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## RESEARCH ARTICLE

# Insulin-like peptide genes in honey bee fat body respond differently to manipulation of social behavioral physiology

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## SUMMARY

**Nutrient sensitive insulin-like peptides (ILPs) have profound effects on invertebrate metabolism, nutrient storage, fertility and aging. Many insects transcribe ILPs in specialized neurosecretory cells at changing levels correlated with life history. However, the major site of insect metabolism and nutrient storage is not the brain, but rather the fat body, where functions of ILP expression are rarely studied and poorly understood. Fat body is analogous to mammalian liver and adipose tissue, with nutrient stores that often correlate with behavior. We used the honey bee (*Apis mellifera*), an insect with complex behavior, to test whether ILP genes in fat body respond to experimentally induced changes of behavioral physiology. Honey bee fat body influences endocrine state and behavior by secreting the yolk protein precursor vitellogenin (Vg), which suppresses lipophilic juvenile hormone and social foraging behavior. In a two-factorial experiment, we used RNA interference (RNAi)-mediated *vg* gene knockdown and amino acid nutrient enrichment of hemolymph (blood) to perturb this regulatory module. We document factor-specific changes in fat body *ilp1* and *ilp2* mRNA, the bee's ILP-encoding genes, and confirm that our protocol affects social behavior. We show that *ilp1* and *ilp2* are regulated independently and differently and diverge in their specific expression-localization between fat body oenocyte and trophocyte cells. Insect *ilp* functions may be better understood by broadening research to account for expression in fat body and not only brain.**

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/214/9/1488/DC1

Key words: peripheral insulin-like signaling, vitellogenin, division of labor, nutrition, RNA-interference.

## INTRODUCTION

The insulin-like peptides (ILPs) of invertebrates are central life-history regulators, functionally homologous to insulin and insulin-like growth factor 1 (IGF1) ligands in mammals (for a review, see Flatt et al., 2005). In contrast to the mammalian insulin, produced by beta-pancreatic cells, many insects produce ILPs in neurons. Much research has centered on this pattern (reviewed by Broughton and Partridge, 2009), but ILPs are also expressed in peripheral insect tissues, e.g. in *Drosophila melanogaster*, *Bombyx mori* and *Locusta migratoria* (Badisco et al., 2008). This finding suggests that ILPs produced by non-neural insect cells may act as paracrine and/or endocrine signal substances, resembling IGFs and many other growth factors known from vertebrates.

Recently, peripheral *ilp* expression in the migratory locust *Schistocerca gregaria* was shown to be associated with complex behaviors (Badisco et al., 2008). In fat body, an organ analogous to vertebrate liver and adipose tissues, transcript levels of the locust ILP, Scg-IRP, were found to diverge between solitary and gregarious locust phenotypes that differ in reproductive strategy and behavior. In the honey bee, correlative relationships between brain *ilp* mRNA levels and complex behavior have been studied, but it is unknown how or whether peripheral expression of *ilp* genes influences insect behavior (Corona et al., 2007; Ament et al., 2008).

Honey bees are advanced social insects that show a reproductive division of labor, where most eggs are laid by queens while essentially sterile female workers perform the remaining behavioral tasks required for colony maintenance. Honey bee workers conduct within-nest tasks as young adults and later forage in the field (Seeley, 1982). This task partitioning through division of labor between the workers is a hallmark of complex sociality, and correlates with differences in metabolic physiology and aging between individuals with different behavior (Elekonich and Roberts, 2005; Toth and Robinson, 2005; Wolschin and Amdam, 2007).

Between their nest and forager life stages, workers differ in many physiological traits, including levels of gene and protein expression, endocrine activity, innate immunity, metabolism, stress resistance, and stored proteins and lipids (for reviews see Amdam et al., 2009; Ament et al., 2010). While conducting tasks in the nest, workers are discouraged from foraging by vitellogenin (Vg; accession no. AJ517411), a phosphoglycolipo-storage protein synthesized by fat body. Vg production requires sufficient nutrient availability, and circulating titers are closely tied to protein consumption (Bitondi and Simoes, 1996). Protein consumption in workers is at its highest during nursing behavior, typically between 5 and 8 days of age (Haydak, 1970), around the time that Vg titers typically reach peak levels (Fluri et al., 1982).

As foragers, honey bee workers have depleted fat and nutrient reserves (Toth and Robinson, 2005). Low individual nutrient availability may also induce foraging behavior in the pre-foraging nest bees by reducing Vg (Amdam and Omholt, 2003). At high levels, Vg confers several of the traits characteristic of nest bees: immunity, stress resistance and low systemic juvenile hormone (JH) titers (Amdam et al., 2005; Seehuus et al., 2006). JH is a stress-sensitive central endocrine factor and metabolic regulator that typically becomes elevated during the workers' forager life stage (Robinson et al., 1992). Once elevated, JH can feed back to reduce Vg further (Pinto et al., 2000).

The relationships between nutrition, Vg, JH and behavior in worker bees have been connected to insulin/insulin-like signaling (IIS) that involves ILP signal transduction (Corona et al., 2007), as well as to the intersecting target of rapamycin (TOR, a nutrient sensing kinase) pathway that can be upstream of Vg (Patel et al., 2007). IIS may include two *ilp* genes in honey bees, *ilp1* (accession no. GB17332-PA) and *ilp2* (accession no. GB10174-PA), which are expressed in worker neural and peripheral tissues (Ament et al., 2008; Corona et al., 2007). Not much is currently known about these genes, except that *ilp1* may increase with JH and be transcribed at higher levels in brains of nutritionally depleted bees and foragers (Ament et al., 2008). However, perhaps because of seasonal or social factors, neural *ilp1* levels can drop while foraging continues, making the connection to behavior ambiguous (Corona et al., 2007; Ament et al., 2008).

Here, we conduct the first study of *ilp1* and *ilp2* mRNA expression patterns in honey bee peripheral fat body using a two-factorial design, combining a reduction of *vg* expression by means of RNA interference (RNAi)-mediated gene knockdown and amino acid enrichment of hemolymph to perturb worker behavioral physiology. RNAi is an established method to reduce Vg levels over 20 days or longer, to increase JH and to release precocious foraging behavior in worker bees (Guidugli et al., 2005; Nelson et al., 2007). Amino acid enrichment of hemolymph, reciprocally, was expected to encourage Vg synthesis (Bitondi and Simoes, 1996) and, thereby, confer low JH levels and delayed foraging onset (Amdam and Omholt, 2003). We anticipated that the two-factorial combination of these treatments would allow an in-depth study of *ilp1* and *ilp2* responses while relationships between nutrient availability, Vg and JH were perturbed or partly decoupled.

We hypothesized that *ilp* genes expressed in honey bee fat body are associated with physiology that influences behavior, and predicted that *ilp1* and *ilp2* expression, therefore, could respond to our factorial treatments to reveal factor-specific connections between ILPs, nutrient availability, Vg and JH. We verified behavioral outcomes by observing the onset of foraging behavior for experimental bees. Our results indicate that *ilp1* and *ilp2* peptide products take part in separate regulatory processes in the fat body and differ in their roles in honey bee behavioral physiology. This possibility suggests that a broadening of research to account for *ilp* expression in the insect fat body, and not just in the brain, can lead to new progress in the understanding of ILP functions.

