. A., Remolina, S., Moran-Lauter, A., Wang, Y., Hughes, K. A. and Robinson, G. E. (2007). Vitellogenin, juvenile hormone, insulin signaling, and queen honey bee kongevity. *Proc. Natl. Acad. Sci. USA* **104**, 7128-7133.

Crailsheim, K. (1992a). The flow of jelly within a honeybee colony. *J. Comp. Physiol. B* **162**, 681-689.

Crailsheim, K., Schneider, L. H. W., Hrassnigg, N., Buhlmann, G., Brosch, U., Gmeinbauer, R. and Schoffmann, B. (1992b). Pollen consumption and utilization in worker honeybees (*Apis mellifers carnica*) – dependence on individual age and function. *J. Insect Physiol.* **38**, 409-419.

Dennis, G., Sherman, B., Hosack, D., Yang, J., Gao, W., Lane, H. C. and Lempicki, R. (2003). DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol.* **4,** P3.

Emilsson, V., Thorleifsson, G., Zhang, B., Leonardson, A., Zink, F. and Zhu, J. (2008). Genetics of gene expression and its effect on disease. *Nature* **452**, 423-428.

Fischer, P. and Grozinger, C. (2008). Pheromonal regulation of starvation resistance in honey bee workers (*Apis mellifera*). *Naturwissenschaften* **95**, 723-729.

Flatt, T., Moroz, L. L., Tatar, M. and Heyland, A. (2006). Comparing thyroid and insect hormone signaling. *Integr. Comp. Biol.* **46**, 777-794.

Foster, L. J., De Hoog, C. L. and Mann, M. (2003). Unbiased quantitative proteomics of lipid rafts reveals high specificity for signaling factors. *Proc. Natl. Acad. Sci. USA* **100**, 5813-5818.

Grozinger, C. M. and Robinson, G. E. (2007). Endocrine modulation of a pheromone-responsive gene in the honey bee brain. *J. Comp. Physiol. A* **193**, 461-470.

Grozinger, C. M., Sharabash, N. M., Whitfield, C. W. and Robinson, G. E. (2003). Pheromone-mediated gene expression in the honey bee brain. *Proc. Natl. Acad. Sci. USA* **100**, 14519-14525.

Guidugli, K. R., Nascimento, A. M., Amdam, G. V., Barchuk, A. R., Omholt, S., Simoes, Z. L. and Hartfelder, K. (2005). Vitellogenin regulates hormonal dynamics in the worker caste of a eusocial insect. *FEBS Lett.* **579**, 4961-4965.

Hahn, D. A. and Denlinger, D. L. (2007). Meeting the energetic demands of insect diapause: nutrient storage and utilization. *J. Insect Physiol.* **53**, 760-773.

Harrison, J. M. (1986). Caste-specific changes in honeybee flight capacity. *Physiol. Zool.* **59**, 175-187.

Haslam, D. W. and James, W. P. (2005). Obesity. *Lancet* **366**, 1197-1209.

Honeybee Genome Sequencing Consortium (2006). Insights into social insects from the genome of the honeybee *Apis mellifera*. *Nature* **443**, 931-949.

Hoover, S. E., Keeling, C. I., Winston, M. L. and Slessor, K. N. (2003). The effect of queen pheromones on worker honey bee ovary development. *Naturwissenschaften* **90**, 477-480.

Hoover, S. E., Higo, H. A. and Winston, M. L. (2006). Worker honey bee ovary development: seasonal variation and the influence of larval and adult nutrition. *J. Comp. Physiol. B* **176**, 55-63.

Jaycox, E. R., Skowrone, W. and Guynn, G. (1974). Behavioral changes in worker honey bees (*Apis mellifera*) induced by injections of a juvenile hormone mimic. *Ann. Entomol. Soc. Am.* **67**, 529-535.

