RESEARCH ARTICLE

The gene vitellogenin affects microRNA regulation in honey bee (Apis mellifera) fat body and brain

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SUMMARY

In honey bees, vitellogenin (Vg) is hypothesized to be a major factor affecting hormone signaling, food-related behavior, immunity, stress resistance and lifespan. MicroRNAs, which play important roles in post-transcriptional gene regulation, likewise affect many biological processes. The actions of microRNAs and Vg are known to intersect in the context of reproduction; however, the role of these associations on social behavior is unknown. The phenotypic effects of Vg knockdown are best established and studied in the forager stage of workers. Thus, we exploited the well-established RNA interference (RNAi) protocol for Vg knockdown to investigate its downstream effects on microRNA population in honey bee foragers’ brain and fat body tissue. To identify microRNAs that are differentially expressed between tissues in control and knockdown foragers, we used μParaflo microfluidic oligonucleotide microRNA microarrays. Our results showed that 76 and 74 microRNAs were expressed in the brain of control and knockdown foragers whereas 66 and 69 microRNAs were expressed in the fat body of control and knockdown foragers, respectively. Target prediction identified potential seed matches for a differentially expressed subset of microRNAs affected by Vg knockdown. These candidate genes are involved in a broad range of biological processes including insulin signaling, juvenile hormone (JH) and ecdysteroid signaling previously shown to affect foraging behavior. Thus, here we demonstrate a causal link between the Vg knockdown forager phenotype and variation in the abundance of microRNAs in different tissues, with possible consequences for the regulation of foraging behavior.

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INTRODUCTION

The gene vitellogenin (Vg) is found in almost all oviparous species and encodes a member of the large lipid transfer protein family. In insects, Vg is generally expressed in the abdominal fat body cells (functionally homologous to vertebrate liver and white adipose tissue) of reproductive females, and the protein product serves as a yolk precursor in egg development (for a review, see Postlethwait and Giorgi, 1985). However, Vg has evolved non-oogenic functions in several species including the honey bee (Apis mellifera, Linnaeus 1758), where the gene is expressed not only by reproductive queens but also by male drones and functionally sterile female workers (Engels, 1974; Rutz and Lüscher, 1974; Trenczek and Engels, 1986; Piulachs et al., 2003). In worker honey bees, Vg protein is found in hypopharyngeal (head) glands and brain in addition to fat body and ovary tissue (Seehuus et al., 2007; Münch and Amdam, 2010). In workers, Vg has several functions: it incorporates into the hypopharyngeal glands for synthesis of proteinaceous secretions (jelly) that are fed to larvae, the queen and other adult bees (Amdam et al., 2003a), it promotes immunity, stress resilience and longevity (Amdam et al., 2004a), and it influences hormone levels, behavioral maturation and foraging biases (Guidugli et al., 2005; Nelson et al., 2007).

Honey bee societies are maintained by a highly structured division of labor between queen and workers, and between workers with different phenotypes. Workers display different behavior in an age-related sequence, starting with labor inside the nest and usually ending with foraging activities (Winston, 1987). A worker’s transition from nest tasks to foraging is mediated by decreasing Vg levels and increasing juvenile hormone (JH). Vg and JH have also been causally linked to transcriptional, physiological and metabolic changes in fat body and brain (Robinson, 1987; Huang et al., 1991; Nilsen et al., 2011; Wang et al., 2012a).

RNA interference (RNAi) has been used to untangle causal relationships between fat body signaling, brain and honey bee behavior (Amdam et al., 2003b; Patel et al., 2007; Nelson et al., 2007; Nunes and Simões, 2009; Ament et al., 2011). RNAi-mediated gene knockdown of Vg revealed a number of the protein’s effects in honey bee workers, including that Vg slows the onset of foraging, promotes pollen collection, and increases immunity, oxidative stress resilience and lifespan (Amdam et al., 2003a;
Amdam et al., 2004a; Nelson et al., 2007). In contrast, JH is a terpenoid compound and cannot be directly targeted with the RNAi method. However, the molecular mechanisms associated with Vg’s pleiotropic actions, including that of JH regulation, are currently largely unknown in honey bees.

