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. Likewise microRNAs play important roles in posttranscriptional gene regulation and affect many biological processes. The action 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 show 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 regulation of foraging behavior.

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 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). JH, on the other hand, is a terpenoid compound and cannot be directly targeted with the RNAi method. However, 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 the 3´ untranslated region (3´UTR) of target mRNAs (for review see Bartel, 2009). Interestingly, many reported roles for microRNAs show parallels to Vg's functions in worker honey bees. MicroRNAs are able to act in the regulation of gene expression within (Chen et al., 2007) as well as between tissues (Liu et al., 2010). In many organisms, they participate in the regulation of complex behavioral phenotypes, such as migratory behavior of butterflies (Zhan et al., 2011), circadian rhythms of flies (Kadener et al., 2009), food-choice in giant pandas

(Jin et al., 2011), and song communication in zebra finches (Gunaratne et al., 2011). Moreover, they are linked to oxidative stress (Hulsmans et al., 2011) as well as immunity (Garbuzov and Tatar, 2010; Fullaondo and Lee, 2012) and lifespan in *Drosophila melanogaster* (Liu et al., 2012). In addition, the functions of specific microRNAs are closely related to oogenesis, including vitellogenesis, in both holometabolous and hemimetabolous insect species (Bryant et al., 2010; Tanaka and Piulachs, 2012). While reproductive pathways link microRNAs and Vg functions, these associations have not been explored in the context of insect social behavior. This evidence led us to ask whether the microRNA population could be working in concert with Vg to affect the behavior of adult workers.

A number of microRNA surveys have been performed in honey bees after genome sequencing (Honeybee Genome Sequencing Consortium, 2006) identified a portion of the species' microRNA population (Weaver et al., 2007; Chen et al., 2010). Some microRNAs are differentially expressed between the head, thorax, and abdomen of workers (Weaver et al., 2007), two microRNAs have been implicated in neural functions (Hori et al., 2011) and overexpressed microRNAs in the brain have been correlated with behavioral maturation (Behura and Whitfield, 2010). These studies show that research on honey bee microRNAs is worthwhile.

We investigated the effect of *Vg* knockdown on microRNA regulation in honey bee fat body and brain. We used RNAi to experimentally reduce *Vg* gene expression in worker honey bees. Both *Vg* knockdowns and controls were collected as foragers. We specifically targeted foragers as the behavioral effects of Vg downregulation are best characterized in this phenotype (Nelson et al., 2007; Ihle et al., 2010). Same-aged control and *Vg* knockdown foragers can be expected to have significantly different biases for nectar versus pollen collection, with *Vg* knockdowns collecting significantly less pollen than controls. We then examined the microRNA populations expressed in the foragers' fat body and brain tissues. We identified a subset of microRNAs that respond downstream to *Vg* knockdown and found that the microRNA response to *Vg* downregulation differs between fat body and brain. Our results provide new insights into how behavioral regulation may be achieved.

MATERIAL AND METHODS

dsRNA synthesis

All steps and reagents for the synthesis of double stranded RNA (dsRNA), the molecules that trigger RNAi, were based on a well-established protocol as previously described (for details see Amdam et al., 2006, section 2.2). 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.

Bees

Worker honey bees (*Apis mellifera* L.) 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 a volume of 2 μl (10 μg/μl) of dsRNA-Vg or dsRNA-GFP using a Hamilton micro syringe fitted with a 30G 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 24 h 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 ten-minute periods and counted all returning, marked foragers. We observed 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 tissues dissection. In order to prevent RNA degradation as much as possible, we quickly dissected brain and fat body (both dissections were completed within approximately 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. Abdominal carcass (lined with fat body) and brain of each bee were separately preserved in 500 µl of QIAzol (Qiagen) and stored at -80ºC 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 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 miRNA-enriched fraction, 100% ethanol was added to the flow-through solution and mixed thoroughly by vortexing. The solution was

transferred to a RNeasy MinElute spin column and centrifuged to bind RNA to the membrane. Phenol and other contaminants were removed by washes with buffer RPE (provided by 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 Buffer RWT (provided by the kit). After centrifugation, membrane-bound RNA was treated with DNase I. Contaminants were removed by washes with buffers RWT and RPE. 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 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′-GTTGGAGAGCAACATGCAGA-3′ and 5′-TCGATCCATTCCTTGATGGT-3′) and *actin* (5′-TGCCAACACTGTCCTTTCTG-3′ and 5′-AGAATTGACCCACCAATCCA-3′) amplification were the same as previously used (Amdam et al., 2004b). For each sample, reactions were assembled in triplicate, and each single reaction consisted of 13.5 µl Master Mix (provided by kit), 8.1 µl nuclease-free water, 1.5 µl of each primer (forward and reverse), 0.27 µl of the RT enzyme (provided by kit) and 2 µl (25 ng/µl) of large RNA fraction as a template. Negative controls without addition of RT enzyme were run to check for contamination by genomic DNA. Individual *Vg* mRNA level was log2-transformed and relative quantities were calculated according to $2^{-\Delta\Delta Ct}$ method (Applied Biosystems, user bulletin #2). We log2-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 often very unequal across the samples (Ballman, 2008; Rieu and Powers, 2009).

microRNA microarrays sample preparation

Six biological samples of small RNA fraction were prepared for each treatment group from brain and fat body. Each biological sample was a pool of RNA from 5 individuals

representing all 3 host colonies and 6 days of injection. Brain or fat body-derived RNA from the same 5 individuals was pooled to generate corresponding biological samples for both tissues. Pools were named as "control forager brain" (GFBr), "knockdown forager brain" (VFBr), "control forager fat body" (GFFb), "knockdown forager fat body" (VFFb), followed by a number from 1 to 6 (eg. GFBr-1, VFFb-4), such that GFBr-1 and GFFb-1 represented pooled tissues from the same 5 individuals. Each brain pool contained a total of 1 µg of small RNA fraction, to which each of the 5 individuals contributed equally (200 ng). Each fat body pool contained a total of 2 µg of small RNA fraction, to which each of the 5 individuals contributed equally (400 ng). Pools were sent to LC Sciences (Houston, TX, USA) for microRNA analysis using µParaflo® microfluidic oligonucleotide microarray technology.

