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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%

coefficient between two repeated HPG gland samples using a Pearson correlation coefficient analysis. The calculating formula was:

$$r = \frac{1}{n-1}\sum_{i=1}^{n}\left(\frac{X_i-\overline{X}}{s_X}\right)\left(\frac{Y_i-\overline{Y}}{s_Y}\right)$$

where n is sample number, X_i and Y_i are the copy number of each gene from two replicates (for example AQL samples from colony A and B), Sx and Sy are their standard errors respectively. If the value of Pearson R is >0.8, it suggests the replicates have a high sequencing quality and repeatability. Therefore, the data from different replicates can be combined for further analysis using the NOISeq methods (Tarazona et al., 2011) that estimates the number of clean tag counts of each gene from all replicates, then uses corrected expression values to correct for library size bias. The formula for correcting expression values in the combined replicates is $x^i_{gj} = c^i_{gj} \times 10^{6'}/s_{gj}$, where "$c$" is the number of read counts, "i" is gene, "j" is sample number, "g" is sample group, "Sgj" is SD of the replicates (Tarazona et al., 2011). Analysis of differentially expressed genes, GO and KEGG analyses were then performed on the combined dataset.

Identification of differentially expressed genes

The expression (TPM) of each gene in AQL and AWL samples is shown in Fig. 1. Genes differentially expressed between AQL and AWL samples in gland analyses were identified by a rigorous statistical algorithm (Audic and Claverie, 1997):

$$P(y|x) = \left(\frac{N2}{N1}\right)^{y}\frac{(x+y)!}{x!y!\left(1+\frac{N2}{N1}\right)^{(x+y+1)}}.$$

Denote the number of unambiguous clean tag from AQL and AWL as x and y respectively. The $P(y/x)$ is in the Poisson distribution, and $N1$ and $N2$ indicate the total number of clean tags in AQL and AWL, respectively. Then the false discovery rate (FDR) was used to determine the threshold p-value in multiple tests in this experiment. Genes were identified as differentially expressed if both the FDR <0.001, and the absolute value of the log2 ratio of expression differences >1. R statistical package (R Core Team, Vienna, Austria) was employed to do these statistics. The identified differentially expressed genes were used for GO and KEGG enrichment analysis. We computed Fisher's exact test p-values for over-representation of the selected genes in all GO biological categories, using R statistical package (R Core Team, Vienna, Austria). GO enrichment analysis of functional significance was carried out by a hypergeometric test which mapping all DEGs to terms in GO database (http://www.geneontology.org). The formula was:

$$P = 1-\sum_{i=0}^{m-1}\frac{\binom{M}{i}\binom{N-M}{n-i}}{\binom{N}{n}}$$

where N is the number of all genes with a GO annotation in our dataset; n is the number of differentially expressed genes in N; M is the number of all genes that are annotated to the certain GO terms; m is the number of differentially expressed genes in M. GO terms with $p \leq 0.05$ were considered as significantly enriched. Similarly, KEGG pathway terms with Q-value ≤ 0.05 were considered as significantly enriched. The formula of Q-value is the same as that in GO analysis. Significantly expressed genes between AQL and AWL were mapped into KEGG pathway database (http://www.genome.jp/kegg/) using BLAST 2.2.26 package, USA.

