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Social immunity in honeybees (*Apis mellifera*): transcriptome analysis of varroa-hygienic behaviour

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Abstract

Honeybees have evolved a social immunity consisting in the cooperation of individuals to decrease disease in the hive. We identified a set of genes involved in this social immunity by analyzing the brain transcriptome of highly varroa-hygienic bees, who efficiently detect and remove brood infected with the *Varroa destructor* mite. The function of those candidate genes does not seem to support a higher olfactory sensitivity in hygienic bees, as previously hypothesized. However, comparing their genomic profile with those from other behaviours suggests a link with brood care and the highly varroa-hygienic Africanized honeybees. These results represent a first step toward the identification of genes involved in social immunity and thus provide first insights into the evolution of social immunity.

Introduction

In insects, defence against pathogens relies mainly on an efficient innate immunity that is comprised of both cellular and humoral reactions (e.g. phagocytosis, melanisation and secretion of antimicrobial peptides) (Hoffmann, 2003; Siva-Jothy *et al.*, 2005). However, when living in groups, like social insects, the presence of stored resources and the close living quarters increase both the attractiveness for pathogens and disease transmission (Schmid-Hempel, 1998). Therefore, higher capacities to respond and defend against pathogens could be expected. A genome-wide analysis of immunity in the honeybee *Apis mellifera* actually showed that they possess only one-third the number of immune response genes known for solitary insects (i.e. fruit fly, mosquito and moth) (Evans *et al.*, 2006). Since the reduction in genes involved the different steps of the immune response, honeybees appear to have a reduced capacity to respond and defend against pathogens.

Despite the wide-range of pathogens to which social insects are exposed, they successfully resist disease, suggesting that other defence mechanisms might be involved. Indeed, in addition to the individual defences, social insects developed group-level strategies against parasites and pathogens. Such social immunity includes grooming, the use of antimicrobial materials for nest construction (e.g.

resin) (Christe *et al.*, 2003; Simone *et al.*, 2009), social fever (Starks *et al.*, 2000) or nest hygiene (see (Cremer *et al.*, 2007) for a review). Since their description, many studies explored the behavioural mechanisms of those collective immune defences against pathogens (see the following reviews (Wilson-Rich *et al.*, 2009; Cremer *et al.*, 2007; Cremer & Sixt, 2009)), but the molecular basis and pathways remain largely unknown. The identification of genes that influence social immunity would not only improve our understanding of its mechanisms but also provide new insights into the evolution of collective defence in insect societies. In the honey bee genome, genes involved in social immunity might have replaced genes from individual immunity that have been lost during evolution of sociality and might be key factors for the defence against diseases.

In honeybees, a well-known behavioural trait to fight against pathogens is hygienic behaviour, which involves the identification and removal of dead or infected larvae. Using the honeybee genome, we attempted to identify genes involved in this well-characterized behaviour, a main component of social immunity. Hygienic behaviour is directed toward dead brood, but also brood infected with bacteria or fungi (Boecking & Spivak, 1999), the greater wax moth, *Galleria mellonella* (Villegas & Villa, 2006; Corrêa-Marques & De Jong, 1998), the small hive beetle, *Aethina tumida* (Ellis *et al.*, 2003; Neumann & Härtels, 2004) or the mite *Varroa destructor*, the parasite with the most pronounced effect on honeybee colonies. Indeed, development of the varroa population, reproducing in brood cells, often lead to the death of the colony (Le Conte *et al.*, 2010). Since hygienic behaviour is genetically-controlled (Rothenbuhler, 1964b; Rothenbuhler, 1964a), selective breeding for varroa resistance through increased varroa-hygienic behaviour offers a sustainable means for controlling mite parasitism among the others factors contributing to a stable parasite-host relationship (Rosenkranz *et al.*, 2010). Accordingly, different varroa-hygienic bee lines have been successfully bred (Harbo & Harris, 1999; Büchler *et al.*, 2010; Harbo & Harris, 2005a; Boecking & Spivak, 1999; Spivak & Reuter, 2001a) with one of those characterized by a low percentage of reproducing varroa mites in the hive. Bees from those colonies display an effective removal of varroa-infested pupae from capped brood cells, which limits varroa infestation rate and reproduction (Harbo & Harris, 2005b; Harbo & Harris, 2009). This genetic line called varroa-sensitive hygiene (VSH) represents thus a good model for uncovering genes