In recent years, non-protein-coding RNAs have emerged as a dynamic regulatory layer involved in a wide range of biological processes. In animals, microRNAs are short non-coding transcripts that trigger endogenous gene silencing by partial base-pairing with dsRNA, the molecules that trigger RNAi, were based on a well-established protocol as previously described (see Amdam et al., 2006). We synthesized dsRNA for Vg knockdown (dsRNA-Vg) and for green fluorescent protein (dsRNA-GFP) as the sham control. There are several options for such controls, but dsRNA-GFP is the most frequently used in honey bees (Maori et al., 2009; Jarosch and Moritz, 2011; Kamakura, 2011; Ament et al., 2012; Desai et al., 2012). The dsRNA products were diluted with nuclease-free water (Qiagen, Valencia, CA, USA) to the final concentration of 10 μg μl⁻¹.

**Bees**

Worker honey bees (*Apis mellifera*) were obtained from six wild-type (unselected commercial stock) source colonies maintained at the Honey Bee Research Laboratory located at the Arizona State University Polytechnic Campus (Gilbert, AZ, USA). dsRNA injections were performed on six successive days. On each day, equal numbers of newly emerged bees (<24 h old) from two source colonies were mixed. We used a toggled collection scheme to ensure that the same colony pair (out of the six participating source colonies) was not sampled more than once. After injection, bees were introduced to one of three wild-type host colonies kept at the Arizona State University Main Campus (Tempe, AZ, USA). Bees injected on days 1 and 4 were introduced into host colony 1, bees injected on days 2 and 5 were introduced into host colony 2, and bees injected on days 3 and 6 were introduced into host colony 3.

**Injections**

Before injections, newly emerged bees were anesthetized at 4°C for 5 min and immobilized on a wax substrate using crossed needles. Each day, 100 bees per treatment group were dorsally injected under the 5th abdominal segment with 2 μl of (10 μg μl⁻¹) dsRNA-Vg or dsRNA-GFP using a Hamilton micro-syringe fitted with a 30 gauge needle (Becton Dickinson, Franklin Lakes, NJ, USA). Individuals showing signs of hemolymph leakage after injection were discarded. After injection, bees were paint-marked on the thorax or abdomen to identify treatment group and day of injection. Bees were introduced into a host nucleus colony in perforated cylindrical cages, allowing them to receive social interactions and nourishment. After 24h, bees were released from the cages.

**Foraging observation and bee sampling**

We collected same-age bees to control for effects due to chronological aging. In order to ensure that sufficient numbers of treated bees had initiated foraging in all colonies before collection, we monitored colony entrances to observe returning foragers. Forager counts were performed three times each day during peak foraging hours. We obstructed nest entrances for 10 min periods and counted all returning, marked foragers. We observed a large number of marked bees returning from foraging trips 15 days post-injection and began sample collection. Marked bees returning to the colony from flights were collected for processing. In our sample, we included only workers with pollen and/or nectar loads to ensure all individuals were indeed foragers. Approximately 10 individuals per treatment and injection day were collected from each host colony.