## MATERIALS AND METHODS

### Two-factorial perturbation experiment

#### RNAi-mediated *vg* gene knockdown

Newly emerged (<24 h old) honey bees (*Apis mellifera* L. 1758) from two wild-type colonies were mixed together and randomly assigned to one of two treatments: control injection with green fluorescent protein (GFP)-derived double-stranded RNA (dsRNA) (factorial notation: $vg^+$, $N$=20 for each of three cages), and injection of dsRNA against the *vg* gene (factorial notation: $vg^-$, $N$=20 for each of three cages) (Nelson et al., 2007). Injection of dsRNA against Vg triggers RNAi, resulting in a fat-body-specific reduction in Vg levels (Amdam et al., 2003; Guidugli et al., 2005). Bees were marked by treatment with a dot of paint on the thorax (Testors Enamel; Testors Corporation, Rockford, IL, USA) and caged in two-compartment cages: the experimental bees on one side of a single wire-mesh screen, and 200 wild-type bees brushed from a comb of open brood cell containing larvae on the other. Bees found over open brood are likely to be performing nursing tasks. This setup allowed semi-social interactions and nourishment of the experimental bees by the presumed nurses (Amdam et al., 2007). Both compartments received 30% sucrose solution and fresh pollen dough (Crockett Honey, Tempe, AZ, USA). Three replicate cages were incubated at 33°C and 70% relative humidity.

#### Amino acid enrichment of hemolymph

After 2 days, the experimental bees were collected. Both $vg^+$ and $vg^-$ bees were divided into two groups before receiving new treatments: amino acid supplementation through injection with 2 µl of bovine serum albumin (BSA; Sigma-Aldrich, St Louis, MO, USA), an amino acid donor, dissolved in Grace insect medium (100 µg µl$^{-1}$, Invitrogen, Carlsbad, CA, USA) (factorial notation: $aa^+$), or a control injection with 2 µl of the same batch of BSA solution where BSA was removed after incubation at 60°C for 1 h (factorial notation: $aa^-$) and pelleting at 14,000 ***g*** for 5 min. In insects, BSA causes release (enrichment) of amino acids to hemolymph over many days (Pan and Telfer, 1992) without a triggering of the innate immune response compared with appropriate control (Dettloff et al., 2001). Amino acids are transcriptional enhancers of *vg*, and BSA also increases Vg concentrations in honey bee fat body cultured *in vitro* (S. C. Seehuus and G.V.A., unpublished data).

#### Factorial treatment combinations

The second set of injections completed the two-factorial design, with all combinations of normal *vs* RNAi-mediated repression of Vg in fat body, and normal *vs* BSA-mediated enrichment of amino acid levels in hemolymph. These treatment groups – $vg^-aa^-$, $vg^+aa^+$, $vg^-aa^+$ and $vg^+aa^-$ – were returned to the cages for 5 days. The bees were subsequently processed as 7-day-olds (see below).

### Quantification of residual BSA in hemolymph

In the 7-day-old bees, the quantity of residual BSA was determined by standard gel separation (9% SDS-PAGE) using a standard of 1, 2 and 4 µg BSA. Gels were stained with Coomassie Brilliant Blue before quantification using a Gel-Doc imaging system and Quantity One software (Bio-Rad, Hercules, CA, USA) as described previously (Nelson et al., 2007). All data points represent individual bees.

### JH assays

#### Juvenile hormone binding assay

An *in vitro* assay (Goodman et al., 1978) (W. Goodman, personal communication) with tritiated JH and native PAGE was used to test for putative binding of JH to the injected amino acid donor BSA. Tritiated JH [10-$^3$H(N)]-JH III with a specific activity of 11.8 Ci mmol l$^{-1}$ (PerkinElmer, Waltham, MA, USA) was diluted in Grace insect medium (Invitrogen) to 27.4 Bq µl$^{-1}$. Twenty microliters were added to 250 µl BSA solution (0.4 µg µl$^{-1}$) and incubated for 4 h at 4°C. As a positive control, the same amount of tritiated JH was incubated with 250 µl hemolymph from *Manduca sexta* fourth instar larvae, which contains a 34–36 kDa JH binding protein, JHBP (Goodman et al., 1978; Park et al., 1993; Orth et al., 2003). After

incubation, samples of BSA and *M. sexta* hemolymph were separated in duplicate on 10% native gels at 180 V. Thereafter, gels were cut vertically. Paired lanes for BSA *vs* hemolymph, as well as background/control (lanes run with JH incubated in medium), were sliced in 10 horizontal pieces (gel fractions). Each fraction was added to a scintillation vial and dissolved in 2 ml of Soluene®-350 (Perkin Elmer) at 50°C overnight, before adding 10 ml Hionic-Fluor Scintillation liquid (Perkin Elmer). Samples were counted using a Packard scintillation counter.

### Quantification of JH titer in hemolymph

JH was extracted using an established protocol for honey bee hemolymph (Huang et al., 1994). The antiserum (Goodman et al., 1990) and the protocol were validated as described previously (Guidugli et al., 2005). Briefly, hemolymph samples from individual worker bees were collected into acetonitrile, and 0.9% NaCl and hexane were added to the sample and separated by centrifugation. The hexane phase containing JH was removed, and the extraction was repeated twice. Hexane phases for each sample were pooled and dried and the hormone residue was resuspended in toluene. Antiserum was diluted in phosphate buffered saline (PBS) containing BSA and rabbit immunoglobulin G. The assay used tritiated JH, [10$^{-3}$H(N)]-JH III (NEN Life Science Products, Waltham, MA, USA) and JH III (Fluka, Milwaukee, WI, USA) as non-radioactive ligand. JH titers were calculated by log linear regression analysis against standard curves.

### Quantification of gene expression in fat body

After hemolymph was collected for JH titer measurements, RNA was isolated from the abdominal tissue of individual workers using TRIzol phenol-chloroform extraction combined with the RNeasy kit (Qiagen, Valencia, CA, USA) as described previously (Amdam et al., 2004; Tsuruda et al., 2008). Individual transcript levels of *vg*, *ilp1*, *ilp2* and *TOR* (accession no. XM 625127) were quantified in a random subset of samples relative to β-*actin* (accession no. AB023025) expression using real-time reverse transcription (RT)-PCR as described previously (e.g. Amdam et al., 2004). Primers are listed in supplementary material Table S1. β-*actin* is stably expressed in different honey bee tissues and has been demonstrated to be an effective control gene when measuring gene expression in adult honey bee fat body (Lourenco et al., 2008; Scharlaken et al., 2008). For these reasons, β-*actin* is a commonly used reference in studies of honey bee gene expression (Chen et al., 2005; Wang et al., 2010). Control reactions without reverse transcriptase were preformed for each sample to ensure that purified RNA was not contaminated with genomic DNA.

### Transcript localization by whole-mount fat body *in situ* hybridization

*In situ* hybridization was performed on a set of untreated worker bees not used in other assays. Sense and antisense probes for hybridization were prepared from PCR products using primers specific to *ilp1* and *ilp2* with T7 promoters attached (supplementary material Table S1). Probes were synthesized and labeled with digoxigenin (DIG) using the Roche RNA labeling mix (Roche Applied Science, Indianapolis, IN, USA). Probes were between 200 and 300 bases long.

*In situ* hybridization was performed according to the modified protocol of Osborne and Dearden (Osborne and Dearden, 2005), optimized for honey bee fat body by Wang et al. (Wang et al., 2010). Tissue was fixed overnight at 4°C in 4% paraformaldehyde (4% formaldehyde, 20 mmol l$^{-1}$ $KH_2PO_4$, 90 mmol l$^{-1}$ KCl, 30 mmol l$^{-1}$ NaCl, 4 mmol l$^{-1}$ $MgCl_2$). After fixation, the tissue was washed in PBS before dehydration in a methanol series. Samples were then stored in methanol at –20°C until use.