involved in social immunity. Harbo and Harris (2005a) suggested only a few genes to be involved in VSH behaviour, however the molecular basis still remained to be deciphered. We therefore compared directly brain-specific gene expression profiles of bees selected for their high rate of hygienic behaviour (VSH+) to bees displaying a low rate of hygienic behaviour (VSH-). We used a honeybee oligonucleotide microarray, based on gene predictions and annotation from the honeybee genome sequencing project (Honeybee Genome Sequencing Consortium, 2006). Finally, in order to further characterize the VSH trait, we compared the brain gene expression profile of VSH bees to genomic profiles from other well-defined behavioural phenotypes.

Results

VSH behaviour

Four VSH+ and 4 VSH- colonies were selected from 24 colonies of a breeding program to present high or low rate of varroa sensitive hygienic expression. The percentage of varroa−infected brood removed by workers in each colony is shown in table 1.

VSH genes

A total of 39 transcripts were found to be differentially expressed in the brain of VSH+ and VSH- bees at a FDR<0.05 (table 2). Among them, 14 were significantly upregulated and 25 downregulated in VSH+. The magnitude of the differences in expression ranged from 1.61 to 2.75 for the upregulated genes and from 1.47 to 2.69 for downregulated genes. The significance of the variation in gene expression seemed robust since 3 exons of the *Dscam* gene found to be differentially expressed were consistently downregulated in VSH+. Microarrays were further validated by a quantitave RT-PCR (qRT-PCR) analysis (Figure 1). Ratios of mean expression levels (VSH-/VSH+) from qRT-PCR analyses were similar to microarray ratios: *Antdh*: 1.47, *lop1*: 1.45 and *Arrestin2*: 1.35.

Overlap between VSH and others behavioural gene sets

To further characterize the VSH genomic profile, we compared the brain genomic profile of VSH bees to 8 relevant gene sets that are associated to different behavioural phenotypes: foraging behaviour, performance of vibration signal (behavioural communication), bees stimulated by queen mandibular pheromone, brood pheromone or alarm pheromone and finally genes differentially expressed between Africanized and European honeybees.

From 3 to 12 genes were overlapping between the VSH and one of the behavioural gene sets (table 3). We then determined whether the different overlaps were higher than the number of genes expected to overlap by chance alone. The VSH gene sets significantly overlapped with the gene set that is induced by the brood pheromone and the gene sets of vibrating bees and Africanized honeybees. Because of the "low" number of VSH genes, the number of overlapping genes was small and thus we could not perform statistical tests to determine the directional bias of the different overlaps. However, the table 2 indicates that there is a slight tendency for genes that were upregulated in one of the gene sets to be downregulated in the VSH+ sets and inversely (table 4).

Discussion

Since group-level defence against pathogens mostly involves collective behaviour, analyzing genes involved in social immunity comes down to the identification of behavioural genes. By comparing VSH+ bees, characterized by a high performance level of hygienic, to VSH- bees we expected to find some genes to be differentially expressed in VSH bees. The identification of 39 transcripts that are differentially expressed between VSH+ and VSH- bees confirmed this hypothesis.

Candidate genes for social immunity

VSH bees are characterized by their high ability to detect and remove varroa-parasitized brood (Harbo & Harris, 2005b). Harris (2007) suggested that VSH bees are either more sensitive to olfactory-based stimuli associated with parasitized brood or have a lower response threshold to mite density (initiate hygienic behaviour at a lower mite density), which is not related to a higher olfactory sensitivity. This latter assumption comes from the fact that, at very low mite density, there is no difference in hygienic behaviour between resistant (hygienic) Africanized bees and non-resistant European bees (Vandame *et*

al., 2000). Hygienic behaviour would be initiated when a critical threshold of mite infestation rate is reached (Vandame *et al.*, 2002); threshold that is lower in resistant bees. The identification of genes that are differentially exp ressed between VSH and control bees might give some clues on the mechanisms of hygienic behaviour.