microRNA microarrays design and analysis

Microfluidic chips were customized containing 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 to 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 ribosomal RNA for *Apis mellifera* (6 probes) and *Drosophila melanogaster* (6 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 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 as reported by Zhou et al. (2012). In brief, normalization was performed using LOWESS method whereas T test 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 is 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 (Table A1). 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 (Table A2). The first 8 nucleotides at the 5´end of microRNAs, called seeds, are critical for target recognition and largely used in computational approaches (Bentwich, 2005). We used a conservative criteria based exclusively on perfect Watson-Crick matches of seeds ranging from 6-8 nucleotides (positions 1-8, 1-7, 2-8, 2-7), since they are frequently found in both invertebrate and vertebrate species (Gaidatziz et al., 2007). A network-based graph was constructed using the software Cytoscape, version 2.7.0 (Shannon et al., 2003).

RESULTS

Vitellogenin 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 to the same-aged controls (injected with dsRNA-GFP). Other 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 factors “day of collection” and “qPCR plate” were not significant ($F_{5,45}$=0.2608, p = 0.932 and $F_{9,45}$=0.7881, p = 0.628 respectively) and were dropped from 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

289 treatment group. Following this criterion, 76, 74, 66, and 69 microRNAs were expressed in
290 GFBr, VFBr, GFFb, and VFFb groups, respectively (Fig. 2). Forty-six of these microRNAs
291 were expressed in all groups, while others were shared between 3 groups or less, or were
292 group-specific. Seventy-two of the microRNAs on the array were not detected in our
293 experiments (Table A3).
294 Fluorescence intensity varied from ~20 to ~40,000 intensity units (digitally defined,
295 relative units). Previous microRNA profiling studies using microarrays or deep-sequencing
296 have separated small subset of highly expressed microRNAs from a larger group of
297 microRNAs that are expressed at low levels (Shao et al., 2010; Cristino et al., 2011; Li et al.,
298 2011; Wei et al., 2011). Based on these studies, we assigned a cutoff of 1,000 intensity units
299 above which microRNAs would be considered as highly expressed to generate a subset of
300 microRNAs for closer comparison. Only approximately 20 microRNAs per treatment/tissue
301 group met this criterion as highly expressed. Thirteen of these microRNAs were highly
302 expressed across both tissue and treatment (Table 1).
303 The data also allowed us to identify a set of microRNAs with stable expression within
304 each tissue (Table A4), serving as potential housekeepers for future studies. Comparing the
305 top 10 stable microRNAs in brain versus fat body, only miR-263 showed similar expression
306 in all 4 groups.
307
308 *microRNAs tissue specific response to vitellogenin downregulation*
309 *Vg* knockdown in forager fat bodies caused downregulation of let-7 and miR-281 and
310 upregulation of miR-3739, miR-3776, miR-3796, miR-316, and miR-3718a (Table 2). The
311 downregulation of miR-750 and upregulation of miR-3749, miR-3745, and miR-133 was
312 suggestive ($p<0.10$) but was not statistically significant.
313 The knockdown of *Vg* in the workers' fat bodies was associated with a parallel
314 decrease in the brain transcript levels of *Vg* as well as decreased expression of miR-252, miR-
315 1, and miR-375 levels, while miR-989, miR-92a, and miR-31a were up-regulated (Table 3).
316 Downregulation of miR-3049 was suggestive ($p<0.07$) but was not statistically significant.
317
318 *Target prediction*
319 Our literature search retrieved 68 protein-coding genes (Table A1) of which 61 have available
320 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 (7 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* (AChE-2), *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; Seehus 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.

Vitellogenin *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 fluorescent 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 (Farooqui 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 haemolymph. This explanation further implies that the microRNA responses we observe in brain can be due to one or more of several explanations: (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 upregulation of Vg in brain when fat body expression of *Vg* was experimentally reduced (reviewed in Münch and Amdam, 2010).

microRNA profiles affected by vitellogenin *knockdown in foragers*

Experimental downregulation of *Vg* induces significant changes in the expression of a restricted number of microRNAs in fat body, the site of Vg synthesis. We also observed effects of treatment on the microRNA expression patterns in the brains of foragers. Possible explanations for the changes in brain microRNA expression were discussed in the above section. Though we are not able to distinguish between these and other possibilities from our data, the predicted targets of the microRNAs along with their potentially conserved functions can suggest answers.

microRNA target prediction in genes associated with foraging behavior

Upregulation of microRNAs is often associated with post-transcriptional suppression of target genes (Bartel et al., 2009); however, a growing body of evidence suggests that microRNAs can also induce upregulation of their targets (reviewed in Vasudevan, 2012). Thus, direct experimentation is necessary not only to confirm the ability of microRNAs to affect predicted target genes, but also to verify the effects under physiologically relevant conditions. Our target prediction identified potential seed matches for microRNAs influenced by *Vg* knockdown 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).