A methanol series was also used to rehydrate the samples before proteinase K treatment. The tissue was digested using 20 μg ml$^{-1}$ proteinase K for 3 min. Digestion was stopped by adding 2 mg ml$^{-1}$ glycine in 0.1% Tween-20 in PBS (PTw). Samples were then rinsed in PTw and post-fixed in 4% paraformaldehyde for 20 min while shaking at room temperature. After post-fixation, the tissue was washed in PTw and transferred to a 1:1 mixture of PTw and hybridization buffer (50% deionized formamide, 5× sodium chloride–sodium phosphate–EDTA buffer, 1 mg ml$^{-1}$ yeast tRNA, 100 μg ml$^{-1}$ salmon sperm DNA, 100 μg ml$^{-1}$ heparin, 1% 100× Denhardt's Solution, 0.1% Tween-20, 5 mmol l$^{-1}$ EDTA). After 10 min incubation in this mixture, samples were transferred into pre-warmed hybridization buffer and prehybridized for 2 h at 60°C.

Hybridization was carried out overnight at 60°C in hybridization buffer with 3 ng μl$^{-1}$ DIG-labeled riboprobe. Following hybridization, unbound probe was removed in a series of washes, and tissue was blocked in PTw with 0.1% sheep serum albumin. Samples were then incubated overnight at 4°C with a 1:2000 dilution of antigen binding fragments (Roche) conjugated with sheep DIG alkaline phosphatase. The tissue was equilibrated with alkaline phosphatase buffer and stained using BM Purple (Roche). For *ilp1*, tissues were stained overnight at 4°C. For *ilp2*, tissues were stained at room temperature for 4 h. Reactions were stopped by PBS washes.

Samples were visualized on an upright microscope (Axio Imager A1, Carl Zeiss Microimaging, Munich, Germany) at 200× magnification and processed with Axiocam MRc5 software (Carl Zeiss Microimaging).

### Anti-Vg immunofluorescence microscopy of fat body tissue

LR-White (Electron Microscopy Sciences, Hartfield, PA, USA)-embedded fat body tissue was sectioned at 1 μm thickness (Reichert Jung ultra-microtome). Immunostaining with primary antibody against Vg (Seehuus et al., 2007) was modified for confocal microscopy (Smedal et al., 2009). The specificity of this antibody was established previously toward preimmune sera (Seehuus et al., 2007), and therefore primary antibody was omitted in the negative control. For detection, a 1:200 dilution of polyclonal Cy3 AffiniPure Goat Anti-Rabbit IgG (Jackson Immuno Research Europe Inc.) was used on all sections for 24 h at 4°C. Images were collected at 40×, zoom 2.8 and visualized on a confocal laser scanning microscope (Leica TCS SP5).

### Behavioral validation in free flight

Newly emerged bees from two wild-type sources were marked, mixed and introduced into two four-frame colonies. Ten days later, marked bees were recaptured and divided into three treatment groups: aa$^+$ ($N$=548) and aa$^-$ ($N$=609) as described above, and a non-injected reference group ($N$=445). This group was used in planned comparisons with the aa$^-$ treatment to monitor handling stress that triggers precocious foraging (Nelson et al., 2007). Although a two-factorial design could not be used because of processing constraints, previous work using the same procedure experimental setup established the behavior of $vg^+$ and $vg^-$ bees relative to a non-injected reference group (Nelson et al., 2007; Ihle et al., 2010).

After treatment, bees were individually tagged and reintroduced to their host colonies. The following day, all bees and frames within each host were transferred into a separate glass-walled observation hive. Foraging onset was recorded daily as described previously in studies using $vg^+$ and $vg^-$ bees (Nelson et al., 2007; Amdam et al.,

2007). We divided the data collected into two equal time windows for analysis: (1) the initial 12 days until bees were 22 days old, when injected BSA is likely present in hemolymph as a supplemental amino acid source (Pan and Telfer, 1992); and (2) the subsequent 12 days when BSA titers are reduced or absent and unlikely to affect behavior (until bees were 34 days old).

### Statistics

Data from the two-factorial laboratory experiment were analyzed using factorial ANOVA and Pearson product-moment correlation. Gene expression data were log-transformed to approximate normality and analyzed using factorial ANOVA (Ament et al., 2008). Foraging onset was analyzed by the non-parametric Kaplan–Meier survival test. For *post hoc* comparison, Cox's *F*-test was used as described previously (Amdam et al., 2007; Ihle et al., 2010). All analyses used Statistica 6.0 (Statistica, StatSoft Inc., Tulsa, OK, USA).

## RESULTS

### Quantitative validation of the two-factorial experimental design

Semiquantitative analysis of hemolymph by SDS-PAGE confirmed that $aa^+$ workers contained significantly higher levels of circulating BSA compared with $aa^-$ controls (one-way ANOVA, $F_{1,60}$=220.79, $P \ll 0.0001$; Fig. 1). We established that cage (replicate 1–3) did not explain variation in JH, residual albumin, *vg*, *ilp1*, *ilp2* or *TOR* mRNA levels (multivariate ANOVA, $F_{10,42}$=2.00, $P$=0.06). We also verified that the two-factorial design ($vg^-aa^-$, $vg^+aa^+$, $vg^-aa^+$ and $vg^+aa^-$) did not interfere with or interact to affect expression of *TOR* (factorial ANOVA, *vg*, $F_{1,29}$=0.15, $P$=0.70; aa, $F_{1,29}$=0.08, $P$=0.77; *vg*–aa interaction, $F_{1,29}$=0.02, $P$=0.88), supporting a specific response of the IIS pathway through ILP regulation. We also verified that BSA did not inhibit JH signaling by JH binding (supplementary material Fig. S1).

We confirmed knockdown of *vg* gene expression (factorial notation: $vg^-$) relative to an established control ($vg^+$) (factorial ANOVA, $F_{1,56}$=4.52, $P$<0.05; Fig. 2G) as described previously (Amdam et al., 2007). Reciprocally, we established that increased *vg* expression (factorial ANOVA, $F_{1,56}$=7.61, $P$<0.01; Fig. 2H) resulted from the inter-abdominal injection of BSA (factorial notation: $aa^+$).

In summary, the experimental design was not confounded by failure to introduce BSA in hemolymph circulation, cage environmental variation, unintended effects on the intersecting pathway of *TOR*, or the disruption of JH by BSA binding. Moreover, we could validate that our bees showed the intended physiological response to the two-factorial treatments: RNAi suppressed *vg* gene activity whereas BSA injection encouraged it.

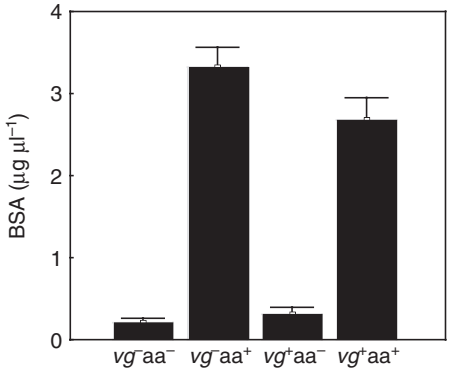


Fig. 1. Hemolymph albumin concentration. Honey bees injected with bovine serum albumin (BSA) ($vg^+aa^+$, $N$=15; $vg^-aa^+$, $N$=14) had significantly higher hemolymph albumin concentrations than unsupplemented bees ($vg^+aa^-$, $N$=14, $vg^-aa^-$, $N$=19). Data are means ± s.e.m. (one-way ANOVA, $P \ll 0.0001$).

### Changes in hemolymph JH titer

As documented previously (Amdam et al., 2007; Guidugli et al., 2005), *vg* knockdown significantly increased the JH titer of workers (factorial ANOVA, *vg*, $F_{1,62}$=5.06, $P$<0.05; Fig. 2A). JH, however, was not affected by the amino acid enrichment protocol that increased *vg* expression (factorial ANOVA, aa, $F_{1,62}$=0.09, $P$=0.76; Fig. 2B), nor did we detect a significant interaction between the treatments (*vg*–aa, $F_{1,62}$<0.01, $P$=0.98). These results are consistent with previous reports, which indicate that *vg* must be suppressed below a threshold level before an endocrine response in JH is detected (reviewed by Amdam et al., 2009).