Among the genes upregulated, *PRL-1* encodes a protein tyrosine phosphatase. In *Drosophila*, its function is unknown but this gene belongs to the category of immediate-early genes (Diamond *et al.*, 1994), which are genes that play an essential role in neural morphogenesis and functioning in mammals (Paul & Lombroso, 2003). This key regulatory component in signal transduction pathways might therefore be important to the development of hygienic behaviour. *CG17323* has been found to be involved in diverse functions like circadian rhythm (Ceriani *et al.*, 2002), aggression (Edwards *et al.*, 2009) and response to ethanol exposure (Morozova *et al.*, 2009). In addition, *CG17323* and *CG31004* (also upregulated in VSH bees) are both affected by nutrient intake (Zinke *et al.*, 2002). But, we don't have enough information on VSH behaviour to establish a link between this behaviour and those functions. The function of the cytochrome P450 *Cyp4g11* is unknown. However, the *Drosophila* ortholog *Cyp4g15* has been found to be predominantly expressed in the brain of *Drosophila* flies, where it might be involved in the ecdysteroid metabolism rather than detoxifying xenobiotics (Maibeche-Coisne *et al.*, 2000). This suggests that in the bee brain *Cyp4g11* might catalyse a reaction in some metabolic pathways that could be involved in hygienic behaviour. Another important gene is *Dscam* (*Down syndrome cell adhesion molecule*), an immunoglobulin superfamily member essential for wiring the brain. The molecular diversity of *Dscam* (38,016 alternative splicing forms in *Drosophila*) is essential for mediating axon guidance and the neuronal wiring specificity (Chen *et al.*, 2006). The significant downregulation of 3 *Dscam* exons suggests therefore a different wiring of neuronal networks in the brain of VSH bees.

Two others genes are both involved in visual signalling: the *long-wavelength sensitive opsin 1* and *arrestin 2* (Dolph *et al.*, 1993). In insects, *arrestin 2* is also expressed in olfactory neurons and is believed to be important for a normal olfactory physiology (Walker *et al.*, 2008; Merrill *et al.*, 2002). Long-wave opsins have been described in the optic lobes (Lampel *et al.*, 2005) but in honeybees this gene is solely expressed in the compound eyes (Velarde *et al.*, 2005), which suggests that small

amount of retinal tissue was associated with the dissected brain tissue. Since, the brood is reared in the dark inside the hive, the downregulation of the visual signalling cascades would indicate that VSH bees spend more time within the hive than non hygienic bees of the same age.

The downregulation of the *odorant binding protein 3* (*obp3*), a member of the *Obp* family first described as carrier of odorant molecule in olfactory tissue, could support a role in the olfactory sensitivity of VSH bees. However, its expression in the brain and in other body parts, with the exception of the antennae (Foret & Maleszka, 2006), suggests that *obp3* is involved in other physiological functions. Similar conclusion can be drawn with *Antdh*, which was first described in the antennae of *Drosophila* as being involved in odorant turnover (Wang *et al.*, 1999). However, the downregulation of *Ets65A*, a candidate gene likely to account for olfactory behaviour in the smell-impaired mutant lines *65A* (Anholt & Mackay, 2001), suggests that VSH bees express a different level of odour-guided behaviour than others bees.

The lack or downregulation of genes involved in olfaction does not seem to support the hypothesis that VSH bees are more sensitive to olfactory-based stimuli associated with parasitized brood. The alternative explanation focusing on the tolerance level to mite density would be more likely. However, to reject definitely the hypothesis of a higher olfactory sensitivity, the analysis of peripheral tissues, like antennae, should be performed. Indeed, insect behaviour can be dramatically affected by changes in expression of genes that are antennal-specific (Wang *et al.*, 2008).