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 significantly up- and down-regulated after *Vg* knockdown. The putative JH receptor *ultraspiracle* (Jones and Sharpe, 1997; Barchuk et al., 2004 (USP)) 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 analogues, 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 *ftz-f1* 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* (PIP5K)

416 are all potential targets for microRNA action downstream of Vg. These genes are also
417 downstream of *IRS*, an insulin and epidermal growth factor pathway gene that can modify
418 honey bee foraging behavior directly (Wang et al., 2010).
419 The regulation of foraging behaviors in honey bees is a complex process that we are
420 only beginning to understand. At the colony level, there are many known factors that
421 influence when an individual worker begins to forage and what she collects as a forager.
422 These factors include cues and signals from larvae, the queen, other workers, and levels of
423 stored food (Pankiw et al., 1998; Dreller and Tarpy, 2000; Amdam et al., 2006). The effects
424 these cues have on individual behavior are mediated by genotype, nutrient stores, and other
425 internal factors (Page and Fondrk, 1995; Pankiw and Page, 2001; Toth et al., 2005).
426 It has been suggested that microRNAs might function in developmental robustness, a
427 process by which an organism compensates for environmental, genetic or other potential
428 disruptions, to maintain a developmental program (Stark et al., 2005; Hornstein et al., 2006;
429 Shomron, 2010). Here we propose that microRNAs may have a similar role in the regulation
430 of honey bee foraging behavior, functioning as integrators of various molecular inputs to
431 maintain and regulate a foraging phenotype in response to a complex network of cues.
432
433 *Promising candidate microRNAs for future studies*
434 Among the microRNAs differentially regulated in response to *Vg knockdown* are promising
435 candidates for future targeted studies on the molecular pathways linked to Vg-microRNA
436 networks directly impacting social behavior.
437 We found that *Vg* knockdown resulted in decreased expression of let-7 in forager fat
438 bodies. This suggests that the decline in let-7 expression observed by Behura and Whitfield
439 (2010) between young nurses and old foragers could be the consequence of reduced Vg levels
440 in the old foragers. Predicted target analysis for let-7 connects this microRNA with genes
441 associated with an increasingly well-studied axis involving, ovarian signaling, JH, and
442 ecdysteroids (Wang et al, 2012b).
443 miR-133 was previously considered to be a muscle-specific microRNA, but it is now
444 known to also have a role in differentiation of murine adipose tissues (Trajkovski et al., 2012
445 and references therein). miR-133 is conserved between vertebrates and invertebrates (see
446 miRBase, http://www.mirbase.org), suggesting that its functions could be phylogenetically
447 retained. miR-133 thus could be associated with the lipid loss observed in foragers and

448 therefore, is a good target for future research on the mechanisms of behavioral progression in
449 bees.

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

455 To date, miR-3739 is only found in honey bees (Chen et al., 2010). Its species-
456 specificity as well as its very high expression in the fat body of *Vg* knockdown foragers
457 (nearly 8 times that of controls, Table 2) suggest that miR-3739 is also a promising candidate
458 for future research.

459

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

461 Three previous studies examined microRNA expression differences in nurse and forager bees
462 (Behura and Whitfield, 2010; Greenberg et al., 2012; Liu et al., 2012). Behura and Whitfield
463 (2010) identified microRNAs differentially expressed in the brains of young nurses versus old
464 foragers, while Greenberg et al. (2012) sequenced microRNA transcriptome from worker
465 heads in the context of division of labor. Liu and colleagues (2012) used whole head extracts
466 of nurses and foragers of unknown ages to make similar comparisons. We found limited
467 overlap between our results and those of the earlier studies.

468 In our study, miR-2796 is among the top 10 highly expressed microRNAs in both
469 brain groups (GFBr and VFBr, with intensities above 7,500 digitally units) and is not affected
470 by *Vg* knockdown. However, the expression of miR-2796 in fat body groups (GFFb and
471 VFFb) is basal (intensity less than 80 digitally units). Greenberg et al. (2012) found that miR-
472 2796 expression in forager heads is three times higher than in nurse heads, and that miR-2796
473 is enriched in the brain relative to all other head tissues. Consistent with previous research
474 identifying elevated miR-133 expression in foragers (Liu et al., 2012), we found that
475 expression of miR-133 was suggestively elevated ($p<0.10$) in response to *Vg* knockdown in
476 forager fat body. The increased brain expression of miR-92a in our study mirrors the
477 increased expression in the brains of old foragers (Behura and Whitfield, 2010) and in whole
478 head forager samples (Liu et al., 2012). Additionally, the reduced expression of let-7 in this
479 study is consistent with increased expression in whole head nurse samples (Liu et al., 2012)

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 those from Liu and colleagues (2012). In our study miR-31a expression was elevated in response to *Vg* knockdown, while Liu et al. (2012) found higher expression in nurses.

The overall lack of agreement between these datasets likely reflects the differences in sample populations. The previous studies compared nurse to forager microRNA expression patterns (Behura and Whitfield, 2010; Greenberg et al., 2012; Liu et al., 2012). 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 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 could be due 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 (2010) may reflect age-associated changes in microRNA populations rather than genes associated with behavioral maturation *per se*. The influence of age versus behavioral phenotype can be further elucidated through functional studies as well as experiments that use demographic manipulations to compare expression profiles of old foragers, precocious foragers, young nurses, and old bees who have reverted from a foraging to nursing behavioral phenotypes.

Concluding remarks

Behavioral maturation in honey bees is a remarkably plastic process that can be separated from chronological age or even reversed (Huang and Robinson, 1996). The mechanism governing the transition from nurse to forager phenotype likely involves the integration of signals from several remote tissues by the brain (Ament et al., 2008; Nilsen et al., 2010). Our challenge is to understand how those remote signals are integrated. Recent work suggests that tissues as diverse as brain and ovary are regulated by the same systemic factors such as

juvenile hormone, ecdysteroids, and Vg that form a global network that modulates individual behavior (Wang et al., 2012a; Wang et al., 2012b). Our findings suggest that microRNAs can be part of this network, potentially acting both within tissues as local signals and between tissues as remote signals. The microRNAs identified in this study provide a starting point for functional tests of specific microRNAs and their targets as we build a more comprehensive understanding of the regulation of complex social behaviors.