### Quantification and localization of fat body *ilp1* and *ilp2* mRNA

The mRNA level of *ilp1* was not influenced by *vg* suppression (factorial ANOVA, *vg*, $F_{1,38}$=0.03, $P$=0.87; Fig. 2C), but it increased strongly (up to 90-fold) in response to BSA injection (factorial ANOVA, *vg*, $F_{1,39}$=9.86, $P$<0.005; Fig. 2D). *ilp2* expression remained unaffected by both *vg* knockdown (factorial ANOVA, *vg*, $F_{1,39}$=0.08, $P$=0.78; Fig. 2E) and amino acid supplementation (factorial ANOVA, aa, $F_{1,39}$=0.66, $P$=0.42; *vg*–aa, $F_{1,39}$=0.35, $P$=0.85; Fig. 2F). An interaction between treatments was suggested but not significant for *ilp1* (factorial ANOVA, *vg*–aa, $F_{1,38}$=3.36, $P$=0.07), indicating that the *ilp1* response to circulating amino acids could be affected by the Vg status of a worker bee. These very different transcriptional responses of *ilp1* and *ilp2* to the factorial treatments suggested that the encoded peptides take part in largely separate paracrine and/or endocrine signaling pathways in fat body.

We confirmed transcription of *ilp1* and *ilp2* in fat body cells by *in situ* hybridization. *ilp1* mRNA was detected in fat body oenocytes (Fig. 3A,B) whereas *ilp2* transcript occurred in both oenocyte and trophocyte cells (Fig. 3C,D). Oenocytes store lipids and may be a site of lipid and lipoprotein synthesis (Paes-de-Oliveira and Cruz-Landim, 2003). Trophocytes, the second major cell type in fat body, are storage sites for lipids, proteins and carbohydrates (Paes-de-Oliveira et al., 2008). These are among the most metabolically active cells in worker bees and are the primary location of Vg synthesis and protein production overall (Landim, 1985). These results show that *ilp1* and *ilp2* can be differentially expressed at a spatial scale that is functionally relevant in fat body, corroborating the proposition that the corresponding peptides may take part in largely separate processes.

### Interactions between *ilp1* and *vg* levels in workers

In contrast to *ilp2*, *ilp1* transcripts responded strongly to the amino acid enrichment protocol that increased *vg* gene expression (see above). We found that *ilp1* and *vg* mRNA levels were tightly linked in the data set overall ($N$=41, $r$=0.54, $P$<0.005), and that the gene transcripts remained correlated in three of four factorial treatment groups independent of the concurrent changes in JH and *ilp2* ($vg^+aa^-$, $N$=10, $r$=0.77, $P$<0.01; $vg^+aa^+$, $N$=13, $r$=0.23, $P$=0.45; $vg^-aa^-$, $N$=11, $r$=0.59, $P$=0.05; $vg^-aa^+$, $N$=7, $r$=0.95, $P$<0.0005; Fig. 4A–D). These correlative relationships helped clarify the suggestive *vg*–aa interaction ($P$=0.07) in our initial analysis of *ilp1*: significant scaling of *ilp1* to *vg* expression is disrupted by amino acid supplementation in the absence of *vg* knockdown (Fig. 4B), indicating that transcriptional enhancement of *ilp1* and *vg* can occur independently. Moreover, the correlation between *ilp1* and *vg* in *vg* knockdowns implies that *ilp1* is reduced in some workers with low

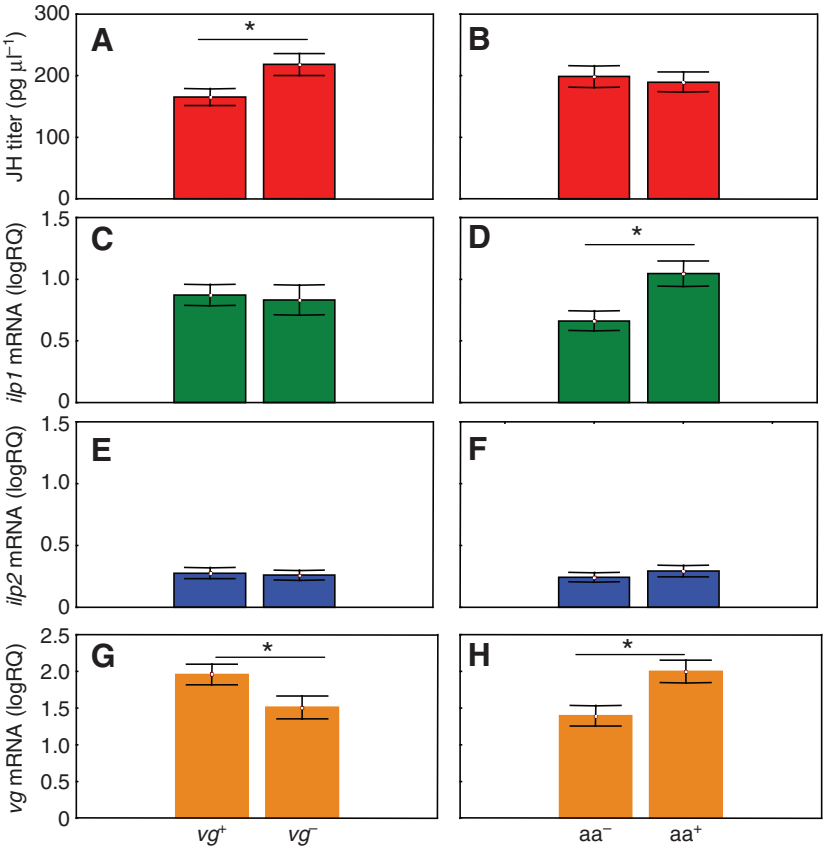


Fig. 2. The main effects of the factorial experiment on honey bee juvenile hormone (JH), *ilp1* and *ilp2*. (A) Relative to control ($vg^+$, $N$=30), *vg* RNAi ($vg^-$, $N$=36) caused an increase in JH, whereas (B) hemolymph amino acid supplementation did not affect the hormone titer [control=$aa^-$ ($N$=34) *vs* supplemented with BSA=$aa^+$ ($N$=32)]. (C) *ilp1* was unaffected by *vg* knockdown ($vg^+$, $N$=23; $vg^-$, $N$=19) but (D) responded significantly to amino acid supplementation ($aa^-$, $N$=21; $aa^+$, $N$=21), whereas (E,F) *ilp2* did not show main effects of the treatments ($vg^+$, $N$=15; $vg^-$, $N$=28; $aa^-$, $N$=23; $aa^+$, $N$=20) and (G) *vg* RNAi significantly reduced vitellogenin (Vg) expression in treated bees [control=$vg^+$ ($N$=26) knockdown=$vg^-$ ($N$=34)]. (H) Amino acid supplementation resulted in significantly higher Vg expression ($aa^-$, $N$=29; $aa^+$, $N$=31). Data are means ± s.e.m. Asterisks denote significant differences (factorial ANOVA, $P$<0.05). Gene expression levels are given as log-transformed quantities relative to β-*actin* expression (logRQ).

transcript levels of *vg*. However, our analysis did not detect a direct effect of *vg* knockdown on *ilp1* (Fig. 2C).