Relationship between VSH and others behavioural phenotypes

The overlap analyses between different genomic profiles provided a better characterization of VSH behaviour. We did not find a significant overlap between VSH and forager brain gene expression profile. This could be due to age differences between our VSH samples and old foragers, however our finding that those two phenotypes are not linked is supported by Goode *et al.* (2006), who found that hygienic behaviour is independent of foraging ontogeny. Interestingly, the number of genes overlapping between VSH and vibrating bees was higher than expected by chance alone. This "modulatory communication signal" induces a non-specific increase in worker activity (Schneider &

Lewis, 2004) and is produced by a restricted number of bees, primarily successful forager collecting food outside the hive (Schneider & Lewis, 2004). Since VSH bees tend to display an inverse gene expression pattern than vibrating bees, this suggests that hygienic bees might spend more time in inside-hive activity. Another interesting result is the significant overlap between the genomic profiles of VSH bees and bees stimulated by BP. This pheromone emitted by larvae stimulates brood care (feeding) (Le Conte *et al.*, 2001) but also the capping of brood cells containing mature larvae (Le Conte *et al.*, 1990). This raises the question of whether BP can affect hygienic behaviour. Interestingly, BP tends to inhibit genes that are upregulated in VSH bees and inversely, which suggests that hygienic bees don't spend time on feeding larvae and/or have a higher propensity to not cap opened brood cells. Indeed, hygienic behaviour is performed on mite infested pupae, especially young pupae (3–5 days post capping) (Harris, 2007). Since, the QMP regulate similar behaviour than BP (Alaux *et al.*, 2010), similar conclusion could be drawn with this pheromone. Finally, our results indicate that Africanized honeybees (AHB) and VSH genomic profiles share a significant number of genes despite the age difference between bees from both data sets. This is particularly appealing given that AHB are tolerant to varroa compared to European honeybee (EHB). One explanation highlights the fact that, similarly to VSH bees, AHB are more able to remove infested brood than EHB (Guzmán-Novoa *et al.*, 1999; Vandame *et al.*, 2000) but see (Mondragon *et al.*, 2005). The shared behavioural and molecular traits would suggest that the mechanism underlying the tolerance to varroa is similar between AHB and VSH bees. But among the overlapping genes, few are regulated in the same direction in both AHB and VSH bees. Genes that are common to both strains and follow the same pattern of regulation might represent key factors of the molecular resistance to varroa.

Comparisons to others studies

A previous study identified genes associated to another type of mite resistance displayed by some French colonies (Navajas *et al.*, 2008). Only two genes were both differentially expressed in VSH and those *Varroa* surviving bees (VSB) that are naturally tolerant to *Varroa* infestation. *BB160006A10F07* and *Dscam exon 10.9 upregulated and* downregulated respectively in VSH were both downregulated in VSB+ compared to VSB-. While this low number was surprising, on one hand the analysis of VSB

individuals was performed on whole pupae and the present study was performed with bee brains and on the other hand the mechanisms of varroa-tolerance in the French strains hasn't been clearly established yet and it is possible that it differs from the hygienic behaviour performed by VSH bees. Nevertheless, since *Dscam exon 10.9* was also downregulated in VSH bees, this gene might be important for the resistance to varroa parasitism.

A different approach based on genetic mapping has been used in order to identify the genetic component of hygienic behaviour. By performing a quantitative trait loci (QTLs) analysis, Lapidge *et al.* (2002) first found that hygienic behaviour is influenced by many different loci but recently, in an attempt to provide marker-assisted selection for hygienic behaviour, Oxley *et al.* (2010) identify three QTLs that influence the propensity of workers to perform hygienic tasks. QTLs are phenotypically-defined genomic regions associated with variation in a phenotypic trait, which can be large and contain hundreds of candidate genes. However, QTL analysis does not indicate the expression pattern of these genes. Quantitative expression studies like microarray analysis can be used to systematically reduce the list of candidate loci and reveal regulatory variation in genes and pathway signalling. So, combining QTL mapping with transcriptome promises to identify positional candidate genes for a phenotype of interest whose expression varies between lines (Wayne & McIntyre, 2002; Jansen & Nap, 2001; Li & Burmeister, 2005). Unfortunately, none of the genes from those QTLs were found to be differentially expressed in VSH bee indicating that further studies are needed to understand the genetic background of such behaviour. This lack of overlap could come from the different breeding lines of bees that were used in both studies. The QTL mapping was performed with the Minnesota (USA) Hygienic bee stock (Boecking & Spivak, 1999; Spivak & Reuter, 2001a) and the transcriptome analysis with VSH bees from Lousiana (USA) (Harbo & Harris, 1999). In addition, each line was obtained with different methods of selection: the VSH line was specifically selected for its resistance to varroa but the selection of the Minnesota line was not varroa specific and included a broad spectrum of pathogens causing notably the American foulbrood (Spivak & Reuter, 2001b) and chalkbrood diseases (Spivak & Reuter, 1998). So, combining both techniques on the same bee lines would promise a finer identification of candidate genes involved in hygienic behaviour.