Kaatz, H. H., Hildebrandt, H. and Engels, W. (1992). Primer effect of queen pheromone on juvenile hormone biosynthesis in adult worker honey bees. *J. Comp. Physiol. B* **162**, 588-592.

Kanehisa, M. and Goto, S. (2000). KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Res.* **28**, 27-30.

Kaun, K. R. and Sokolowski, M. B. (2009). cGMP-dependent protein kinase: linking foraging to energy homeostasis. *Genome* **52**, 1-7.

Kim, S. K. and Rulifson, E. J. (2004). Conserved mechanisms of glucose sensing and regulation by *Drosophila* corpora cardiaca cells. *Nature* **431**, 316-320.

Klowden, M. J. (2002). *Physiological Systems in Insects*. San Diego: Academic Press.

Le Conte, Y., Mohammedi, A. and Robinson, G. E. (2001). Primer effects of a brood pheromone on honeybee behavioural development. *Proc. Biol. Sci.* **268**, 163-168.

Leopold, P. and Perrimon, N. (2007). *Drosophila* and the genetics of the internal milieu. *Nature* **450**, 186-188.

Li, L., Edgar, B. A. and Grewal, S. S. (2010). Nutritional control of gene expression in *Drosophila* larvae via TOR, *Myc* and a novel *cis*-regulatory element. *BMC Cell Biol.* **11**, 7.

Lindemans, M., Liu, F., Janssen, T., Husson, S. J., Mertens, I., Gäde, G. and Schoofs, L. (2009). Adipokinetic hormone signaling through the gonadotropin-releasing hormone receptor modulates egg-laying in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* **106**, 1642-1647.

Morton, G. J., Cummings, D. E., Baskin, D. G., Barsh, G. S. and Schwartz, M. W. (2006). Central nervous system control of food intake and body weight. *Nature* **443**, 289-295.

Nelson, C. M., Ihle, K. E., Fondrk, M. K., Page, R. E. and Amdam, G. V. (2007). The gene vitellogenin has multiple coordinating effects on social organization. *PLoS Biol.* **5**, 673-677.

Nilsen, K. A., Ihle, K. E., Frederick, K., Fondrk, M. K., Smedal, B., Hartfelder, K. and Amdam, G. V. (2011). Insulin-like peptide genes in honey bee fat body respond differently to manipulation of social behavioral physiology. *J. Exp. Biol.* **214**, 1488-1497.

Pankiw, T. and Page, R. E. (1999). The effect of genotype, age, sex, and caste on response thresholds to sucrose and foraging behavior of honey bees (*Apis mellifera* L.). *J. Comp. Physiol. A* **185**, 207-213.

Pankiw, T., Winston, M. L. and Slessor, K. N. (1994). Variation in worker response to honey bee (*Apis mellifera*, L.) queen mandibular pheromone (Hymenoptera, Apidae). *J. Insect Behav.* **7**, 1-15.