**Interactions between JH, *ilp2* and *vg* levels in worker bees**

As Vg repression causes JH to increase whereas elevated JH has been connected to reduced *ilp2* transcript levels in neural tissue (Ament et al., 2008), we anticipated that *ilp2* could decrease in the *vg* knockdown groups (Guidugli et al., 2005; Amdam et al., 2007). However, our analysis did not detect a response in *ilp2* transcript to *vg* knockdown (Fig. 2E). We therefore studied the correlative relationships between JH and *ilp2* in detail. In agreement with previous findings, *ilp2* transcript levels correlated with JH in the *vg* RNAi controls ($vg^+$,

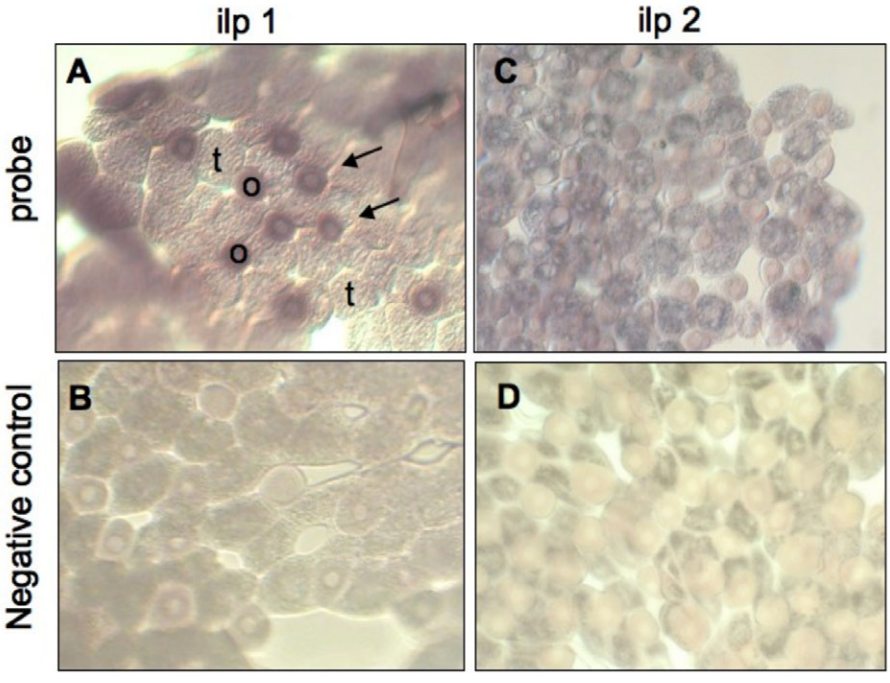


Fig. 3. Specific staining of *ilp* transcripts in honey bee fat body cells using *in situ* hybridization. (A) *ilp1* is expressed in fat body oenocytes; (B) negative control for *ilp1* demonstrates specificity of staining in A. (C) *ilp2* is expressed in both oenocytes and trophocytes in honey bee fat body; (D) negative control for *ilp2* demonstrates specificity of staining in C. o, oenocyte; t, trophocyte.

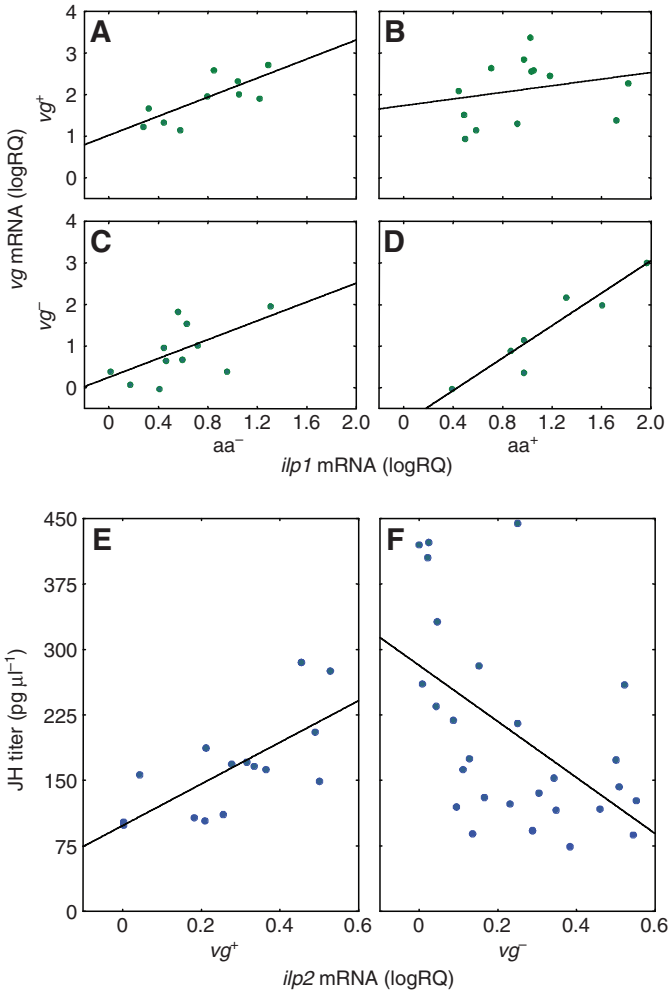


Fig. 4. Complex relationships between endocrine factors and gene transcripts in honey bees. (A–D) The correlative relationship between *ilp1* and *vg* is positive ($vg^+aa^-$, *N*=10; $vg^-aa^+$, *N*=8; $vg^-aa^-$, *N*=11), with the exception of RNAi controls that received amino acid ($aa^+$) supplementation ($vg^+aa^+$, *N*=13; B). Gene expression levels are given as log-transformed quantities relative to β-*actin* expression (logRQ). (E,F) The correlative relationship between *ilp2* and JH is conditional on *vg*. (E) For $vg^+$ controls ($vg^+aa^+$, *N*=7; $vg^+aa^-$, *N*=8), *ilp2* and JH are positively correlated. (F) After *vg* RNAi ($vg^-aa^+$, *N*=13; $vg^-aa^-$, *N*=15), however, the relationship is inversed and *ilp2* and JH are negatively correlated.

*n*=15, *r*=0.71, *P*<0.005; Fig. 4E). With RNAi, however, we established that this positive association was inverted after *vg* knockdown, so *ilp2* and JH became negatively correlated ($vg^-$, *N*=27, *r*=–0.45, *P*=0.02; Fig. 4F). These results are generally consistent with the proposition that JH titers and *ilp2* mRNA levels are linked in worker honey bees, although our data reveal new dynamic properties.

### Changes in fat body Vg storage

To better understand the correlative relationship between *ilp1* and *vg*, fat body was sectioned and Vg protein was visualized using immunofluorescence confocal microscopy (*N*=5). Accumulation of Vg occurred exclusively in fat bodies from the $vg^+aa^+$ factorial treatment (Fig. 5), the group in which the association between *ilp1* and *vg* was decoupled (Fig. 4B). This could suggest that levels of stored Vg feed back to affect the level of Vg production in fat body through intracellular signaling, as hypothesized previously (Amdam et al., 2007). Meanwhile, and similar to IGF signaling, *ilp1* responds to levels of available amino acids (Clemmons and Underwood, 1991). To test the predicted relationship between available amino acids and the expression levels of *vg* and *ilp1*, we measured the correlations of *vg* and *ilp1*, respectively, with the amount of residual BSA (as a marker of amino acid availability) in workers that were not subject to *vg* RNAi (i.e. $vg^+aa^+$ and $vg^+aa^-$). As predicted, expression of *ilp1* more closely tracked variation in BSA levels than did *vg* level (*ilp1*, *N*=22, *r*=0.36, *P*=0.10 *vs vg*, *N*=25, *r*=–0.05, *P*=0.80), but this association, as well as a difference test on the regression coefficients ($r_{ilp2}$ *vs* $r_{vg}$, *P*=0.09), was only significant if a one-sided test-criterion was used.