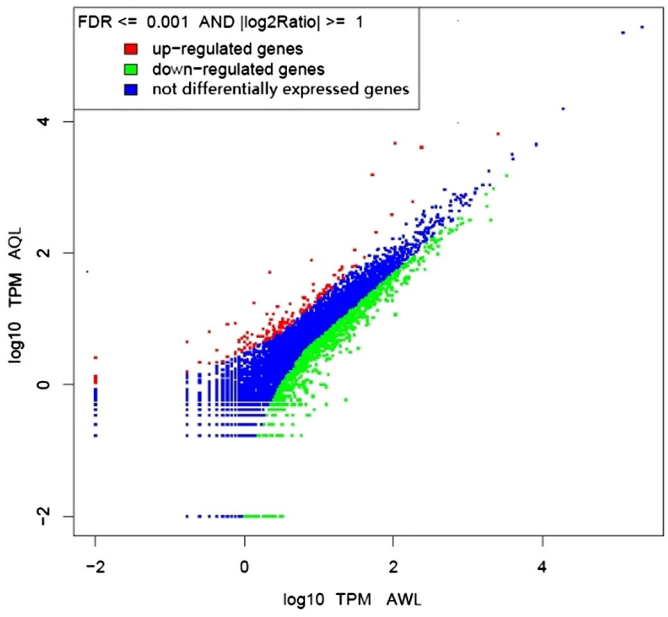

Fig. 1. Volcano plots of gene expression in AQL plotted against AWL samples in gland samples. Genes were identified as differentially expressed if both the FDR < 0.001, and the absolute value of the log2 ratio >1 by a rigorous statistical algorithm. Each point is one gene. Red points were significantly up-regulated genes in AWL, blue points were not differentially expressed and green points were significantly down-regulated genes in AQL compared to AWL. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Nursing behavior observation

A colony with four frames and a naturally mated queen was kept in a glass observation hive. Of the four frames, one contained hundreds of day-old worker larvae and 36 queen larvae (housed in 36 custom-made plastic queen cells), where larvae were collected from the observation colony. This frame was replaced every three days with a similar one from another colony throughout the whole experimental period, in order to provide enough young queen and worker larvae for the nurses. Throughout the experiment the queen was restrained in a small single-mesh cage to prevent her laying so that all eggs and larvae were of a known age and restricted to a single frame of the observation hive.

Frames of emerging brood were removed from the colony and held overnight in an incubator at 34.4 °C and 80% RH. Newly emerged adult bees were marked with numbered plastic tags and returned to the observation hive. AQL and AWL were identified as bees with their heads entered into cells containing queen or worker larvae that are less than 3 days old. Observation hives were watched for 3 h per day from 9:00 am to 10:30 am and 3:00 pm to 4:30 pm, and any incidences of tagged bees attending queen or worker larvae were recorded. Bees' nursing activities were recorded for 12 days. This experiment was repeated with 3 colonies. For the first replicate we marked 150 day-old bees, but for replicates 2 and 3 we marked 200.

In order to identify whether there were some nurses that were more likely to attend queen larvae, the number of times each marked individual was seen attending either queen or worker larvae was recorded. We calculated the numbers of each of three types of nurse bees, who only attended worker larvae, only attended queen larvae and attended both worker and queen larvae respectively, ANOVA test was used to test the total bee numbers of those three groups. Numbers of bees who attended queen larvae at least once in each day were separated into four groups: days 1–3, days 4–6, days 7–9, days 10–12. Data of these four groups was compared with a Mann–Whitney U-test. Moreover, total nursing activity of bees seen attending queen larvae at least once and bees never seen attending queen larvae was compared with a Mann–Whitney U-test. All analyses were performed with the statview 5.0 package, SAS Institute, Gary, NC, USA.

Results

Statistics of raw data

We sequenced the DGE libraries of RNA extracted from gland samples. A summary of the number of DGE tags and their mapping to the reference database is presented in Table 1. For all of our samples greater than 65% of tag could be matched unambiguously to reference genes and less than 8% of tags were unknown. In total, 11,861,361 (AWL) and 11,882,536(AQL) clean tags were sequenced. This corresponded to 114,357(AWL) and 104,538 (AQL) distinct clean tags in these two samples. In this study, the tag sequences of the the DGE library was mapped to *Apis mellifera* L. transcripts (OGS 1) (ftp.ncbi.nih.gov/genomes/Apis_mellifera/RNA/rna.fa.gz) and to the honeybee genome (Amel 4.5) (ftp://ftp.ncbi.nih.gov/genomes/Apis_mellifera/).

Saturation analysis of sequencing

To validate whether we had sufficient depth of sequencing, we performed sequencing saturation analysis (Fig. S1). As shown in Fig. S1, these AQL and AWL samples showed similar trends on saturation. The rate of identification of novel genes reached a plateau at 23.7 million tags. Since we exceeded this number for all four samples we are confident our coverage was sufficient to detect all honeybee genes expressed in the tissues. Further, the Pearson correlation coefficient between two repeated HPG gland samples from two colonies were both >0.99, as is illustrated in Fig. S2, indicating that there was a high sequencing quality and repeatability between two HPG gland samples.