LIST OF ABBREVIATIONS

3´UTR - 3´ untranslated region
AChE-2 - acetylcholinesterase
ANOVA - analysis of variance
dsRNA - double stranded RNA
ftz-f1 - fushi tarazu factor 1
GEO - Gene Expression Omnibus database
GFBr - control forager brain
GFFb - control forager fat body
GFP - green fluorescent protein
HR46 - hormone receptor-like in 46
IRS - insulin receptor substrate
JH - juvenile hormone
Jhi-26 - juvenile hormone inducible protein 26
Map - mapmodulin
MIAME - Minimum Information About a Microarray Experiment
miR - microRNA
NCBI - National Center for Biotechnology Information
PDK1 - phosphoinositide-dependent kinase-1
PI3K - phosphoinositide 3-kinase
PIP5K - phosphatidylinositol-4-phosphate-5-kinase
PTEN - phosphatase and tensin homolog
RNAi - RNA interference
SD - standard deviation
SE - standard error

TYR - tyrosinase receptor

USP - ultraspiracle

VFBr - knockdown forager brain

VFFb - knockdown forager fat body

Vg – vitellogenin

APPENDIX

Table A1 Honey bee protein-coding genes described to be involved in behavioral maturation. Their predicted or validated 3´ untranslated region (UTR) were recovered from NCBI-GenBank and used for microRNA target prediction.

Table A2 Sequences of the most relevant honey bee mature microRNAs found in this study and used for target prediction analysis. Sequences were recovered from miRBase, release 17 (http://www.mirbase.org).

Table A3 List of 72 honey bee microRNAs undetected in this study (based on information available in miRBase release 17).

Table A4 Top 10 housekeeper microRNA candidates in brain and fat body tissues. By means of T test (see p-value) and Log2 ratio, expression level (Mean±SD) of each microRNA is similar between compared groups of the same tissue. However, although expression levels of the highlighted microRNAs (with black background) are similar between both tissues, they are differentially expressed according to an ANOVA comparison considering $p<0.05$. The only exception is ame-miR-263, the expression of which is similar when all 4 groups were compared (ANOVA, $p>0.09$).

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AUTHOR CONTRIBUTIONS

The study was conceived by FMFN, NSM, ZLPS and GVA. FMFN, NSM and GVA designed research. FMFN and NSM conducted the study. FMFN, KEI, NSM and GVA analyzed the data. FMFN, KEI and GVA wrote the manuscript. All authors contributed to discussions of the data and edited the manuscript. All authors read and approved the final version of the manuscript.

COMPETING INTERESTS

No competing interests declared.

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Table 1 Highly expressed microRNAs (averaged signal above 1,000 intensity units) in brain (Br) and fat body (Fb) tissues for control (GF) and *Vg* knockdown (VF) groups. MicroRNAs that are highly expressed in a non-tissue specific manner are marked in bold text.

GFBr	Mean	VFBr	Mean	GFFb	Mean	VFFb	Mean
miR-252	41,511	**miR-252**	30,582	**miR-8**	39,176	**miR-8**	39,658
miR-87	21,817	**miR-184**	18,716	**miR-276**	27,282	**miR-276**	28,091
miR-184	21,038	**miR-87**	17,790	**miR-2**	14,010	**miR-2**	15,804
miR-276	15,041	**miR-276**	15,982	**miR-307**	10,916	**miR-307**	10,974
miR-317	13,069	**miR-317**	14,047	**miR-317**	8,859	**miR-317**	8,880
miR-2796	7,638	miR-2796	9,850	**miR-277**	7,932	**miR-277**	7,522
miR-277	6,042	**miR-34**	6,853	**bantam**	5,919	**miR-34**	5,366
miR-2	5,749	**miR-2**	6,314	**miR-87**	5,148	**bantam**	5,363
miR-14	4,795	**miR-277**	5,654	**miR-34**	4,942	**miR-87**	4,912
miR-34	4,709	miR-210	5,122	miR-12	3,779	**miR-13b**	3,156
miR-13b	4,234	**miR-11**	3,899	**miR-184**	3,622	miR-12	3,116
miR-7	3,416	**miR-13b**	3,344	miR-1	3,298	**miR-184**	3,076
miR-11	3,138	miR-14	3,306	**miR-13b**	2,937	miR-3739	2,885
miR-307	2,570	miR-7	3,042	miR-275	2,334	miR-275	2,580
miR-210	2,542	**miR-307**	2,573	miR-750	2,115	miR-279c	2,218
miR-8	1,929	**miR-8**	1,658	miR-279c	1,956	miR-1	2,077
miR-29b	1,464	miR-29b	1,268	**miR-11**	1,876	**miR-11**	1,936
bantam	1,299	**bantam**	1,132	**miR-252**	1,384	**miR-252**	1,360
let-7	1,106	-	-	miR-279	1,234	miR-279	1,330
miR-932	1,086	-	-	miR-3477	1,082	miR-316	1,144
-	-	-	-	-	-	miR-305	1,041

Table 2 Differentially expressed microRNAs between vitellogenin-supressed fat bodies of forager workers (VFFb) and their respective same-aged controls (GFFb). T-tests were used to calculate statistical significance (p-value).

Effect	microRNA	p-value	GFFb	VFFb	Fold change
			Mean	Mean	
Down-regulated	ame-let-7	7.79E-03	555	345	1.6
	ame-miR-281	4.32E-02	160	78	2.0
	ame-miR-750	9.60E-02	2,115	727	2.9
Up-regulated	ame-miR-3739	1.19E-03	371	2,885	7.8
	ame-miR-3776	1.41E-03	64	373	5.9
	ame-miR-3796	9.51E-03	42	94	2.3
	ame-miR-316	1.07E-02	759	1,144	1.5
	ame-miR-3718a	3.74E-02	103	138	1.3
	ame-miR-3749	8.08E-02	36	59	1.6
	ame-miR-3745	8.84E-02	93	148	1.6
	ame-miR-133	9.12E-02	406	551	1.4

Table 3 Differentially expressed microRNAs between vitellogenin-supressed brains of forager workers (VFBr) and their respective same-aged controls (GFBr). T-tests were used to calculate statistical significance (p-value).