Conclusion

It has been shown in ants and honeybees, that social defence reduces the investment of individuals in their own immune function (Castella *et al.*, 2008; Simone *et al.*, 2009). This indicates that the evolution of sociality might have lead to the loss of immune genes, as indicated by the analysis of the honeybee genome (Evans *et al.*, 2006), to the expense of behavioural genes involved in group level defence. Since behavioural genes are often pleiotropic (Greenspan, 2001; Sokolowski, 2001), those genes might be both involved in social immunity and other behavioral phenotypes (see table 3 and 4), which might reduce the physiological investment in the defence against pathogens compared to less-pleiotropic immune genes. This study represents a first step towards understanding the genomic basis of social immunity. Future research will have to test the functions of those candidate genes in collective defences.

Experimental procedures

Honeybee rearing and selective breeding

The honeybee colonies used in this study presenting high or low rate of varroa-hygienic behaviour were the same than those previously obtained by Harbo and Harris for studying the responses to varroa by honeybees with different levels of varroa-sensitive hygiene (Harbo and Harris, 2009). Briefly, we produced a group of 26 colonies of European mix of *A. mellifera* subspecies typically found in North America that presented different levels of varroa-sensitive hygiene. Fourteen colonies had queens produced from a line with 100% expression of VSH (high line, H), and twelve had queens produced from a line that did not express VSH (low line, L). Each of the 26 queens was backcrossed to a single drone produced by an HL queen (a daughter of both lines H and L). Since the HL queen had half of the VSH alleles, she produced drones that ranged from having 0 to 100% of the VSH alleles. With this design, the fourteen colonies in group H should have 50 – 100% of the alleles for VSH and the twelve in group L should have 0 – 50%. Therefore, we speculated that the lowest of the low group had none of the genes for VSH and the highest of the high group had all of the genes for

VSH. Each queen was introduced into colonies that were evaluated for varroa hygienic ability as in (Harbo and Harris, 2009), after the test queens had been laying in their colonies for at least 6 weeks. In this way all worker bees that were between 0 and 3 weeks old were daughters of the test queen. The varroa hygienic ability corresponded to the percentage of mite infested pupae that were removed by the colony. Thanks to this method, we were able to sample high and low varroa hygienic bees for gene expression analysis. Some bees were marked at the emergence and returned to their colonies of origin. We then collected and flash froze in liquid nitrogen 14 days-old bees from 4 VSH+ colonies and 4 VSH- colonies (control) (table 1). To avoid any bias toward a specific behaviour, bees found on the brood were randomly collected. VSH+/VSH- colonies were directly compared with microarrays analysis using a dye swap method.

Brain dissection and mRNA extraction

Whole heads were partially freeze-dried at -80°C (0.0005 mmbar for 140 min) to facilitate brain dissection. Dissections were performed on dry ice to prevent brain thawing. For each sample, 10 frozen bee brains were pooled and grounded on dry ice. We added 600µl of RLT buffer containing 6 µl of ß-Mercaptoethanol to the powder to disrupt the tissue. RNA extraction was carried out as indicated in the Qiagen RNeasy kit for total RNA (Qiagen, Courtaboeuf, France). RNA isolated from 10 pooled brains then used for microarrays analysis.