**Dissection and RNA isolation**

Bees were anesthetized at 4°C for 5 min and kept on ice prior to tissue dissection. In order to prevent RNA degradation as far as possible, we quickly dissected brain and fat body (both dissections were completed within ~1 min after the bee was killed) from each bee collected. In brief, brains were dissected under a stereomicroscope and carefully cleaned inside a drop of nuclease-free water for a complete elimination of adjacent glands and tissues. Abdominal carcasses were separated from digestive, reproductive...
and venom systems by pulling the sting apparatus. The abdominal carcases (lined with fat body) and brain of each bee were separately preserved in 500 μl of QIAzol (Qiagen) and stored at −80°C until RNA isolation. In order to separate large (>200 nucleotides, for knockdown validation) and small (<200 nucleotides, rich in microRNAs, for array experiments) RNA fractions, miRNeasy kit (Qiagen) and RNeasy MinElute Cleanup kit (Qiagen) were combined, following the manufacturer’s instructions. In brief, each individual tissue was homogenized in QIAzol Lysis Reagent, a monophasic solution of phenol and guanidine thiocyanate. Chloroform was added to obtain an aqueous phase containing RNA partitions, which was mixed with a 70% ethanol solution and pipetted into a RNeasy Mini spin column for centrifugation. At this point, large RNAs were retained on the column membrane, while the flow-through contained small RNAs. From here, the isolation of small RNA (round 1) and large RNA (round 2) followed different protocols. Round 1: in order to purify the microRNA-enriched fraction, 100% ethanol was added to the flow-through solution and mixed thoroughly by vortexing. The solution was transferred to an RNeasy MinElute spin column and centrifuged to bind RNA to the membrane. Phenol and other contaminants were removed by washes with RPE buffer (provided in the kit) and 80% ethanol solution. The microRNA-enriched fraction was then eluted in RNase-free water. Round 2: in order to purify the large RNA fraction, the column membrane was washed with RWT buffer (provided in the kit). After centrifugation, membrane-bound RNA was treated with DNase I. Contaminants were removed by washes with RWT and RPE buffer. The large RNA fraction was then eluted in RNase-free water. All samples were quantified using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA).

### Quantitative RT-qPCR for knockdown validation

To validate Vg knockdown, one-step reverse transcription-polymerase chain reaction (RT-qPCR) for Vg and actin (as the reference gene, GenBank accession number AB023025) were performed for all brain (N=30 for dsRNA-Vg and N=30 for dsRNA-GFP) and fat body (N=30 for dsRNA-Vg and N=30 for dsRNA-GFP) samples using QuantiTect SYBR Green RT-PCR Master Mix kit (Qiagen) and ABI Prism 7500 (Applied Biosystems, Foster City, CA, USA). Primers for Vg (5′-GGTGGAGAGCAACATCGAGA-3′ and 5′-TCATCCATTCTTGTGTTG-3′) and actin (5′- TGCCAAACAGTGTCTCTCTG-3′ and 5′-AGAATTGGACCCA CCAATTCCA-3′) amplification were the same as previously used (Ammdam et al., 2004b). For each sample, reactions were assembled in triplicate, and each single reaction consisted of 13.5 μl Master Mix (provided in the kit), 8.1 μl nuclelease-free water, 1.5 μl of each primer (forward and reverse), 0.27 μl of the RT enzyme (provided in the kit) and 2 μl (25 ng μl−1) of the large RNA fraction as a template. Negative controls without the addition of RT enzyme were run to check for contamination by genomic DNA. Individual Vg mRNA level was log 2-transformed and relative quantities were calculated according to the 2−ΔΔCt method (Applied Biosystems, user bulletin no. 2). We log 2-transformed the data in order to approximate normality as is often done with gene expression data sets, as these data are non-linear and the variance is very unequal across the samples (Ballman, 2008; Rieu and Powers, 2009).

### microRNA microarray sample preparation

Six biological samples of the small RNA fraction were prepared for each treatment group from the brains and fat bodies. Each biological sample was a pool of RNA from five individuals representing all three host colonies and 6 days of injection. Brain- or fat body-derived RNA from the same five individuals was pooled to generate corresponding biological samples for both tissues. Pools were named as ‘control forager brain’ (GFB), ‘knockdown forager brain’ (VFB), ‘control forager fat body’ (GFFb) and ‘knockdown forager fat body’ (VFFb), followed by a number from one to six (e.g. GFB-1, VFB-4), such that GFB-1 and GFFb-1 represented pooled tissues from the same five individuals. Each brain pool contained a total of 1 μg of the small RNA fraction, to which each of the five individuals contributed equally (200 ng). Each fat body pool contained a total of 2 μg of the small RNA fraction, to which each of the five individuals contributed equally (400 ng). Pools were sent to LC Sciences (Houston, TX, USA) for microRNA analysis using microfluidic oligonucleotide microarray technology.