Effect	microRNA	p-value	GFBr	VFBr	Fold change
			Mean	Mean	
Down-regulated	ame-miR-252	1.54E-02	41,511	30,582	1.4
	ame-miR-1	3.80E-02	319	148	2.2
	ame-miR-375	2.99E-02	53	36	1.5
Up-regulated	ame-miR-989	3.35E-02	89	135	1.5
	ame-miR-92a	4.42E-02	50	68	1.4
	ame-miR-31a	4.68E-02	95	160	1.7
	ame-miR-3049	6.18E-02	250	377	1.5

Figure captions

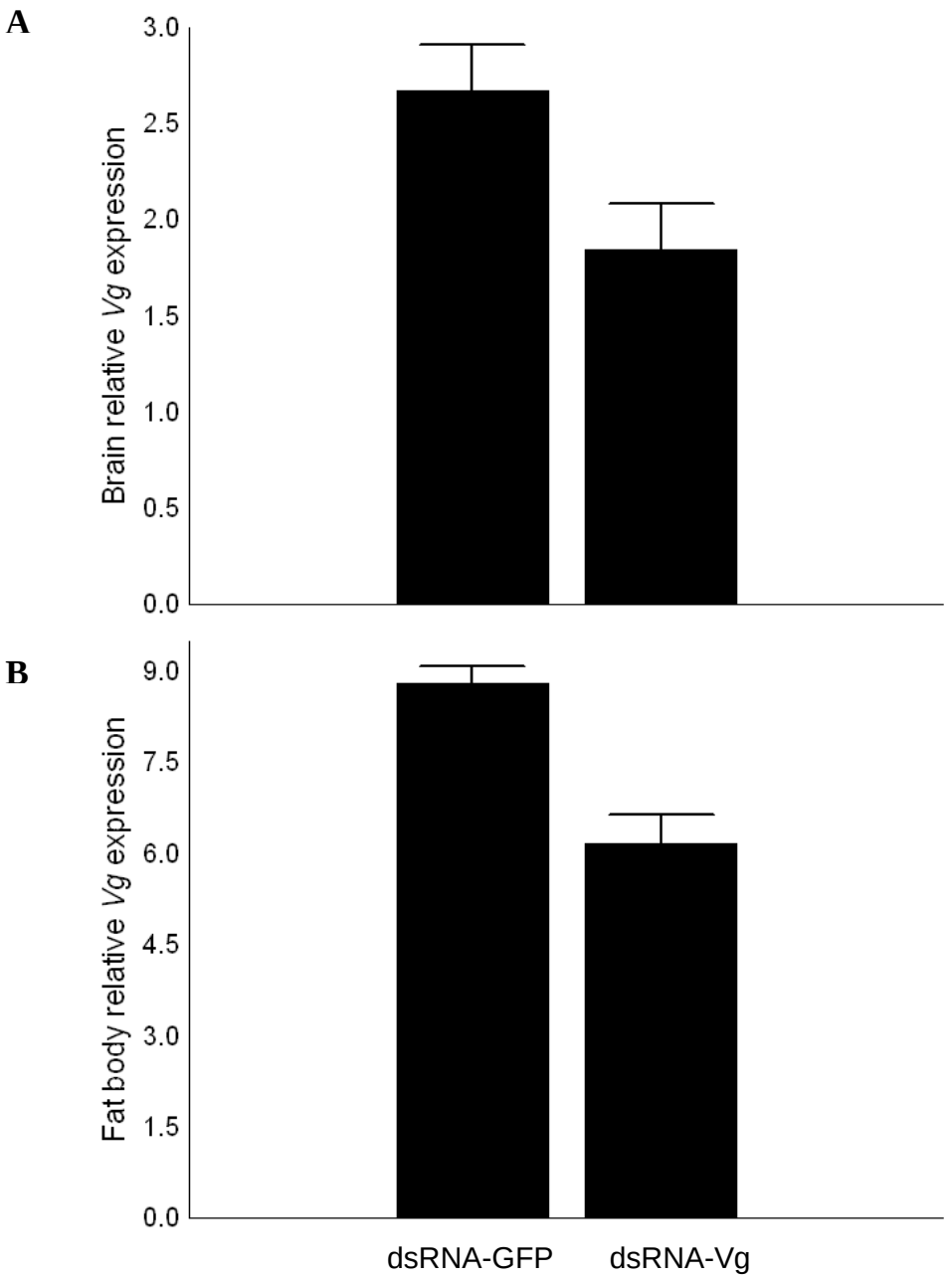
Fig. 1 RNAi-mediated *vitellogenin* silencing. *Vitellogenin* mRNA levels (Mean ± SE) in (A) brain (n=30 for dsRNA-GFP and dsRNA-Vg) and (B) fat body (n=30 for dsRNA-GFP and dsRNA-Vg) tissues of foragers are shown as log2-transformed relative quantities (RQ). See text for statistics.

Fig. 2 Number of microRNAs with common or treatment- or tissue-specific expression: GFBr (control forager brain), VFBr (knockdown forager brain), GFFb (control forager fat body), and VFFb (knockdown forager fat body). Venn diagram was generated using http://bioinfogp.cnb.csic.es/tools/venny/index.html and microRNA microarray-based data (this study).