Microarrays and data analysis

For the preparation of the labelled Cy3- and Cy5- aRNA target, total RNA (1 µg) was amplified with the Amino Allyl MessageAmp™ II aRNA Amplifcation kit (Ambion, Courtaboeuf, France), according to manufacturer's instructions. Then, aaRNA samples were dried in SpeedVac and resuspended in 9 µl Coupling buffer (0.1M carbonate buffer pH 9). We added to each sample 11 µl of one of the N-Hydroxysuccinimide ester Dye (Cy3 and Cy5) diluted in DMSO (CyDye Post-Labelling Reactive Dye, GE Healthcare, Montpellier, France). Samples were incubated at room temp in the dark for 30 min with shaking and 4.5 µl 4 M hydroxylamine was added to each sample. Samples were incubated at room temperature for 15 min in the dark. Nuclease-free water was added to each sample

to bring the volume to 100 µl. Then, we added 350 µl of aRNA Binding Buffer and 250 µl 100% ethanol to each aRNA sample. Samples were applied on the column and centrifuge 10,000 g for 1 minute. We added 650 µl Wash Buffer and centrifuge 1 minute at 10,000 g. After discarding the flow-through, samples were centrifuged again 1 minute at 10,000 g. Samples were eluted twice in 50 µl nuclease-free water. Cy3 and Cy5 reactions were equally combined and fragmented according to kit instructions.

Before hybridization, slides were passed quickly through steam and placed in a UV linker at 100 mJ. Before pre-hybridization, slides were plunged twice in 0.2% SDS and immediately shaken vigorously for 1 min. They were then washed twice in distilled water for 1 min. The two labelled aRNA were added to 4X hybridization buffer (GE Healthcare, Montpellier, France) in a final concentration of 50% formamide, denaturated at 95°C for 5 min and applied to the microarrays in individual chambers of an automated slide processor (GE Healthcare, Montpellier, France). Hybridization was carried out at 37°C for 16 h. Hybridized slides were washed at 37°C successively with 1X SSC, 0.2% SDS for 20 min, twice with 0.1X SSC, 0.2% SDS for 10 min, with 0.1X SSC for 1 min and with isopropanol before air drying.

Microarrays were immediately scanned at 10 µm resolutions in both Cy3 and Cy5 channels using a GenePix 4200AL scanner (Molecular Devices, St. Grégoire, France). The scanning was done with a variable PMT voltage to obtain maximal signal intensities (<0.1% probe saturation). ArrayVision software (GE Healthcare, Montpellier, France) was used for feature extraction. Spots with high local background or contamination fluorescence were flagged manually. A local background was calculated for each spot as the median values of the fluorescence intensities of 4 squares surrounding the spot. This background was subtracted from the foreground fluorescence intensity.

No background correction was performed. No spatial bias in the quality analysis was detected so a Loess normalisation was performed for all microarrays to correct dyes effect and technical bias. Tests of differential expression were conducted using the Siggenes package from Bioconductor and the

Significance Analysis of Microarrays (SAM) proposed by (Tusher *et al.*, 2001). SAM assigns a score to each gene based on the standard deviation of repeated gene expression measurements. Then, a false discovery rate (FDR) is estimated by permutations of the repeated measurements to obtain a ranking of significantly expressed genes. The Bioarray Software Environment (BASE) (local installation: http://baseprod.igf.cnrs.fr/index.phtml) was used to visualize differential expression for each gene.

Verification by qRT-PCR

In order to validate the microarrays results, qRT-PCR was performed on each sample. The transcript abundance was measured for *lop1, Arrestin2 and Andth* with a Roche LightCycler 480 Real-Time PCR System (Roche Diagnostics, Meylan, France). Their expression levels were then normalized to a housekeeping gene (*BI511718*) and relative to a control sample using $2^{-\Delta\Delta Ct}$ values. Primer sequences (5' to 3') were *lop1* forward: GTTCTCTCTCGGATGGACTA, reverse: GGGACGAAGTAAACCCAAAT; *Arrestin2* forward: CTTGTAAGAGGACGTAAATTGCCTA, reverse: TGAGCATTAACCATTGTCACC; *Antdh* forward: CAATTTAGAAGATTGGCGCTC, reverse: TCCAGGTATGAAAGGCACTC; *BI511718* forward: CTCATCAGTTGTTGGTTCTCCTC, reverse: TCGTTTGGCTCTTCAGTCTTGT.