Pankiw, T., Huang, Z., Winston, M. L. and Robinson, G. E. (1998a). Queen mandibular gland pheromone influences worker honey bee (*Apis mellifera* L.) foraging ontogeny and juvenile hormone titers. *J. Insect Physiol.* **44**, 685-692.
Pankiw, T., Page, R. E. and Fondrk, M. K. (1998b). Brood pheromone stimulates pollen foraging in honey bees (*Apis mellifera*). *Behav. Ecol. Sociobiol.* **44**, 193-198.
Pinto, L. Z., Bitondi, M. M. G. and Simoes, Z. L. P. (2000). Inhibition of vitellogenin synthesis in *Apis mellifera* workers by a juvenile hormone analogue, pyriproxyfen. *J. Insect Physiol.* **46**, 153-160.
Robinson, G. E. (1987). Regulation of honey bee age polyethism by juvenile hormone. *Behav. Ecol. Sociobiol.* **20**, 329-338.
Root, C. M., Ko, K. I., Jafari, A. and Wang, J. W. (2011). Presynaptic facilitation by neuropeptide signaling mediates odor-driven food search. *Cell* **145**, 133-144.
Rousseau, K., Atcha, Z. and Loudon, A. S. I. (2003). Leptin and seasonal mammals. *J. Neuroendocrinol.* **15**, 409-414.
Rulifson, E. J., Kim, S. K. and Nusse, R. (2002). Ablation of insulin-producing neurons in flies: growth and diabetic phenotypes. *Science* **296**, 1118-1120.
Rutz, W. and Luscher, M. (1974). The occurrence of vitellogenin in workers and queens of *Apis mellifica* and the possibility of its transmission to the queen. *J. Insect Physiol.* **20**, 897-909.
Schulz, D. J., Huang, Z. Y. and Robinson, G. E. (1998). Effects of colony food shortage on behavioral development in honey bees. *Behav. Ecol. Sociobiol.* **42**, 295-303.
Seehuus, S., Norberg, K., Gimsa, U., Krekling, T. and Amdam, G. (2006). Reproductive protein protects functionally sterile honey bee workers from oxidative stress. *Proc. Natl. Acad. Sci. USA* **103**, 962-967.
Seeley, T. D. (1982). Adaptive significance of the age polyethism schedule in honeybee colonies. *Behav. Ecol. Sociobiol.* **11**, 287-293.
Sinha, S., Ling, X., Whitfield, C. W., Zhai, C. and Robinson, G. E. (2006). Genome scan for *cis*-regulatory DNA motifs associated with social behavior in honey bees. *Proc. Natl. Acad. Sci. USA* **103**, 16352-16357.
Smedal, B., Brynem, M., Kreibich, C. and Amdam, G. (2009). Brood pheromone suppresses physiology of extreme longevity in honeybees (*Apis mellifera*). *J. Exp. Biol.* **212**, 3795-3801.
Snodgrass, R. E. (1956). *Anatomy of the Honey Bee*. Ithaca, NY: Comstock.
Sullivan, J. P., Fahrbach, S. E. and Robinson, G. E. (2000). Juvenile hormone paces behavioral development in the adult worker honey bee. *Horm. Behav.* **37**, 1-14.
Sullivan, J. P., Fahrbach, S. E., Harrison, J. F., Capaldi, E. A., Fewell, J. H. and Robinson, G. E. (2003). Juvenile hormone and division of labor in honey bee colonies: effects of allatectomy on flight behavior and metabolism. *J. Exp. Biol.* **206**, 2287-2296.
Toth, A. L. and Robinson, G. E. (2005). Worker nutrition and division of labour in honeybees. *Anim. Behav.* **69**, 427-435.
Toth, A. L., Kantarovich, S., Meisel, A. F. and Robinson, G. E. (2005). Nutritional status influences socially regulated foraging ontogeny in honey bees. *J. Exp. Biol.* **208**, 4641-4649.
Wilson, E. O. (1971). *The Insect Societies*. Cambridge, MA: Harvard University Press.
Winston, M. L. (1987). *The Biology of the Honey Bee*. Cambridge, MA: Harvard University Press.
Wu, Q. and Brown, M. R. (2006). Signaling and function of insulin-like peptides in insects. *Annu. Rev. Entomol.* **51**, 1-24.
Wu, Q., Zhao, Z. and Shen, P. (2005). Regulation of aversion to noxious food by *Drosophila* neuropeptide Y- and insulin-like systems. *Nat. Neurosci.* **8**, 1350-1355.
Zhao, Z. W. and Zera, A. J. (2002). Differential lipid biosynthesis underlies a tradeoff between reproduction and flight capability in a wing-polymorphic cricket. *Proc. Natl. Acad. Sci. USA* **99**, 16829-16834.
Zinke, I., Schutz, C. S., Katzenberger, J. D., Bauer, M. and Pankratz, M. J. (2002). Nutrient control of gene expression in *Drosophila*: microarray analysis of starvation and sugar-dependent response. *EMBO J.* **21**, 6162-6173.