Behavioral and molecular studies of quantitative differences in hygienic behavior in honeybees

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Abstract

Background: Hygienic behavior (HB) enables honeybees to tolerate parasites, including infection with the parasitic mite Varroa destructor, and it is a well-known example of a quantitative genetic trait. The understanding of the molecular processes underpinning the quantitative differences in this behavior remains limited.

Results: We performed gene expression studies in worker bees that displayed quantitative genetic differences in HB. We established a high and low genetic source of HB performance and studied the engagements into HB of single worker bees under the same environmental conditions. We found that the percentage of worker bees that engaged in a hygienic behavioral task tripled in the high versus low HB sources, thus suggesting that genetic differences may mediate differences in stimulated states to perform HB. We found 501 differently expressed genes (DEGs) in the brains of hygienic and non-hygienic performing workers in the high HB source bees, and 342 DEGs in the brains of hygienic performing worker bees, relative to the gene expression in non-hygienic worker bees from the low HB source group. “Cell surface receptor ligand signal transduction” in the high and “negative regulation of cell communication” in the low HB source were overrepresented molecular processes, suggesting that these molecular processes in the brain may play a role in the regulation of quantitative differences in HB. Moreover, only 21 HB-associated DEGs were common between the high and low HB sources.

Conclusions: The better HB colony performance is primarily achieved by a high number of bees engaging in the hygienic tasks that associate with distinct molecular processes in the brain. We propose that different gene products and pathways may mediate the quantitative genetic differences of HB.

Keywords: Varroa, Hygienic behavior (HB), Varroa sensitive hygiene (VSH), Gene expression, Quantitative genetic trait

Background

Worker honey bees can detect and remove parasitized brood from their nest, thus reducing the damaging effects of various parasites in the colony. This so-called hygienic behavior (HB), performed by single bees in a colony, plays a major role in the overall resistance of the colony to a number of important pathogens, including Ascosphaera apis (which induces chalkbrood disease) [1, 2], Paenibacillus larvae (which causes American Foulbrood) [3, 4] and the mite Varroa destructor [2, 5, 6]. HB has a genetic component, as inferred by genetic crossing experiments and scoring of hygienic performance [7]. The hygienic task usually involves uncapping behavior that results in the uncapping of the diseased brood cell and removing behavior that involves the removal of the pupae from the cell [7]. Varroa sensitive hygiene (VSH) behaviors specifically refer to the removal of Varroa mite-parasitized pupae [2, 8, 9]. Quantitative genetic studies have identified genes associated with the HB and Varroa sensitive hygiene (VSH) behavior [10–17]. Differently expressed genes (DEGs) have been identified between high and low HB and VSH-selected honeybee lines [10, 11] and between worker bees from a high performing line either displaying VSH behavior or not display such behavior.
Other approaches have correlated expression levels with the quantitative performance of HB and VSH in local honeybee breeding populations [13, 14]. The assembly of genetically high and low HB performing worker bees in different proportions in a single group has enabled the identification of genes indirectly regulated by high performing social neighbors (indirect genetic effects) [15]. Backcrosses from high and low HB and VSH-selected lines have established large genetic mapping populations in which high and low HB or VSH alleles segregate. Genetic mapping of the behavioral performance of worker bees in such backcrosses has allowed for the identification of six and two quantitative trait loci (QTLs) in HB and VSH populations, respectively [16, 17]. The genomic regions and candidate genes have been identified with the help of the genomic sequence [18]. Together, these studies have generated a list of genes that have provided further information about the molecular processes associated with HB and VSH behavior, which can provide a source to detect biomarkers for marker-assisted selective breeding of disease- and parasite-tolerant bees [4, 16, 19, 20]. How the differences in the quantitative genetic traits affect gene expression and thus, the molecular state and the selection of possible markers are unknown.

In the present study, we identified HB-associated DEGs in single worker bees that originated from genetic high and low HB sources. This was achieved through crosses of bees from three colonies with a low quantitative genetic trait for HB and three colonies with a high HB trait. We scored the behavior of the workers in a common social environment and examined HB-associated DEGs from the high and the low HB source.

