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Behavior and molecular physiology of nurses of worker and queen larvae in honey bees (*Apis mellifera*)

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ABSTRACT

In a honey bee colony, worker bees rear a new queen by providing her with a larger cell in which to develop and a large amount of richer food (royal jelly). Royal jelly and worker jelly (fed to developing worker larvae) differ in terms of sugar, vitamin, protein and nucleotide composition. Here we examined whether workers attending queen and worker larvae are separate specialized sub-castes of the nurse bees. We collected nurse bees attending queen larvae (AQL) and worker larvae (AWL) and compared gene expression profiles of hypopharyngeal gland tissues, using Solexa/Illumina digital gene expression tag profiling (DGE). Significant differences in gene expression were found that included a disproportionate number of genes involved in glandular secretion and royal jelly synthesis. However behavioral observations showed that these were not two entirely distinct populations. Nurse workers were observed attending both worker larvae and queen larvae, and there was no evidence of a specialized group of workers that preferentially or exclusively attended developing queens. Nevertheless, AQL attended larvae more frequently compared to AWL, suggesting that nurses sampled attending queen larvae may have been the most active nurses. This study serves as another example of the relationship between differences in gene expression and behavioral specialisation in honey bees.

Introduction

A key reason for the success of the social insect lifestyle is an efficient division of labor between workers. Honey bee workers perform different tasks normally based on an aged-related process of behavioral development (Seeley, 1982), but this is individually highly variable and flexible and responsive to changes in the social environment (Huang and Robinson, 1992, 1996, 1998). Both the behavioral transitions between the nurse and forager states, and the gene expression profiles associated with each behavioral caste are strongly influenced by changes in the colony's social and pheromonal environment (Bloch et al., 2001; Whitfield et al., 2006; Yamazaki et al., 2006).

In a honey bee colony, one of the most critical functions the workers ever perform is the successful rearing of a new queen. The queen is normally the colony's only individual capable of laying fertilized eggs destined to become workers. When the queen becomes old or dies, for the colony to survive a new queen must be raised, and for the colony to reproduce by swarming, new queens must be reared. In a honeybee colony, there is no genetic difference between a queen or worker. The development of young larvae into either a queen or worker depends on the amount and type of food given to them by nurses during early larval development (Kucharski et al., 2008). Previous studies have shown that the royal jelly fed to worker larvae and queen larvae differs in terms of composition (ratio of water-clear and milky-white components) (Haydak, 1970), sugar content (Asencot and Lensky, 1977), amino acid (Brouwers, 1984), vitamin (Brouwers et al., 1987), juvenile hormone (Asencot and Lensky, 1984), and major royal jelly protein content (Kamakura, 2011). These differences suggest that the nurses attending to the queen and worker larvae might represent distinct, but cryptic, sub-castes able to deliver different forms of brood food.

To explore this issue we used digital gene expression tag profiling (DGE) to identify genes differentially expressed in hypopharyngeal glands (HPG glands) between nurses attending queen larvae (AQL) and nurses attending worker larvae (AWL). Moreover, we also observed the behavior of marked bees AQL and AWL over two weeks in a glass-walled four-frame observation hive to examine any behavioral differences between AQL and AWL.

Material and methods

Insect

The standard Chinese commercial strain of Western honey bee (*Apis mellifera*) was used throughout this study. All experiments were

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performed at the Honeybee Research Institute of Jiangxi Agricultural University, Nanchang City, China (28.46° N, 115.49° E).

Digital gene expression analysis of HPG gland samples from AQL and AWL

For transcription analyses, nurse bees were collected from an eight-frame standard Langstroth hive with a naturally mated queen (not been caged). AQL and AWL were identified as bees with their heads inserted into queen- or worker-brood cells respectively. Queen larvae were grafted as eggs into 192 custom-made plastic queen cells (Liu et al., 2009) and inserted into the colony on wooden cross bars. Worker larvae were laid by the queen naturally in worker cells. In each bee colony, sixty of each type of nurse (AQL and AWL) were caught with forceps while they were attending young larvae (3rd instar or less). The nurses for two biological replicates of AQL and AWL were collected from two independent colonies. HPG glands were dissected from the heads while still frozen over dry ice under a dissecting microscope, and immediately flash-frozen in liquid nitrogen. HPG glands from 57 honeybees were pooled for each sample for RNA extraction, yielding 6 μg total RNA. The four HPG gland samples (AQL and AWL from two colonies) were then stored at −80 °C until further processing.