### microRNA microarray design and analysis

Microfluidic chips were custom designed to contain 18 repeated probes for each of 168 known honey bee mature microRNAs available at miRBase, release 17 (Kozomara and Griffiths-Jones, 2011). In addition, the manufacturer added 56 control probes (each one repeated 4–16 times) for quality control of chip fabrication, RNA integrity, RNA labeling reaction and experiment conditions. In particular, these controls included spike-in RNA sequences and probes targeting different sections of conserved 5S RNA for Apis mellifera (six probes) and Drosophila melanogaster (six probes). To avoid dye-related bias, a simple-sample assay was performed so that 500 ng of each small RNA pool was Cy3-labeled at the 3′ ends, and each labeled pool was hybridized to one chip. All microarray reagents and detailed steps used for labeling, hybridization, image acquisition, normalization and data analysis were identical to those reported previously (Zhou et al., 2012). In brief, normalization was performed using the LOWESS method, whereas t-tests were applied to evaluate statistical significance of differentially expressed microRNAs within tested tissues. In compliance with MIAME standards (Brazma et al., 2001), all microarray data are available on the NCBI Gene Expression Omnibus database (GEO) under the accession number GSE44917.

### Target prediction analysis

In order to infer regulatory relationships, we searched for base-pairing between the most relevant mature microRNA sequences found in our dataset and 3′UTR sequences from honey bee protein-coding genes described to be involved in behavioral maturation. We selected a list of genes by performing a literature search on both NCBI-PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) and ISI Web of Knowledge (http://www.webofknowledge.com/) for the terms ‘bee’, ‘foraging behavior’ and ‘gene’. To simplify our analysis, only genes with individually tested associations with foraging behavior were included in our analysis (supplementary material Table S1). For example, this excludes data from microarray or transcription profiling studies in general, but does include the individually validated genes from those studies. Predicted or validated 3′UTR sequences were recovered from NCBI-GenBank, and microRNA mature sequences were extracted from miRBase (supplementary material Table S2). The first eight nucleotides at the 5′ end of microRNAs, called seeds, are crucial for target recognition and are largely used in computational approaches (Bentwich, 2005). We used a conservative criterion based exclusively on perfect Watson–Crick matches of seeds ranging from six to eight nucleotides (positions 1–8, 1–7, 2–8, 2–7), as they are frequently found in both invertebrate and vertebrate species (Gaidatzis et al., 2007). A network-based graph was constructed using the software Cytoscape, version 2.7.0 (Shannon et al., 2003).
Vitellogenin affects miRNA regulation

Vg downregulation in fat body and brain

Intra-abdominally injected dsRNA against Vg in newly emerged bees resulted in a significant reduction of Vg mRNA in forager fat bodies (one-way ANOVA of log2-transformed data, $F_{1,60}=21.9482, P=0.000017$, Fig. 1A) when compared with the same-aged controls (injected with dsRNA-GFP). The factors ‘day of collection’ and ‘qPCR plate’ were not significant ($F_{5,43}=0.0413, P=0.9989$ and $F_{10,43}=0.5315, P=0.8584$, respectively) and thus were dropped from the final analysis. Vg downregulation also occurred in the brain (one-way ANOVA of log2-transformed data, $F_{1,60}=6.1553, P=0.016024$, Fig. 1B). Again, the factors ‘day of collection’ and ‘qPCR plate’ were not significant ($F_{5,45}=0.7881, P=0.932$ and $F_{9,45}=0.5315, P=0.9989$) and were dropped from the analysis.

General findings on microRNA expression

We consider as ‘expressed’ those microRNAs in which the averaged signal from the microarrays was detectable above background in at least two different pools of the same treatment group. Following this criterion, 76, 74, 66 and 69 microRNAs were expressed in GFBr, VFBr, GFFb and VFFb groups, respectively (Fig. 2). Forty-six of these microRNAs were expressed in all groups, while others were shared between three groups or fewer, or were group specific. Seventy-two of the microRNAs on the array were not detected in our experiments (supplementary material Table S3).