Overlap between the VSH and other gene expression profiles

We compared the VSH genomic profile to different gene lists previously identified in other honeybees studies. These brain gene expression profiles are specific to foraging behaviour (Alaux *et al.*, 2009b), bees specialized in vibration communication (strong arousal state) (Alaux *et al.*, 2009a), Africanized honeybees (Alaux *et al.*, 2009c) and finally bees stimulated by queen mandibular pheromone (Grozinger *et al.*, 2003), brood pheromone (Alaux *et al.*, 2009b) or alarm pheromone (Alaux *et al.*, 2009c). We calculated a "representation factor" (the number of observed overlapping genes divided by the expected number of overlapping genes) and used an exact hypergeometric probability test to determine whether the overlap between the VSH and another gene set was statistically significant. The expected number corresponds to the product of the number of genes in each list divided by the total number of genes analyzed (Kim *et al.*, 2001).

Web resource

Gene expression data meet Minimum Information About a Microarray Experiment (MIAME) standards and have been deposited at ArrayExpress (www.ebi.ac.uk/arrayexpress): E-TABM-1002.

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Table 1: Microarray pair comparisons of four VSH+ and VSH− colonies. The percentage of varroa−infected brood removed by workers in each colony is indicated. The baseline population of mites in each colony was estimated in 200 worker-brood cells that were 0-3 days post-capping. Then, when the cells were 7-10 days post-capping, the number of uninfested cells was counted giving the brood removal rate (see Harbo and Harris (2009) for more details).

Pair comparisons	VSH +	VSH −
1	100%	17%
2	90%	30%
3	80%	27%
4	100%	33%

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Table 2. Probes differentially expressed in brains of VSH+ and VSH− bees. Corresponding *Drosophila* orthologs, log2 ratio of expression values (VSH+/VSH−) and gene ontology based on fly orthologs (Flybase) are shown. Positive expression values indicate higher expression in VSH+ bees compared to VSH− bees. The sign / indicates the absence of clear *Drosophila* orthologs.

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Probe ID	Description	*Drosophila* ortholog	log2 ratio (VSH+/VSH−)	Gene Ontology
AM01773	NW_001253565.1 SET: UI_EST BI510059	/	1,46	
AM02265	BB160015A20H04	/	1,17	
AM01177	NW_001253491.1 SET: UI_EST BI504108	/	1,12	
AM01915	BB160006A10F07	/	0,74	
AM02211	BB160014A10B11	/	0,71	
AM04119	GB11499	*CG31004*	0,46	cell−matrix adhesion
AM05646	GB13036	/	0,69	
AM01622	BB170002A10A06	/	0,58	
AM00808	DB773117 RIKEN full-length enriched honeybee cDNA library	/	0,49	
AM02131	BB160011B20H01	/	0,63	
AM03470	GB10845	*PRL−1*	0,54	protein tyrosine phosphatase activity; immediate early gene
AM01666	BB170006B20H07	/	0,77	
AM09325	GB16747	*CG17323*	0,81	glucuronosyltransferase activity; inter−male aggressive behaviour
AM01607	NW_001253063.1 SET: UI_EST BI508396	/	0,69	
AM07547	GB14956	*CG6910*	-0,56	inositol oxygenase activity ; oxidation reduction
AM12206	GB19657 (*long wavelength sensitive opsin 1- lop1*)	*Rhodopsin 6*	-0,79	G−protein coupled photoreceptor activity; phototransduction
AM01085	BB170007A20A04	/	-0,60	
AM02392	NM_001040230.1	/	-1,42	
AM03333	GB10708	/	-0,99	
AM03972	GB12522	*Antdh*	-0,68	carbonyl reductase (NADPH) activity ; oxidation reduction
AM05381	GB12766	*Arrestin 2*	-0,74	adaptation of rhodopsin mediated signalling
AM00612	DB739042 RIKEN full-length enriched honeybee cDNA library	/	-0,77	
AM09032	GB16453	*CG32645*	-0,70	transferase activity
AM00604	DB738421 RIKEN full-length enriched honeybee cDNA library	/	-0,69	
AM00955	DB752711 RIKEN full-length enriched honeybee cDNA library	/	-0,61	
AM06645	GB30234	*Ets65A*	-0,72	transcription factor activity
AM12005	GB30242 (*Odorant binding protein 3 - Obp3)*	/	-0,58	odorant binding ; sensory perception of chemical stimulus
AM03226	GB10599	/	-0,80	
AM06202	GB13602	*CG10175*	-0,56	carboxylesterase activity; metabolic process
AM10277	GB17704	/	-0,50	
AM02039	BB160009B10D09	/	-0,74	
AM00103	GB30209 (*Dscam exon 3*)	*Dscam*	-0,77	axon guidance; mushroom body development
AM00166	GB15141 (*Dscam exon 10.9*)	*Dscam*	-1,08	axon guidance; mushroom body development
AM04590	GB11973 (*Cyp4g11*)	*Cyp4g15*	-0,93	electron carrier activity ; steroid biosynthetic process
AM01535	BB170004B10F01	/	-1,11	
AM01750	BB170027A10E09	/	-1,43	
AM01173	BB170009B20C11	/	-1,52	
AM00167	GB15141 (*Dscam exon 10.10*)	*Dscam*	-1,62	axon guidance; mushroom body development
AM01360	BB170030B10C09	/	-1,43	