RNA extraction and digital gene expression library preparation and sequencing

Each tissue sample was homogenized and vortexed with chloroform, and total RNA was extracted using a standard method of SV Total RNA isolation System (Promega, USA). cDNA libraries were constructed using the Illumina gene expression sample preparation kit (developed by the Beijing Genomics Institute-Shenzhen) according to the standard protocol. Briefly, poly(A)+ RNA was purified from 6 μg of total RNA using oligo(dT) magnetic beads. Single-strand cDNA was directly synthesized against the poly(A)+ RNA-bound beads, then the complementary cDNA strand was synthesized. cDNAs were then digested with NlaIII, which recognizes the CATG site. The digested cDNA fragments containing 3′ ends were purified from the magnetic beads, and then the Illumina adaptor1 was added to the 5′ ends of these cDNA fragments. These fragments were further digested by another endonuclease, MmeI, which recognizes the junction of the Illumina adaptor1 and the CATG site and cuts at 17 bp downstream of the CATG site producing 21 bp tags containing the adaptor1 sequence. After removing the cleaved 3′ end sequences with magnetic bead precipitation, the Illumina adaptor2 was ligated to the 3′ ends of the tags to create a tag library containing tags with the different Illumina adaptors on both ends. The library was then amplified by PCR for 15 cycles. PCR products were separated on 6% PAGE gel electrophoresis, and the 95 bp fragments were chosen and purified for sequencing. Double-stranded DNA fragments were denatured, and the single-stranded molecules were bound to the Illumina sequencing chip (hiseq2000) for sequencing (sequencing strategy was 50 SE). Each element within the chip (flowcell) generated millions of raw tags with a length of 49 bp. This sequencing analysis was completed by the Beijing Genomic Institute-Shenzhen.

Analysis and mapping of DGE tags to genes and the Apis mellifera genome

Raw sequences were filtered using the following steps: 1, removal of adaptor sequences (since tags are only 21 nucleotides long while the sequencing reads are 49 nucleotides long, raw sequences include the 3′ adaptor sequences); 2, removal of empty tags (no tag sequence between the adaptors); 3, removal of low quality tags (tags with any unknown nucleotide "N"); 4, removal of tags with only one copy number (which might result from sequencing errors); and 5, removal of tags which are too long or too short. After filtration, the remaining 'clean tags' each contained CATG and were 21 bp long.

Before mapping, a tag library containing all the possible CATG + 17-nt tag sequences was created by reference to all the available mRNA sequences and genome sequences of *A. mellifera* within the Genbank database (version OGS 1, (ftp.ncbi.nih.gov/genomes/Apis_mellifera/RNA/rna.fa.gz)) and Amel 4.5 (ftp://ftp.ncbi.nih.gov/genomes/Apis_mellifera/) respectively. All clean tags were mapped to the reference database with only one nucleotide mismatch being allowed. Clean tags that mapped to multiple possible genes were excluded from further analysis. The remaining clean tags were designated as unambiguous clean tags. For gene expression analysis, the number of unambiguous clean tags for each gene was calculated and normalized to number of transcripts per million clean tags (TPM). Clean tag numbers, and numbers of genes identified in each sample are summarized in Table 1.

Combined analysis of the DGE data from two replicates of HPG gland samples

For the two replicated HPG gland samples, we combined the DGE data from two colonies together according to a NOISeq algorithm developed by Tarazona et al. (2011). Firstly, we tested the correlation

Table 1
Summary of DGE profiles and their mapping to the reference genes.

Summary		AQL	AWL
Raw data	Total	12,000,000	12,000,000
Raw data	Distinct tag	233,897	206,990
Clean tag	Total number	11,861,361	11,882,536
Clean tag	Distinct tag number	114,357	104,538
All tag mapping to gene	Total number	9,449,476	10,064,830
All tag mapping to gene	Total % of clean tag	79.67%	84.70%
All tag mapping to gene	Distinct tag number	47,629	48,014
All tag mapping to gene	Distinct tag % of clean tag	41.65%	45.93%
Unambiguous tag mapping to gene	Total number	7,115,873	8,351,000
Unambiguous tag mapping to gene	Total % of clean tag	59.99%	70.28%
Unambiguous tag mapping to gene	Distinct tag number	46,559	46,830
Unambiguous tag mapping to gene	Distinct tag % of clean tag	40.71%	44.80%
All tag-mapped genes	Number	8233	7786
All tag-mapped genes	% of ref genes	74.44%	70.40%
Unambiguous tag-mapped genes	Number	7984	7558
Unambiguous tag-mapped genes	% of ref genes	72.19%	68.34%
Mapping to genome	Total number	1,502,698	984,578
Mapping to genome	Total % of clean tag	12.67%	8.29%
Mapping to genome	Distinct tag number	50,529	43,889
Mapping to genome	Distinct tag % of clean tag	44.19%	41.98%
Unknown tag	Total number	909,187	833,128
Unknown tag	Total % of clean tag	7.67%	7.01%
Unknown tag	Distinct tag number	16,199	12,635
Unknown tag	Distinct tag % of clean tag	14.17%	12.09%