### Behavioral experiment

Because *vg* knockdown ($vg^-$) releases precocious foraging behavior (Nelson et al., 2007; Marco Antonio et al., 2008), we predicted that enhanced *vg* expression facilitated by amino acid enrichment ($aa^+$, Fig. 2H) would delay foraging onset. In the subset of bees that initiated foraging during the first 12-day period post-injection when bees were 10–22 days old, and BSA was likely still present in the hemolymph of supplemented bees, the $aa^+$ bees (*N*=113) showed a significant delay in foraging onset compared with the $aa^-$ controls (*N*=164) (Kaplan–Meier, 10–22 d, *P*<0.0001; Cox's *F*-test, *P*=0.02; Fig. 6A,B). This pattern was consistent between the two replicate observation hives. Using an uninjected experimental group (a handling reference, *N*=162; Fig. 6A,B), we also confirmed that the injection procedure *per se* released precocious foraging behavior in the workers, as shown in a previous study (Nelson et al., 2007). It is important to note, however, that this stress effect was controlled for in our analysis. Stress of injection was common to both $aa^+$ and $aa^-$ treatment groups, allowing us to partition out the separate and statistically significant effect of amino acid supplementation on foraging onset from any effect of handling. Over the remaining 12 days of the study, the age of bees that initiated foraging was not different between the treatment groups (data not shown, Kaplan–Maier, 23–34 days, *P*=0.68; Cox's *F*-test, *P*=0.22).

## DISCUSSION

We demonstrate that in honey bee fat body, ILP-encoding genes are perturbed by experimental changes in the phosphoglycolipo-storage protein Vg, the lipophilic endocrine factor JH and amino acid availability.

### *ilp1*

We document a positive relationship between the fat body expression levels of *ilp1* and *vg* in worker honey bees. This linkage is independent of *ilp2* and JH, and may result from a common effect of amino acid availability on *ilp1* and *vg* transcription. Although *ilp1* expression was correlated with *vg* transcript abundance, *ilp1* expression was unaffected by *vg* knockdown. This finding may indicate that ilp1 peptide production tracks a broad measure of nutritional status in the fat body, of which the Vg protein is one component.

The outcome of our *in situ* hybridization screen of fat body suggests that *ilp1* expression is restricted to oenocytes, cells that function in lipid storage and metabolism in fruit flies (Gutierrez et al., 2007). This relationship could point to a role for *ilp1* transcript changes in lipid mobilizing pathways. The ilp1 peptide might, for example, take part in the release of stored lipids during metabolic challenges, such as the extremely energetically demanding flight activity of honey bee workers (Harrison and Fewell, 2002). The bees' physiological response to hugely elevated flight metabolism has also been linked to increased JH synthesis (Sullivan et al., 2003). Taken together, these

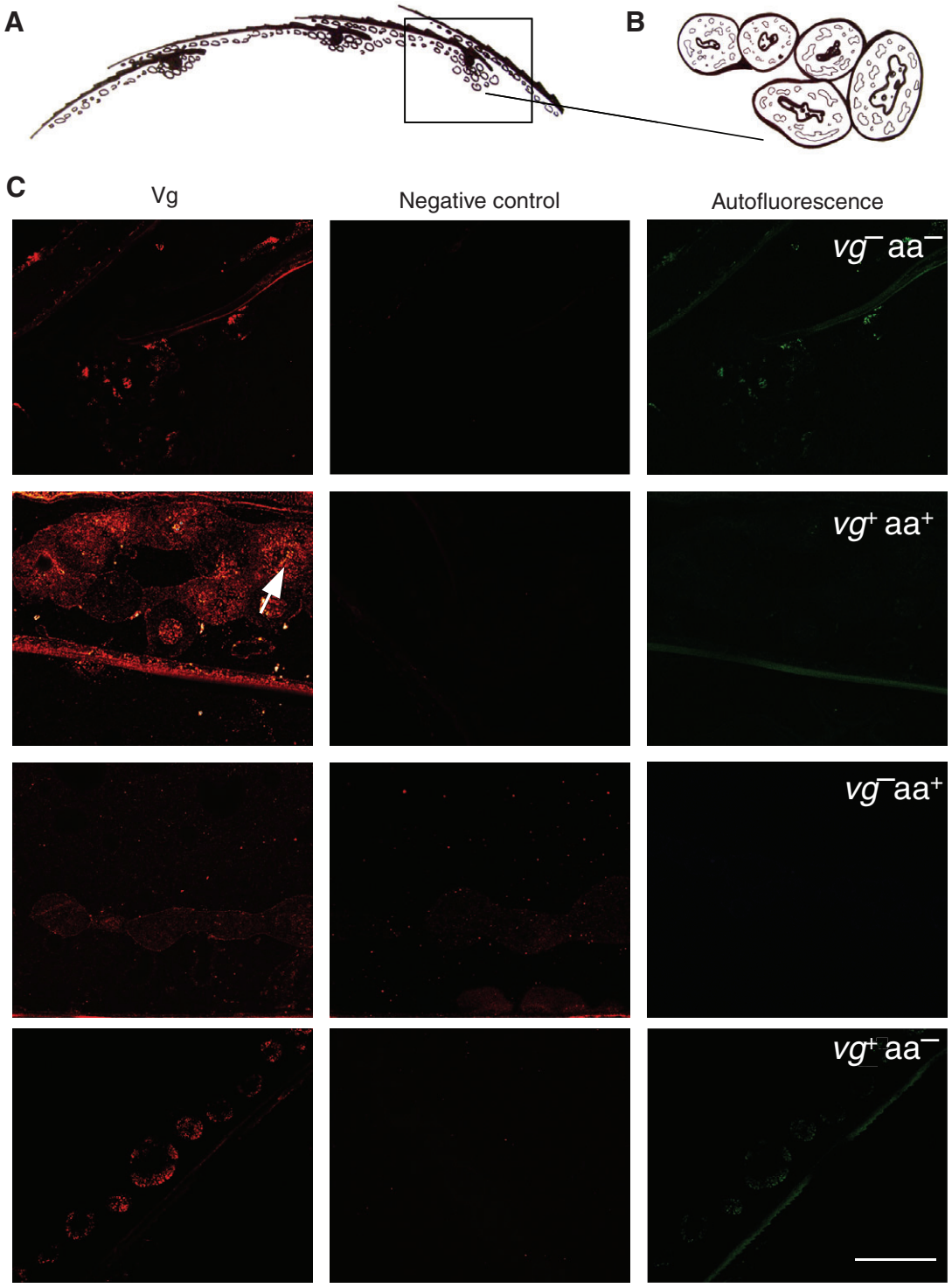


Fig. 5. Immunofluorescence localization of Vg in honey bee fat body. Micrographs represent the four treatment groups of the two-factorial experimental design. Schematic cross-sections of the (A) abdomen and (B) fat body cells indicate where the optical sections shown in C were obtained. (C) Fluorescence images of Vg immune reactivity demonstrate that Vg protein was exclusively detected in $vg^{+}aa^{+}$ workers (e.g. arrow). Scale bar, 30 μm.

indications imply that *ilp1* and JH may share a common association to flight, but average *ilp1* levels did not increase in parallel with average JH changes in our study. Why? A reasonable explanation may be that bees did not fly in our experiment. Workers cannot engage in energetically demanding foraging flights in small laboratory cages, and *ad libitum* access to food in captivity may render further mobilization of stored fat unnecessary. Our caged bees, consequently, may not have revealed some relationships between *ilp1* and JH that may be observed under more natural conditions (Ament et al., 2008). In other words, caged bees can have lower activity levels and larger nutrient stores than free-living workers, despite being primed by for flight by elevated JH titers and reduced Vg synthesis. This behavioral