Table 3. Significance of overlap between the VSH and others behavioural gene sets. Expected #: the number of genes expected to overlap between two gene sets by chance alone; *RF*: representation factor. Since the gene sets regulated by the queen mandibular pheromone was determined by using a different microarray platform (cDNA mircroarrays generated from brain expressed sequenced tags), its overlap with the VSH gene sets could not be calculated. Guard and forager describe specific tasks of bees: the first guard the hive at the nest entrance, the second is the first to react to a threat. NA: not available.

	Expected #	**Observed #**	***RF***	***P*-value**
Forager	4.3	5	1.1	0.44
Vibrating bee	3	10	3.3	<0.001
Queen mandibular pheromone	NA	8	NA	NA
Brood pheromone	1.1	7	6.5	<0.001
Alarm pheromone	1.5	3	1.9	0.2
AHB guard	0.8	6	7.5	<0.001
AHB soldier	1.8	12	6.7	<0.001
AHB forager	0.19	5	26.5	<0.001

Table 4. Overlap between the VSH and others behavioural gene sets. The upper and lower part of the table shows probes that are up- and downregulated in VSH+ bees, respectively. “up” and “down” indicates whether probes are up- or downregulated in the corresponding behavioural phenotype. QMP: queen mandibular pheromone, BP: brood pheromone, AHB: Africanized honeybees.

Oligo ID	Foraging bee	Vibrating bee	QMP	BP	Alarm pheromone	AHB guard	AHB soldier	AHB forager
AM01773	down							up
AM02265								
AM01177		down					down	
AM01915		down				down	down	
AM02211								
AM04119								
AM05646								
AM01622								
AM00808								
AM02131			down				up	
AM03470	up	up		up				
AM01666								
AM09325						down	down	
AM01607								
AM07547	up	up	up				up	
AM12206		up	down		up			
AM01085	down	up						
AM02392				down	up	down	down	down
AM03333		up		up			up	
AM03972			down					
AM05381	up	down			up	up	down	
AM00612								
AM09032								
AM00604								
AM00955		up					down	
AM06645								
AM12005								
AM03226							up	
AM06202			up					
AM10277								
AM02039				up		up		
AM00103				up				
AM00166			up					up
AM04590			up					
AM01535							down	
AM01750				up				up
AM01173								
AM00167							up	up
AM01360		up	up	up		up		

Figure legend

Figure 1. Validation of microarray results with real-time quantitative qRT-PCR. Brain expression levels of 3 genes (*Arrestin 2*, *lop1* and *Antdh*) identified by the microarray study as being differentially expressed between VSH- and VSH+ bees. Individuals from VSH- and VSH+ colonies used for the arrays were tested. Each bar represents a colony sample (pool of 10 bees). Significant differences were found for each gene using a Mann-Whitney U test ($P < 0.05$).

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