Fig. 3 Integrative mRNA-microRNA predictive networks. Coding-genes are represented by squares containing gene name abbreviations. MicroRNAs are indicated by circles containing microRNA identification numbers. Down-regulated microRNAs in knockdown forager fat body are marked in green. Up-regulated microRNAs in knockdown forager fat body are marked in red. Down-regulated microRNAs in knockdown forager brain are marked in yellow. Up-regulated microRNAs in knockdown forager fat body are marked in blue.

875 **Fig. 1**

876



880 **Fig. 2**

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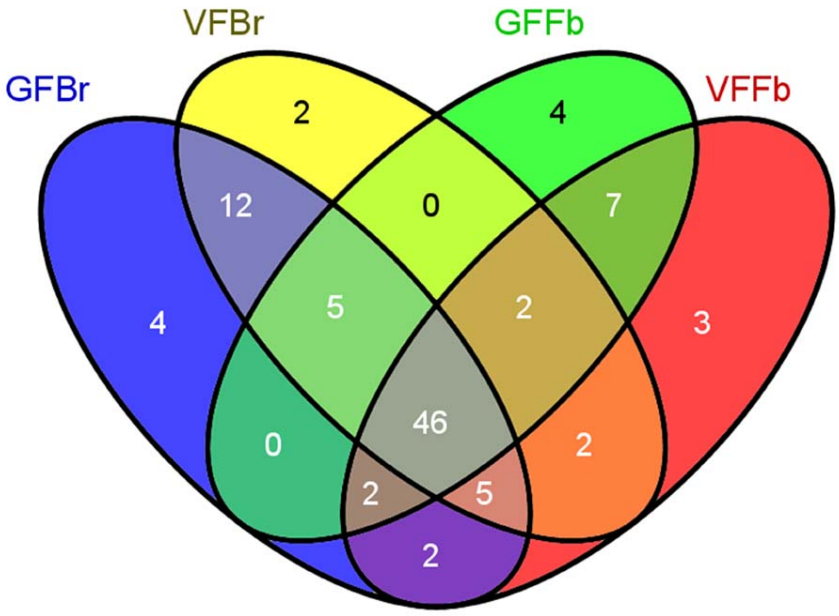

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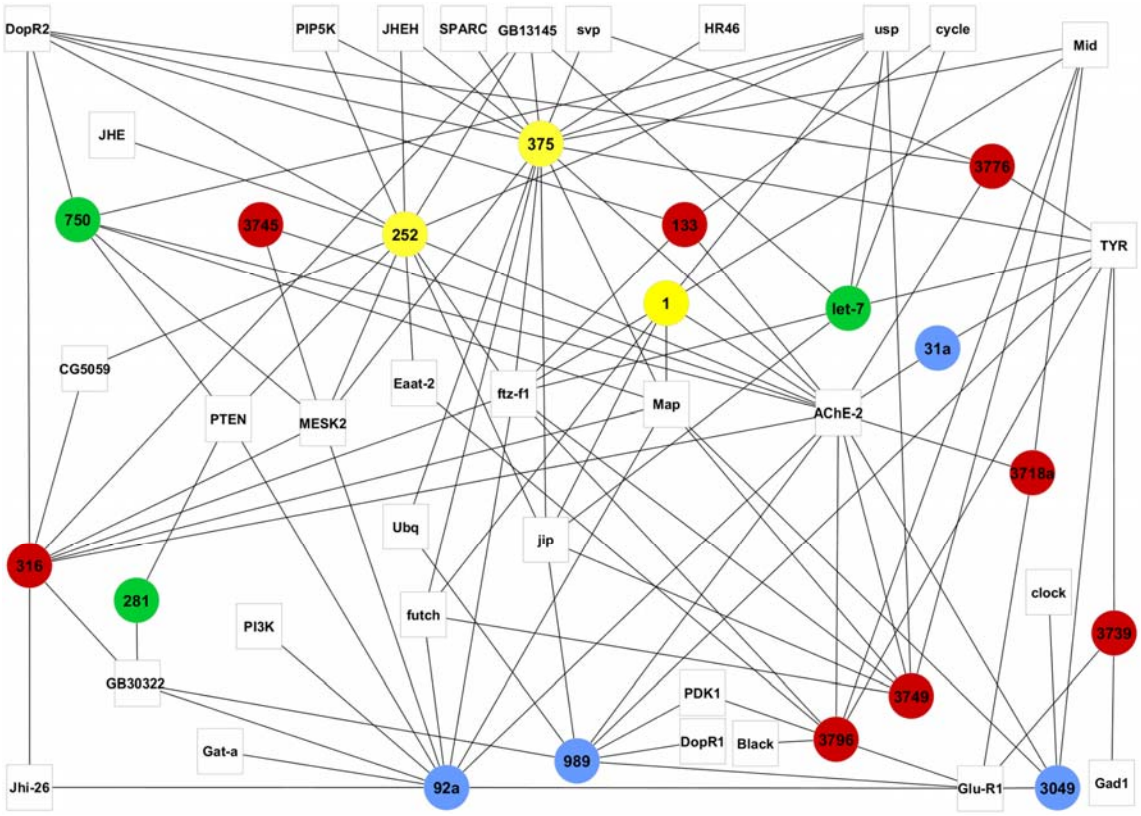
883 **Fig. 3**