**Results**

**Three times more worker bees from the high source group, compared with the low source group, engaged in HB**

We generated three high and three low HB performing colonies (Fig. 1) through crossings of progeny selected from an ongoing breeding program for Varroa-tolerant bees, as identified on the basis of overall colony performance in the removal of dead brood (pin-test [21]). To identify the workers that performed HB, we assembled 300 newly emerged worker bees from each of the three low and high HB colonies and marked each bee. At the age of 12 days old (an age at which worker bees usually can perform HB) ~1800 worker bees were confined on a brood comb containing ~33 pin-killed pupae [22]. We recorded the behavior of the worker bees by video for 12 h. We studied four biological replicate groups, resulting in a total of ~7200 worker bees included in our analysis. We obtained hygienic behavioral information (whether the worker bees engaged in uncapping or removing behavior) from 3938 worker bees. For the high HB source, we found that 8.8 % (median) of worker bees per cross and replicate (Fig. 2) engaged in HB (190 out of the 1912 worker bees). For the low HB source, 3.3 % (median) of worker bees performed HB activity (69 out of the 2026 worker bees), which was significantly lower than the values observed for the worker bees from the high HB sources ($MWU$-test, $P < 0.01$, Fig. 2a). This result indicated that the portion of workers that engaged in HB among the high HB performing worker bees was nearly three times
that of the worker bees from the low HB source. Because worker bees from the high and low HB colonies experienced the same environment (we assembled them into a single group), we concluded that worker bees from the high HB source displayed a higher frequency of HB engagement. However, the number of hygienic activities per worker bee was not measurably different between the high and the low HB sources (Fig. 2b). This result suggests that the average frequency in engagement of single bees in HB was not affected by the source (high vs low HB source).

HB performing worker bees from the high and low HB sources have distinct molecular states in their brains

To identify molecular processes in the brain that are associated with HB in bees from the high and low HB sources, we repeatedly measured transcription profiles of 13,440 genes. We used 82 two-color microarrays that were hybridized in a loop design [23] (Additional file 1: Table S1) to identify DEGs between the different behavior and genetic source conditions. Worker bees from the three high and the three low sources were similarly represented (Additional file 1: Table S1). We identified 501 genes (3.7 % of the genes present on the microarray) that were differently expressed between the HB-performing versus the non-performing bees from the high HB source (Table 1; Additional file 2: Table S2). We identified 342 differently expressed genes (2.5 %) between the behavioral states in worker bees from the low HB source (Table 1; Additional file 3: Table S3). We observed that more genes were upregulated in bees from the high HB source than in bees from the low HB source (Table 1; $\chi^2$, df = 1, $P < 0.0001$), suggesting that HB-associated genes were more often upregulated in worker bees from the high HB source. We also found that 480 HB-associated genes were observed in only the high HB source, and 320 were observed in only the low HB source, whereas only 21 genes were common between the two (Fig. 3). The gene expression patterns and molecular states associated with HB markedly differ between the high and low HB sources. Furthermore, we detected only 5 genes (Additional file 4: Table S4) that were differently expressed due to the different genetic backgrounds between the high and low HB sources, suggesting that the different genetic sources only marginally affect gene expression.

We then performed gene ontology (GO) analysis of the 501 and 342 HB-associated genes from the two sources. Genes were assigned to their orthologs in *Drosophila melanogaster*, and functional clustering was performed

| # of DEGs in the HB sources* | High | Low |
|-----------------------------|------|-----|
| Upregulated                 | ↑    | 339 | 155 |
| Downregulated               | ↓    | 162 | 187 |
| Sum                         | Σ    | 501 | 342 |

* $P < 0.01$ was adjusted for multiple testing
using DAVID [24, 25]. For the high HB source, we found a single cluster with an enrichment score above 2, which included 11–47 genes. The GO terms “cell surface receptor ligand signal transduction” and “sensory perception of smell” were overrepresented. Proteins that were predicted to integrate into the membrane or possess an “olfactory receptor” domain or that carry glycosylation were also overrepresented (Additional file 5: Table S5). For the HB-associated genes in the low source, we found a small cluster of genes (a cluster comprising 8 to 3 genes above an enrichment score greater than 2). The GO term “negative regulation of cell communication” was overrepresented, and some of the genes within this cluster are predicted to regulate protein kinase pathways (Additional file 6: Table S6). Together, the results of the GO analysis suggest that different molecular processes in the high and the low source groups are associated with HB performance.

Discussion

We produced three high and three low HB worker bee sources by genetic crossing. The colonies that produced the parents were selected according to the display of high or low quantitative genetic differences associated with the number of dead pupae removed from the colony in pin-tests [7]. Our results suggested that the quantitative differences in hygienic performance at the colony level were achieved by different percentages of workers engaging in the hygienic task. The percentage of worker bees engaging in a hygienic behavioral task tripled in the high versus the low HB source groups (Fig. 3), whereas the average frequency of HB engagement of a single worker was not detectably different between the two sources. These results suggested that better HB colony performance is primarily achieved by a high number of bees engaging in the hygienic tasks, rather than by a subset of highly specialized worker bees repeatedly performing that task. This finding is consistent with the view that all bees in a colony are capable of performing HB [26]. However, in the high HB backcross, more worker bees were stimulated to perform HB, possibly because of the lower level of stimulus perception and processing (see below) through olfaction [27–29]. Hence, the partitioning among hygienic and non-hygienic tasks can be modulated by various genetic propensities for performing HB, consistent with the response threshold model of task allocation [30, 31]. The proposed function of the DEGs associated with HB performance further suggests that further downstream neuronal processes in addition to perception using the antennae may also affect HB. We found that in the brain, signal transduction in the high HB source, and cell communication was overrepresented in the low HB source were overrepresented molecular processes. We speculate that these molecular processes of the brain may influence the quantitative genetic differences of HB at the phenotypic level.