Table 1. Highly expressed microRNAs (averaged signal above 1000 intensity units) in brain (Br) and fat body (Fb) tissues for control (GF) and vitellogenin (Vg) knockdown (VF) groups

<table>
<thead>
<tr>
<th>MicroRNA</th>
<th>Brain Mean</th>
<th>Brain SD</th>
<th>Vg Mean</th>
<th>Vg SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-252</td>
<td>41,511</td>
<td>5122</td>
<td>30,582</td>
<td>2576</td>
</tr>
<tr>
<td>miR-808</td>
<td>21,817</td>
<td>2348</td>
<td>18,716</td>
<td>2176</td>
</tr>
<tr>
<td>miR-124</td>
<td>21,038</td>
<td>2103</td>
<td>17,790</td>
<td>2076</td>
</tr>
<tr>
<td>miR-276</td>
<td>15,041</td>
<td>1490</td>
<td>15,982</td>
<td>1576</td>
</tr>
<tr>
<td>miR-317</td>
<td>13,069</td>
<td>1290</td>
<td>14,047</td>
<td>1376</td>
</tr>
<tr>
<td>miR-276</td>
<td>7638</td>
<td>7549</td>
<td>8580</td>
<td>8376</td>
</tr>
<tr>
<td>miR-277</td>
<td>6042</td>
<td>6425</td>
<td>6853</td>
<td>6576</td>
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<tr>
<td>miR-34</td>
<td>5749</td>
<td>5425</td>
<td>6314</td>
<td>5876</td>
</tr>
<tr>
<td>miR-15</td>
<td>4795</td>
<td>4749</td>
<td>5664</td>
<td>5363</td>
</tr>
<tr>
<td>miR-34</td>
<td>4709</td>
<td>4697</td>
<td>5122</td>
<td>5197</td>
</tr>
<tr>
<td>miR-13b</td>
<td>4324</td>
<td>4294</td>
<td>3899</td>
<td>3862</td>
</tr>
<tr>
<td>miR-7</td>
<td>3416</td>
<td>3393</td>
<td>3344</td>
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</tr>
<tr>
<td>miR-11</td>
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<td>3117</td>
<td>3306</td>
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<td>miR-307</td>
<td>2570</td>
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<td>3042</td>
<td>2912</td>
</tr>
<tr>
<td>miR-210</td>
<td>2542</td>
<td>2524</td>
<td>3073</td>
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</tr>
<tr>
<td>miR-8</td>
<td>1929</td>
<td>1890</td>
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</tr>
<tr>
<td>miR-29b</td>
<td>1464</td>
<td>1445</td>
<td>1268</td>
<td>1176</td>
</tr>
<tr>
<td>bantam</td>
<td>1299</td>
<td>1286</td>
<td>1132</td>
<td>1076</td>
</tr>
<tr>
<td>let-7</td>
<td>1106</td>
<td>1096</td>
<td>1234</td>
<td>1176</td>
</tr>
<tr>
<td>miR-932</td>
<td>1026</td>
<td>1024</td>
<td>1082</td>
<td>1036</td>
</tr>
</tbody>
</table>

MicroRNAs that are highly expressed in a non-tissue-specific manner are marked in bold.

Fluorescence intensity varied from ~20 to ~40,000 intensity units (digitally defined, relative units). Previous microRNA profiling studies using microarrays or deep-sequencing have separated a small subset of highly expressed microRNAs from a larger group of microRNAs that are expressed at low levels (Shao et al., 2010; Cristino et al., 2011; Li et al., 2011; Wei et al., 2011). Based on these studies, we assigned a cutoff of 1000 intensity units above which microRNAs would be considered as highly expressed to generate a subset of microRNAs for closer comparison. Only ~20 microRNAs per treatment/tissue group met this criterion as highly expressed. Thirteen of these microRNAs were highly expressed across both tissue and treatment (Table I).
The data also allowed us to identify a set of microRNAs with stable expression within each tissue (supplementary material Table S4), serving as potential housekeepers for future studies. Comparing the top 10 stable microRNAs in brain versus fat body, only miR-263 showed similar expression in all four groups.

**microRNA tissue-specific response to Vg downregulation**

Vg knockdown in forager fat bodies caused downregulation of let-7 and miR-281 and upregulation of miR-3739, miR-3776, miR-3796, miR-316 and miR-3718a (Table 2). The downregulation of miR-750 and upregulation of miR-3749, miR-3745 and miR-133 was suggestive ($P<0.10$) but was not statistically significant.