884



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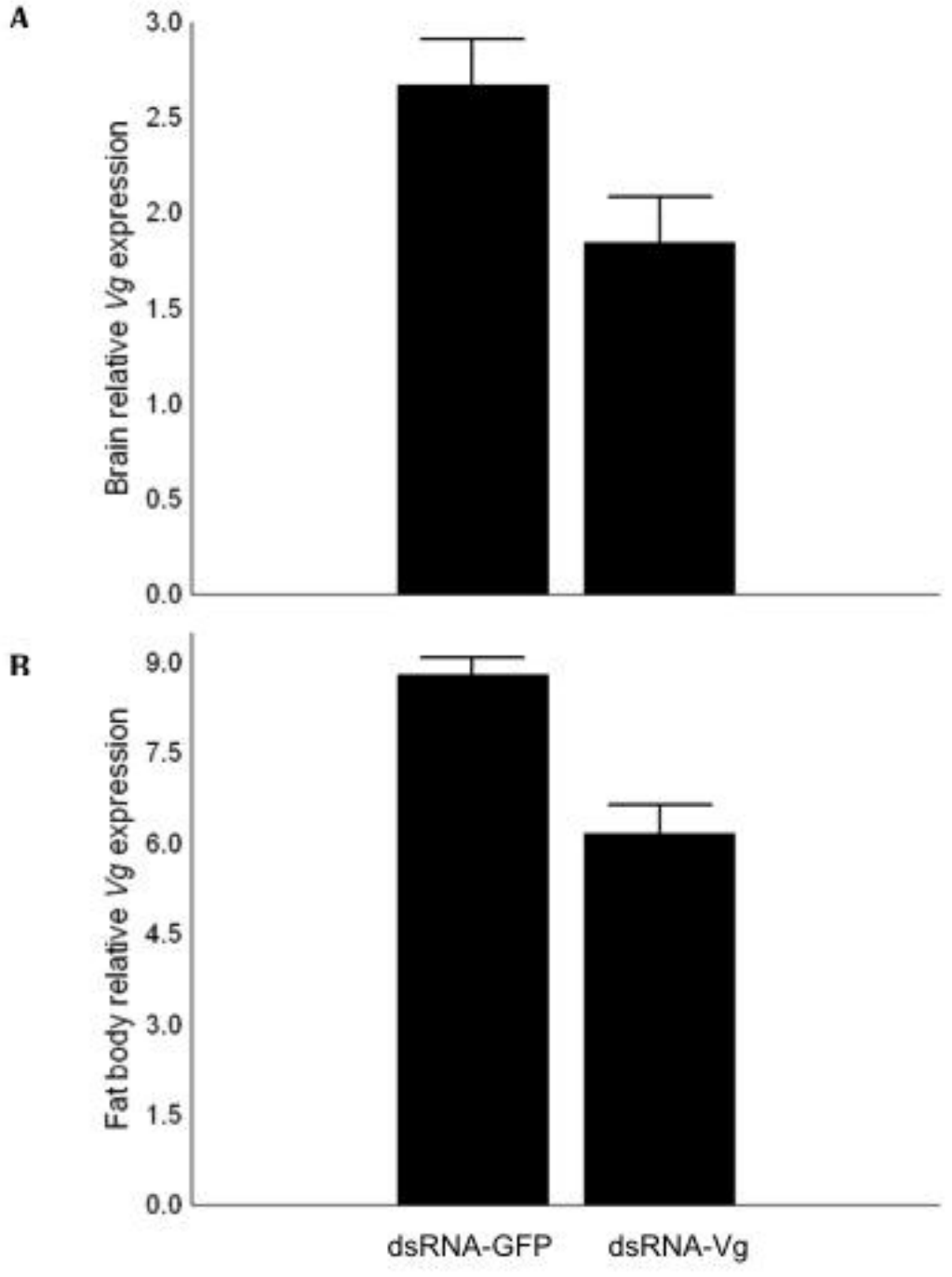
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Brain relative Vg expression
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2.0
1.5
1.0
0.5
0.0
B
Fat body relative Vg expression
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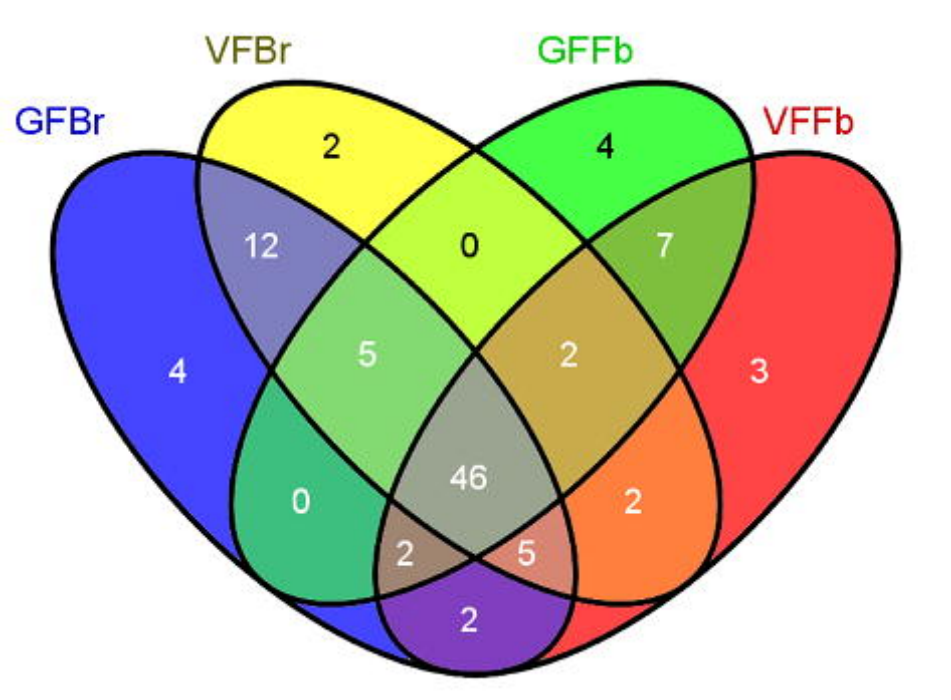
7.5
6.0
4.5
3.0
1.5
0.0
dsRNA-GFP
dsRNA-Vg


VFBr
GFFb
GFBr
VFFb
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7
4
5
2
3
46
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2
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5
2