Further, we found that the molecular processes in the brain associated with quantitative genetic differences involved in the engagement in HB can be markedly different. From the 501 and 342 HB-associated DEGs that we identified in the two sources, only 21 genes were common between the two groups of bees. Furthermore, GO analysis revealed no overlap in the assigned GO terms and gene functions for the two sets of DEGs. We conclude that the molecular states in the brain that associate with HB worker performance are variable. We speculate that some of the molecular patterns that are confined to the HB worker source may relate to the more stimulated state of those worker bees coming from the HB sources. However, it remains unclear whether those genes and their gene products are the cause of the higher stimulated state or are the product of the hygienic behavioral engagement. Irrespective of the underlying cause, our results suggest that the relationship between gene products and the quantitative differences in worker engagement into the hygienic task may be associated with distinct molecular processes.

We next investigated whether the DEGs that we identified have also been identified in other HB and VSH studies (Table 2; Additional file 7: Table S7). We speculated that more of the DEGs from the high HB source group in our study would match the gene candidates in previous studies than those DEGs from the low HB source because they should associate with the high HB or VSH performance evaluated in previous studies. We found that 7 % (34 genes) of our high and 9 % (29) of our low source DEGs have been previously associated with HB (Table 2). Hence, the DEGs in the high HB source ($\chi^2, \text{df } = 1, P > 0.39$) were not overrepresented among the genes reported in previous studies. A possible explanation for this discrepancy is that the design of the experiments, the characterized genetic differences (QTL versus gene expression differences (DEGs)) and the scored phenotypes differed among studies. Some DEGs studies have compared the DEGs in pupae or in adult brains between selected VSH high and low lines [10, 11]. One study has identified DEGs in a selected VSH line by comparing DEGs in the antennae, which were collected from workers that were or were not performing VSH behaviors [12]. Others have correlated expression levels with the quantitative HB and VSH
performance in a local breeding population [13, 14]. One study has identified DEGs associated with relatively high HB activity that were induced by the social neighbors (indirect genetic effects) [15]. QTL mapping identified different genomic loci that associated with HB or VSH [16, 17]. In this study we found that the DEGs and the GO terms differed between our low and high performing sources. Only few genes were shared despite the fact that the same methods were used. The different molecular states in worker brains (large set of different DEGs and associated GO terms in high and low HB performing worker bees) suggest that possibly different molecular routes are involved in the regulation of quantitative differences in HB behavior. If validated by further experiments, a different molecular basis for quantitative differences in HB would have broader implications for selective breeding strategies and for understanding of the evolution and molecular control of social behaviors in honeybees.

**Methods**

**Sources of honeybees**

We identified colonies (*Apis mellifera*) that displayed varying degrees of HB performance, using a large database from an ongoing breeding program (www.beebreed.eu). The breeding program calculate breeding values for HB based on group performance using the pin-test [32]. The breeding values were estimated by using the BLUP (Best Linear Unbiased Method) approach [33]. We crossed three queens with a single drone that derived from colonies that had the highest breeding values for HB in our sample (values see Additional file 7: Table S7). The offspring of those three crosses established the workers in the high HB source group in our experiment. We also crossed three queens with drones that derived from colonies with the lowest calculated breeding values in our sample (Additional file 8: Table S8). The offspring of those three crosses established the workers in the low HB source group in our experiment. Five drones derived from different mothers and grandfather (not related) one drone had the same mother and grandfather (a replicate). Crosses were performed by instrumental insemination using the semen from the different, single drones.