The knockdown of Vg in the workers’ fat bodies was associated with a parallel decrease in the brain transcript levels of Vg as well as decreased expression of miR-252, miR-1 and miR-375 levels, while miR-989, miR-92a and miR-31a were upregulated (Table 3). The downregulation of miR-3049 was suggestive ($P<0.07$) but was not statistically significant.

**Target prediction**

Our literature search retrieved 68 protein-coding genes (supplementary material Table S1) of which 61 have available 3′UTR information in NCBI-GenBank. We compared those 3′UTR sequences against 18 mature microRNA sequences, resulting from our analysis in fat bodies (11 microRNAs, see Table 2) and brains (seven microRNAs, see Table 3). Thirty-two out of 61 coding genes presented one or multiple seed sites in their 3′ ends for one or more microRNAs. Both miR-3745 and miR-184 had no target genes. The most microRNA-connected genes were acetylcholinesterase (ACHE2), fushi tarazu factor 1 (ftz-f1), tyrosinase receptor (TYR) and mapmodulin (Map). miR-375, miR-252, miR92a and miR-316 presented the greater number of target genes (Fig. 3).

**DISCUSSION**

The pleiotropic influence of Vg as a key regulator of honey bee social behavior has been previously demonstrated by different scientific approaches (Amdam et al., 2004a; Seeuws et al., 2006; Nelson et al., 2007; Marco Antonio et al., 2008). However, a detailed understanding of the molecular mechanisms that link Vg to behavior is in its infancy. To date, the investigation on the regulation of foraging behavior in honey bee workers has mainly explored the roles of protein-coding genes and their physiological connections. But the recent emergence of non-coding RNAs highlights the complexity of the gene expression networks that regulate many biological processes (Mattick and Gagen, 2001). Accordingly, a growing body of evidence has linked microRNA expression and behavioral traits (Kadener et al., 2009; Gunaratne et al., 2011; Jin et al., 2011; Zhan et al., 2011). We herein investigated whether microRNAs may work downstream of Vg in order to effect social changes. Toward this end, we knocked down Vg expression in adult workers to identify potential consequences on the microRNA populations in tissues central to bee behavior: the brain and the fat body.

**Vg mRNA levels decrease in brain in parallel with its knockdown in the fat body of foragers**

The data showed that Vg transcript abundance was reduced in the brain when the gene was targeted for downregulation in the fat body. Vg knockdown in fat body was expected as successful Vg RNAi is routinely achieved in this tissue (Amdam et al., 2003b; Guidugli et al., 2005; Amdam et al., 2006; Nelson et al., 2007; Nunes and Simões, 2009; Ihle et al., 2010). However, to our knowledge, this is the first detection of a concomitant reduction in the head. Several studies suggest that direct RNAi effects are difficult to achieve in the honey bee brain (Wang et al., 2010; Jarosch and Moritz, 2011). After
abdominal injection of fluorescently labeled siRNAs (small interfering RNAs), no fluorescence signals were detectable in head tissue in worker honey bees, which could suggest that the siRNAs do not pass the neurilemma, an insect blood–brain barrier (Jarosch and Moritz, 2011). When RNAi-induced gene knockdowns have been effective in honey bees, it has required local injections directly into brain tissue (Faroqui et al., 2003; Farooqui et al., 2004; Mustard et al., 2010).

It is therefore possible, and even probable, that our observation of reduced Vg transcript levels in the brain is not caused by RNAi directly, but rather is the product of a global or brain-specific response to peripheral signaling following Vg RNAi in the fat body, such as reduced Vg levels in the hemolymph. This explanation further implies that the microRNA responses we observe in brain could be due to one or more of several factors: (i) they are caused by remote signaling triggered by RNAi in the fat body, (ii) they are caused by secondary Vg reduction in the brain, (iii) they are part of the machinery that causes secondary Vg reduction in the brain, (iv) they are the result of longer foraging experience due to RNAi-triggered precocious foraging. However, more studies are required to elucidate how fat body Vg RNAi affects brain and the role of microRNAs in that process. Such experimentation might also reconcile our findings with previous work, performed on a different genetic stock of honey bees, which recorded a compensatory response to peripheral signaling following Vg RNAi in an increasingly well-studied axis involving JH, ecdysteroids and the insulin receptor substrate (IRS) gene known to affect honey bee foraging behavior (Velarde et al., 2009; Wang et al., 2009; Wang et al., 2012a).