Table 1

GFBr	Mean	VFBr	Mean	GFFb	Mean	VFFb	Mean
miR-252	41,511	**miR-252**	30,582	**miR-8**	39,176	**miR-8**	39,658
miR-87	21,817	**miR-184**	18,716	**miR-276**	27,282	**miR-276**	28,091
miR-184	21,038	**miR-87**	17,790	**miR-2**	14,010	**miR-2**	15,804
miR-276	15,041	**miR-276**	15,982	**miR-307**	10,916	**miR-307**	10,974
miR-317	13,069	**miR-317**	14,047	**miR-317**	8,859	**miR-317**	8,880
miR-2796	7,638	miR-2796	9,850	**miR-277**	7,932	**miR-277**	7,522
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miR-932	1,086	-	-	miR-3477	1,082	miR-316	1,144
-	-	-	-	-	-	miR-305	1,041

Table 2

Effect	microRNA	p-value	GFFb	VFFb	Fold change
			Mean	Mean	
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	ame-miR-750	9.60E-02	2,115	727	2.9
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	ame-miR-3776	1.41E-03	64	373	5.9
	ame-miR-3796	9.51E-03	42	94	2.3
	ame-miR-316	1.07E-02	759	1,144	1.5
	ame-miR-3718a	3.74E-02	103	138	1.3
	ame-miR-3749	8.08E-02	36	59	1.6
	ame-miR-3745	8.84E-02	93	148	1.6
	ame-miR-133	9.12E-02	406	551	1.4

Table 3

Effect	microRNA	p-value	GFBr	VFBr	Fold change
			Mean	Mean	
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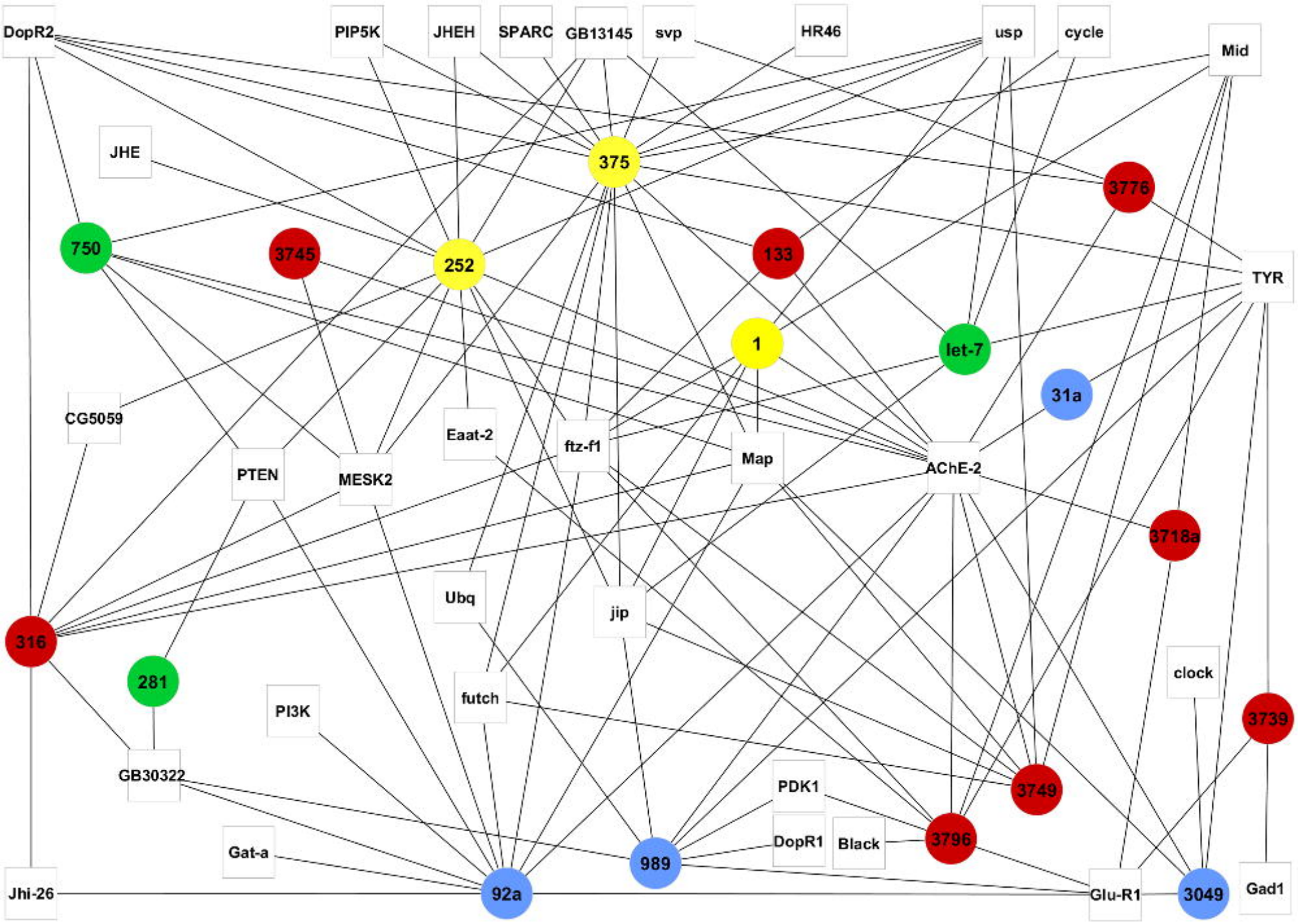
	ame-miR-375	2.99E-02	53	36	1.5
Up-regulated	ame-miR-989	3.35E-02	89	135	1.5
	ame-miR-92a	4.42E-02	50	68	1.4
	ame-miR-31a	4.68E-02	95	160	1.7
	ame-miR-3049	6.18E-02	250	377	1.5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ftz-f1
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