**Behavioral assay**

Hygienic behavior (HB) was evaluated in individualized age-standardized worker bees (the progeny of the F1 generation, see Fig. 1) using pin-killed brood (the so called “pin-test”). Directly after eclosion, the worker bees were individually labeled with small colored, numbered tags (Opalith-Plättchen) assembled in equal proportions (~300 individuals per backcross line, ~1800 altogether) and kept in a queen right host colony until the bees reached the age of 12 days, which is the period of time during which worker bees engage in HB at the highest frequency [34]. Throughout the experiment,

| Table 2 The HB associated DEGs found also in previous HB and VSH studies |
|-----------------------------------------------|
| **This study** | **Previous studies** | |
| HB sources | **Sum of common genes** | **Gempe 2012** | **Navajas 2008** | **LeConte 2011** | **Tsuruda 2012** | **Oxley 2010** | **Parker 2012** | **Mondet 2015** |
| | | pupae Mª | brain Mª | Qª | Qª | (antenna, larvae Pª) | antenna Rª |
| High | 34 (7 %) | 20 (3 %) | 2 (2 %) | 3 (16 %) | 2 (2 %) | 1 (3 %) | 0 | 9 (5 %) |
| Low | 29 (9 %) | 20 (3 %) | 2 (2 %) | 1 (5 %) | 2 (2 %) | 2 (7 %) | 0 | 4 (2 %) |

ª Microarray analysis
ª QTL analysis
ª Proteome analysis
ª RNAseq analysis
the worker bees were kept as a separate group using a
gauze mesh cage that was supplied with honey and pol-
len, which was inserted into the queen right colony. The
worker bees had physical and olfactory contact with
the host colony which was standard colony which was
not involved in the selection program. At the onset of
the experiment, the worker bees were transferred to
an experimental comb supplied with brood, honey and
pollen, in which an average of 21.5 pupae in sealed
brood cells within a square of 11 × 10 cells had been
pierced at regular intervals; approximately 90 non-
pierced pupae in sealed brood cells were used as non-
treated controls. Brood combs for the pin-test assay
were derived from several randomly chosen colonies.
The experimental comb was inserted into a cage with
a glass front on one side, opposite of the manipulated
area, and a gauze mesh on the other side, allowing for
physical and olfactory contact with the host colony.
The glass front of the cage faced an infrared-sensitive
camera [22]. During the experiment, the comb was
illuminated with infrared light, a wavelength that the
bees cannot perceive, allowing them to behave as they
would in the natural darkness of the hive (LEDs: OSA
Opto-Light GmbH, Germany, Type: OIS 330,880). The
experiment lasted 12 h, during which the movements
of the worker bees on the brood area were monitored
by video recording using an infrared-sensitive camera
(Panasonic WV-NP1004 megapixel color network IP).
Directly after the experiment, the worker bees were
anaesthetized with CO2, shock-frozen in liquid nitro-
gen and stored at −70 °C until further analysis. The
experiment was replicated four times with another set
of worker bees acquired from each backcross.

Worker bees that performed once or several
times at one or more cells was scored. Bees that per-
erformed HB that a single worker bee performed once or several
times at one or more cells was scored. Bees that per-
erformed HB that a single worker bee performed once or several
times at one or more cells was scored. Bees that per-

Microarray analysis

The honeybee whole-genome oligonucleotide microar-
ray (Design: UIUC Honey bee oligo 13 K v1, Accession:
A-MEXP-755) contains 28,800 oligos representing 13,440
genes derived from annotations of the entire honeybee

genetic sequence [18]. A total of 82 microarrays were

Table S1). We randomly chose bees from each backcross
comb and experimental replicate. Total RNA was isolated from

All microarray data are

from the UIUC Honey Bee oligo 13 Kv1 annotation file.

Additional files

Additional file 1: Table S1. The design of the microarray experiments that comprise 82 hybridizations.

Additional file 2: Table S2. DEGs associated with hygienic behavior (HB) in the high HB source. A modified t-test was performed on the log-transformed transcription ratios gained from 82 hybridizations between 138 hygienic worker bees and 138 non-hygienic siblings. Oligo Ids, Gene Ids, and Comments are quoted from the UIUC Honey bee oligo 13 K v1 annotation file (May 2007).

Additional file 3: Table S3. DEGs associated with hygienic behavior (HB) in the low HB source. A modified t-test was performed on the log-transformed transcription ratios gained from 82 hybridizations between 138 hygienic worker bees and 138 non-hygienic siblings. Oligo Ids, Gene Ids, and Comments are quoted from the UIUC Honey bee oligo 13 K v1 annotation file (May 2007).

Additional file 4: Table S4. DEGs associated with the HB sources but not with the behavior. A modified t-test was performed on the log-transformed transcription ratios gained from 82 hybridizations. Oligo Ids, Gene Ids, and Comments are quoted from the UIUC Honey bee oligo 13 K v1 annotation file (May 2007).

Additional file 5: Table S5. The enrichment analysis for HB associated DEGs from the high HB source. Enrichments above the score 2.0 are presented.

Additional file 6: Table S6. The GO enrichment analysis for HB associated DEGs from the low HB source. Enrichments above score 2.0 are presented.

Additional file 7: Table S7. List of HB associated DEGs from high and low sources found in previous studies.

Additional file 8: Table S8. The breeding values of HB for the drones.

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