The JH response to Vg reduction is likely involved in the regulation of the forager phenotype of Vg knockdowns (Guidugli et al., 2005; Marco Antonio et al., 2008; Ihle et al., 2010), but the mechanism by which Vg titers influence JH levels are currently not well understood. We have identified JH-associated genes that are potential targets of microRNAs as being significantly upregulated and downregulated after Vg knockdown. The putative JH receptor ultraspiracle (USP) (Jones and Sharp, 1997; Barchuk et al., 2004) is a likely target of microRNAs, with increased and reduced expression in response to Vg reduction in both brain and fat body. Juvenile hormone inducible protein 26 (Jhi-26), which is expressed in response to JH or its analogs, is a potential target of microRNAs upregulated in response to Vg knockdown in both brain and fat body. microRNAs that are significantly downregulated in the brains of Vg knockdown foragers potentially target genes encoding JH degrading enzymes, JH esterase and JH epoxide hydrolase. These results could suggest a role of microRNAs in the suppressive effect of Vg on JH, and the release of JH synthesis when Vg is knocked down.

New evidence makes it increasingly likely that ecdysteroids influence honey bee social behaviors (Velarde et al., 2009; Wang et al., 2009; Wang et al., 2012b), and we have identified microRNAs...
that may be part of this pathway. Hormone receptor-like in 46 (HR46) and fzd-fl are predicted targets of microRNAs that are themselves affected by Vg knockdown.

Nutrient status is associated with behavioral phenotype and roles for nutrient-sensing pathways including the insulin/insulin-like signaling pathway have been identified (Toth et al., 2005; Ament et al., 2008; Wang et al., 2010). Our target analysis predicts a role for several microRNAs with expression modulated by Vg knockdown or its downstream effects. Phosphoinositide-dependent kinase-1 (PDK1), Phosphatase and tensin homolog (PTEN), Phosphoinositide 3-kinase (PI3K) and Phosphatidylinositol-4-phosphate-5-kinase (PIPK5) are all potential targets for microRNA action downstream of Vg. These genes are also downstream of IRS, an insulin and epidermal growth factor pathway gene that can modify honey bee foraging behavior directly (Wang et al., 2010).

The regulation of foraging behaviors in honey bees is a complex process that we are only beginning to understand. At the colony level, there are many known factors that influence when an individual worker begins to forage and what she collects as a forager. These factors include cues and signals from larvae, the queen, other workers and levels of stored food (Pankiw et al., 1998; Dreller and Tarpy, 2000; Amdam et al., 2006). The effects these cues have on individual behavior are mediated by genotype, nutrient stores and other internal factors (Page and Fondrk, 1995; Pankiw and Page, 2001; Toth et al., 2005).

It has been suggested that microRNAs might function in developmental robustness, a process by which an organism compensates for environmental, genetic or other potential disruptions, to maintain a developmental program (Stark et al., 2005; Hornstein and Shomron, 2006; Shomron, 2010). Here, we propose that microRNAs may have a similar role in the regulation of honey bee foraging behavior, functioning as integrators of various molecular inputs to maintain and regulate a foraging phenotype in response to a complex network of cues.

Promising candidate microRNAs for future studies

Among the microRNAs differentially regulated in response to Vg knockdown are promising candidates for future targeted studies on the molecular pathways linked to Vg–microRNA networks directly impacting social behavior.

We found that Vg knockdown resulted in decreased expression of let-7 in forager fat bodies. This suggests that the decline in let-7 expression observed by Behura and Whitfield (Behura and Whitfield, 2010) between young nurses and old foragers could be the consequence of reduced Vg levels in the old foragers. Predicted target analysis for let-7 connects this microRNA with genes associated with an increasingly well-studied axis involving ovarian signaling, JH and ecdysteroids (Wang et al., 2012b).

miR-133 was previously considered to be a muscle-specific microRNA, but it is now known to also have a role in differentiation of murine adipose tissues (Trakovsky et al., 2012; and references therein). miR-133 is conserved between vertebrates and invertebrates (see miRBase, http://www.mirbase.org), suggesting that its functions could be phylogenetically retained. miR-133 thus could be associated with the lipid loss observed in foragers and, therefore, is a good target for future research on the mechanisms of behavioral progression in bees.