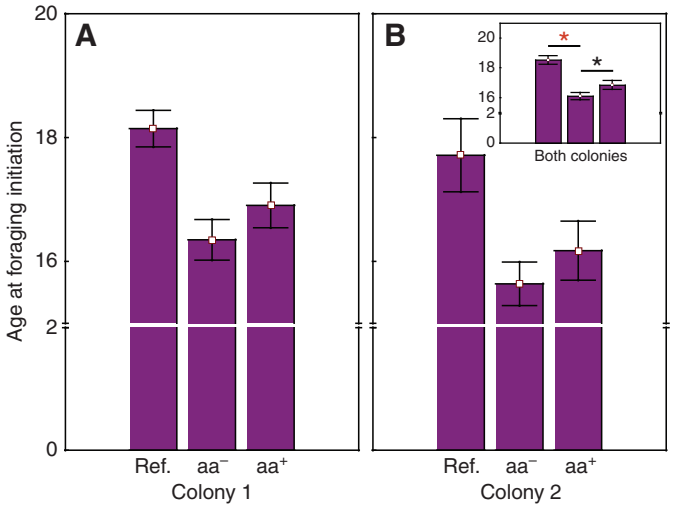


Fig. 6. Behavioral consequences of amino acid supplementation. (A,B) The effect of amino acid supplementation on foraging behavior revealed by comparing uninjected reference (Ref.), control-injected ($aa^-$) and BSA-injected ($aa^+$) honey bees. Worker bees respond to handling stress with behavioral changes, and therefore an injected control (here, $aa^-$) was used (Nelson et al., 2007). Bees were treated at 10 days of age. The *y*-axis reflects the chronological age of the worker bees. To visualize the stress effect, this control is contrasted to the reference group (inset in B, red asterisk). To establish the treatment effect, the injected control, $aa^-$, is contrasted to $aa^+$ (inset in B, black asterisk). Data are means ± s.e.m. Asterisks denote significance (Cox's *F*-test, $P<0.05$).

physiology may not encourage ilp1 signaling, and *ilp1* expression may even be suppressed to protect the bees' lipid stores.

Our hypotheses on the dynamic associations between *ilp1* and JH could help to explain previous work showing that although *ilp1* expression in is often positively correlated with JH and foraging behavior in honey bees, the amount of *ilp1* mRNA in brain and the titer of JH in hemolymph are sensitive to seasonal and/or social factors (Ament et al., 2008). A specific example is that JH and *ilp1* can be suppressed in some workers that forage during late summer and fall (Huang and Robinson, 1995; Ament et al., 2008). Their phenotype is poorly understood, but is hypothesized to result from food deprivation that may result in low adiposity in early life stages (Ament et al., 2008). Instead, we suggest that such late season foragers may resemble our caged bees except that, as they are not subjected to *vg* RNAi, they have high Vg levels (Fluri et al., 1977). High Vg levels would confer low JH titers whereas reduced work load because of seasonal constraints on flight activity would encourage adiposity and low *ilp1* levels.

### *ilp2*

Our results show that *ilp2* expression is closely linked to the JH level, but this dynamic is conditional on *vg*. When *vg* expression is 'on' ($vg^+$), *ilp2* and JH are positively correlated; conversely, when *vg* transcript levels are suppressed from adult emergence onwards ($vg^-$), *ilp2* and JH are negatively correlated. As with *ilp2* expression, the relationship between JH titer and foraging behavior is not always consistent. Although in general, foraging is associated with high JH titer (Huang et al., 1994), workers can forage with low JH titers in the fall (Huang and Robinson, 1995) and also initiate foraging after surgical removal of the corpora allata, the glands that produce JH (Sullivan et al., 2003). Similarly, *vg* knockdown can release foraging behavior in some workers without increasing JH (Marco Antonio et al., 2008). Some of these relationships may be explained if the role of JH is to physiologically 'lock' workers into the forager state rather than to induce foraging behavior (Amdam and Omholt, 2003). JH is an integrator of the metabolic physiology of insect flight (Mayer and Candy, 1969; Sullivan et al., 2003), and the rise in JH that occurs in response to Vg suppression may prepare a bee for foraging. Indeed, bees in which the corpora allata is surgically removed have lower flying metabolic rates than sham controls, and methoprene, a JH analog, can partially rescue a normal flying metabolic rate in these animals (Sullivan et al., 2003).

Expression of *ilp2* within both the trophocytes and oenocytes of the honey bee fat body suggest that *ilp2* signaling acts as a broader indicator of nutrient status than *ilp1*, encompassing both protein and fat levels in this insect. In insects in general, trophocytes provide the major site of amino acid turnover for synthesis and build-up of storage proteins, including Vg (Landim, 1985). In agreement with this finding, the high-nutrition phenotype of worker bees – the nurse – is characterized by higher *ilp2* expression levels in fat body than the foragers, which represent a low-nutrition phenotype (K.E.I., R. E. Page and G.V.A., unpublished data). Here, we do not show whether or how *ilp2* (or *ilp1*) can signal available nutrients, but future studies that directly measure peptide levels and downstream signal transduction changes in response to macronutrients are likely to enhance our understanding of honey bee IIS.

### Behavioral observations

Our behavioral data complement results on precocious foraging in *vg* knockdowns (Marco Antonio et al., 2008; Nelson et al., 2007) in showing that a reciprocal treatment (i.e. one that increases *vg* expression) delays foraging onset. Because of limits to the number of bees that could be processed with the double-injection protocol of the full two-factorial design (see Materials and methods), only the groups corresponding to the $vg^+aa^+$ and $vg^+aa^-$ treatments of the initial cage experiment were contrasted in our test of behavior. Therefore, although the main treatment effect is significant, and the effects of Vg repression are known from a previous study (Nelson et al., 2007), our data cannot reveal the possible interactions between *vg* downregulation and BSA injection. For more detailed results, future studies can combine a two-factorial design with alternative protocols that overcome logistical and technological difficulties for hemolymph amino acid enrichment or *vg* upregulation. Currently, such protocols are not available for honey bees. These results can be further bolstered by immunocytochemistry after antibodies specific to ilp1 and ilp2 are developed.

### Model of ILP action

We propose a model of ILP action in honey bee workers that outlines how connections between levels of IIS, JH and nutritional status including Vg can produce nurse and forager phenotypes (Fig. 7). Two insulin receptor genes (*InR1*/*InR2*) are annotated for the honey bee genome (Weinstock et al., 2006) and are tightly correlated in their expression levels, whereas expression of the two ILP-encoding genes can vary more independently (de Azevedo and Hartfelder, 2008). Although it is tempting to speculate that each ILP has its own receptor, in our model, ilp1 and ilp2 act as the agonist and antagonist, respectively, of the honey bee InR proteins (Amdam, 2011). In a setting of tightly correlated *InR1*/*InR2* expression, this model might better explain associations observed in free-flying bees; between *ilp1*, nutrient depletion and foraging (Corona et al., 2007; Ament et al., 2008), and between *ilp2*, nutrient surplus and nursing (K.E.I., R. E. Page and G.V.A., unpublished data). A similar agonist/antagonist relationship is shown for the ILPs INS-7 and INS-1 of the nematode

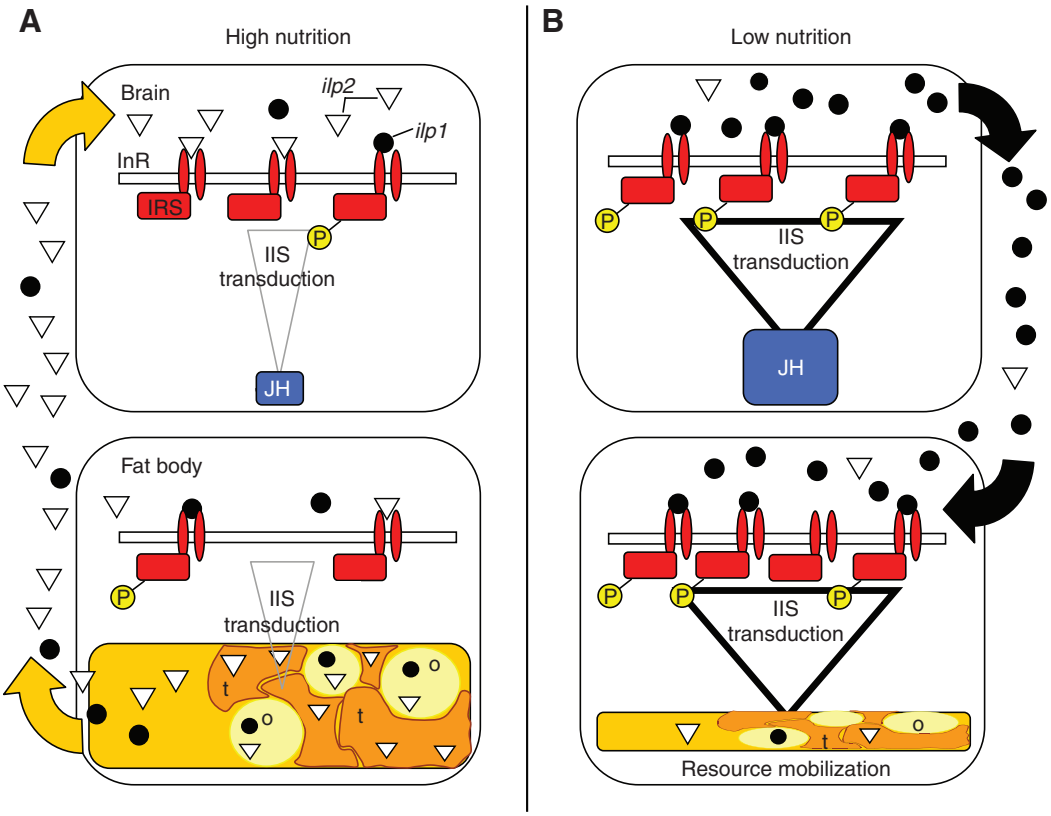


Fig. 7. Model for insulin-like protein (ILP) action on behavioral physiology of honey bees. Brain and fat body signaling are illustrated for (A) a high nutrition and (B) a low nutrition phenotype. Levels of nutrient stores are indicated by the size of the trophocytes (t; irregular orange shapes) and oenocytes (o; light yellow ovals) that make up the fat body. We hypothesize that *ilp1* (filled circles) acts as an agonist of the honey bee insulin receptors (InR, red indicators) whereas *ilp2* (small open triangles) is an antagonist of the InR. Nutritional status, of which Vg is one component, influences the interplay between ilp1 and ilp2 action. During periods of high nutritional status (A), such as nursing, when honey bees have large nutrient stores and high titers of Vg, the fat body is less sensitive to ILP action as *InR* expression is low, leading to less InR incorporated in cell membranes. The high *ilp2* expression in the fat body acts remotely to reduce transduction (phosphorylation of InR, encircled 'P') of the insulin/insulin-like signaling (IIS) pathway in the brain, a tissue with relatively constant *InR* expression. IIS transduction suppresses synthesis of JH, a downstream target of IIS. When honey bees have depleted nutrient stores and low Vg titers (foragers, B), fat body *InR* expression is high, suggesting that increased sensitivity to IIS in that tissue accompanies increased metabolic needs. Increased *ilp1* expression in the brain increases IIS transduction and acts remotely to mobilize stored nutrient reserves in the fat body. These changes meet energetic demands and may 'lock' individuals into the foraging behavioral state by further depleting nutrient stores and suppressing Vg synthesis.

*Caenorhabditis elegans* (Pierce et al., 2001; Murphy et al., 2003). However, unlike the honey bee with its two ILPs, *C. elegans* has 37 insulin-like ligands, and *D. melanogaster* has seven (Pierce et al., 2001; Grönke et al., 2010). The relative simplicity of the honey bee InR ligand system, therefore, could provide a general resource for studying the roles of IIS in animal physiology and behavior.

Our model is supported by the general insight that the sensitivity of the honey bee fat body to ILP signals appears to vary with behavioral state: expression of *InR1* and *InR2* is elevated in forager fat body relative to nurses, whereas in the brain, *InR* expression tends to remain constant between the two phenotypes (Ament et al., 2008). These data suggest that forager fat body is more sensitive to ILP signaling than that of workers. We propose that in nurse bees, ILP2 signals high nutrient status in fat body to the brain, where this peptide may reduce IIS and JH synthesis, a downstream target of the IIS pathway. As workers age and nutrient stores decline, fat body *ilp2* expression decreases (K.E.I., R. E. Page and G.V.A., unpublished data) whereas brain *ilp1* expression increases (Ament et al., 2008). The ILP1 product increases brain IIS and releases JH, and could, combined with higher *InR* expression in fat body, lead to mobilization of stored nutrients. These coordinated changes would result in a forager phenotype.

## Conclusions

We show that the gene-specific transcription pattern of *ilp1* and *ilp2* in fat body responds differently to perturbations of honey bee behavioral physiology. This finding suggests that the *ilp1* and *ilp2* gene products are part of separate molecular response systems, a hypothesis supported by the different spatial expression of the peptides in fat body cell types, shown here for the first time.

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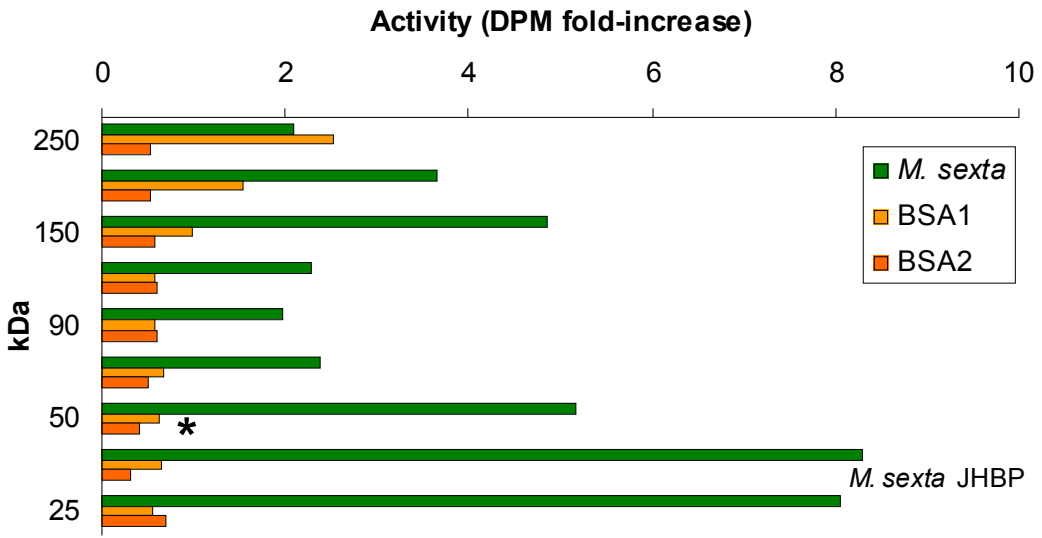
Activity (DPM fold-increase)
0
2
4
6
8
10
kDa
250
150
90
50
25
*
M. sexta
BSA1
BSA2
M. sexta JHBP

Table S1. List of primers and accession numbers

| Gene name | Primer sequence | Accession number |
|---|---|---|
| β-actin | F: 5′-TGCCAACACTGTCCTTTCTG-3′<br>R: 5′-AGAATTGACCCACCAATCCA-3′ | AB023025 |
| insulin-like peptide 1 | F: 5′-CGATAGTCCTGGTCGGTTTG-3′<br>R: 5′-CAAGCTGAGCATAGCTGCAC-3′ | GB17332-PA |
| insulin-like peptide 2 | F: 5′-TTCCAGAAATGGAGATGGATG-3′<br>R: 5′-TAGGAGCGCAACTCCTCTGT-3′ | GB10174-PA |
| target of rapamycin | F: 5′-AACAACTGTTGCTGACGGTG-3′<br>R: 5′-GTTGCAGTCCAGGCTTTTTG-3′ | XM 625127 |
| vitellogenin | F: 5′-GTTGGAGAGCAACATGCAGA-3′<br>R: 5′-TCGATCCATTCCTTGATGGT-3′ | AJ517411 |