Here, we found miR-252 is the highest expressed microRNA in brains (Table 1) and is downregulated in brains of Vg knockdown foragers compared with controls (Table 3). Moreover, miR-252 showed a large number of target genes in flies (Marrone et al., 2012) and was one of the most connected microRNAs in our network (Fig. 3). Together, this evidence suggests that miR-252 is a key brain regulator that deserves future attention.

To date, miR-3739 is only found in honey bees (Chen et al., 2010). Its species-specificity as well as its very high expression in the fat body of Vg knockdown foragers (nearly eight times that of controls, Table 2) suggest that miR-3739 is also a promising candidate for future research.

A role for microRNAs in the regulation of honey bee behavioral maturation

Three previous studies examined microRNA expression differences in nurse and forager bees (Behura and Whitfield, 2010; Greenberg et al., 2012; Liu et al., 2012a). Behura and Whitfield (Behura and Whitfield, 2010) identified microRNAs differentially expressed in the brains of young nurses versus old foragers, while Greenberg and colleagues (Greenberg et al., 2012) sequenced microRNA transcriptome from worker heads in the context of division of labor. Liu and colleagues (Liu et al., 2012a) used whole-head extracts of nurses and foragers of unknown ages to make similar comparisons. We found limited overlap between our results and those of the earlier studies.

In our study, miR-2796 is among the top 10 highly expressed microRNAs in both brain groups (GFBr and VFBr, with intensities above 7500 intensity units) and is not affected by Vg knockdown. However, the expression of miR-2796 in fat body groups (GFFb and VFFb) is basal (intensity less than 80 intensity units). Greenberg and colleagues (Greenberg et al., 2012) found that miR-2796 expression in forager heads is three times higher than that in nurse heads, and that miR-2796 is enriched in the brain relative to all other head tissues. Consistent with previous research identifying elevated miR-133 expression in foragers (Liu et al., 2012a), we found that expression of miR-133 was slightly (but not significantly; P<0.10) elevated in response to Vg knockdown in forager fat body. The increased brain expression of miR-92a in our study mirrors the increased expression in the brains of old foragers (Behura and Whitfield, 2010) and in whole-head forager samples (Liu et al., 2012a). Additionally, the reduced expression of let-7 in this study is consistent with increased expression in whole-head nurse samples (Liu et al., 2012a) and in the brains of young nurses (Behura and Whitfield, 2010). This agreement offers strong support for a role for miR-2796, miR-133, miR-92a and let-7 in the regulation of behavioral maturation in honey bees.

The expression pattern for miR-31a in this study is inconsistent with that found by Liu and colleagues (Liu et al., 2012a). In our study, miR-31a expression was elevated in response to Vg knockdown, while Liu and colleagues (Liu et al., 2012a) found higher expression in nurses.

The overall lack of agreement between these data sets likely reflects the differences in sample populations. The previous studies compared nurse with forager microRNA expression patterns (Behura and Whitfield, 2010; Greenberg et al., 2012; Liu et al., 2012a). Our focus on a forager phenotype triggered by Vg suppression was designed to identify microRNAs associated with the behavioral phenotype induced by Vg knockdown independent of aging, as our target individuals were all the same chronological age. All of the individuals in our sample had already transitioned to foraging behavior; thus, it is likely that many of the microRNAs that we identified are part of the mechanism by which Vg knockdown induces changes in foraging bias for nectar versus pollen collection. However, as we do not know the ages at which the bees in this study initiated foraging behavior, we cannot rule out the possibility that some of the changes that we observed were due to differences.
in the amount of foraging experience between the Vg knockdown and control groups (Nelson et al., 2007; Ihle et al., 2010). The young nurse and old forager sampling design of Behura and Whitfield (Behura and Whitfield, 2010) may reflect age-associated changes in behavio