Differentially expressed genes in HPG gland samples

Summaries of DGE sequencing analyses are showed in Table 1. Approximately 15.5 million clean tags were sequenced in the HPG glands of AQL and AWL. For this comparision, 1141 (Table 2) genes were found to be significantly differentially expressed between AQL and AWL. When comparing expression level in AQL with AWL, fewer genes were up-regulated than down-regulated (143 vs 1098) (Table 2).

In Table 3, a list of 20 genes with significantly differential expression and highest copy counts were collated for the DGE sample. These genes were significantly up-regulated in AQL or AWL. It can be seen clearly that the most up-regulated genes were the major royal jelly protein genes (MRJP3, MRJP5, MRJP7 and MRJP3-like), whereas those genes in AWL were the ribosomal protein genes (e.g. 40S ribosomal protein SA, 40S ribosomal protein S13, 40S ribosomal protein S23-like, 40S ribosomal protein S27a-like, 60S ribosomal protein L10 isoform 1, 60S ribosomal protein L7, 40S ribosomal protein S15Aa-like isoform 3, 60S ribosomal protein L26 isoform 1 and Ribosomal protein S15).

Gene ontology functional enrichment and KEGG pathway analysis

In Table S2, 387 out of 1141 differently expressed genes were within GO cellular component categories, 16 genes (4.1%) were significantly enriched in metal ion transmembrane transporter activity ($p = 0.002$), 11 were enriched in potassium ion transmembrane transporter activity ($p = 0.007$) and 9 were enriched in potassium channel activity ($p = 0.02$). In KEGG pathways analysis, 1141 differentially expressed genes were involved in 206 pathways, however there was no pathway that the Q-value was >0.05 (Table S3).

Table 2
Overview of differential genes in two contrasted groups.

Group	No. of significantly differentially expressed genes	No. of up-regulated genes (AQL vs AWL)	No. of up-regulated genes (AQL vs AWL)
Gland	1141	143	1098

Behavioral observations

Behavioral observations showed 52.89 ± 6.20% (Mean ± S.D.) nurses attended both worker and queen larvae. 44.00 ± 8.54% nurses attended exclusively worker larvae. Comparatively, significantly less nurses only attended queen larvae (Fig. 2). The age of nurse workers did not showed significantly impacts on their preference for attending queen larvae (Fig. 3). However, bees observed attending queen larvae at least once were observed in more larval attendance events overall than bees that were never observed attending queens (Fig. 4).

Discussion

Numerous prior studies indicated that the flexible caste system of honeybees is highly related to variation in patterns of gene expression, and molecular techniques could prove a fruitful method to identify specialized behavioral castes that may be difficult to detect by other means (Whitfield et al., 2003). In this study a high proportion of MRJP family genes, metabolic genes, ribosomal genes (Table 3 and Table S1) were differentially expressed between AQL and AWL in HPG glands. The most up-regulated genes in AQL were MRJPs (MRJP3, MRJP5, MRJP7 and MRJP3-like) and a metabolic gene (Alpha-glucosidase) whereas ribosomal protein genes were the most significantly down-regulated (Table 3). It may be that AQL tend to have more active HPG glands and a higher body metabolism level to secrete more nutritious food royal jelly (RJ) compared to worker jelly (WJ) (Haydak, 1970; Asencot and Lensky, 1977; 1984; Brouwers, 1984; 1987; Kamakura, 2011), since MRJPs account for 82 to 90% of royal jelly proteins (Sano et al., 2004; Drapeau et al., 2006). Those ribosomal proteins genes were highly expressed in HPG glands of AWL, revealing that AWL's HPG glands were also on a high protein-synthesis level. However, it seems that proteins synthesized in AWL's HPG glands are not only MRJPs but also other proteins. Consequently, these DGE results together with the known differences between WJ and RJ that secreted by AWL and AQL respectively (Haydak, 1970; Asencot and Lensky, 1977; 1984; Brouwers, 1984; 1987; Kamakura, 2011) indicated that AQL and AWL were significantly different in physiological level, and it could be taken as evidence that there are some highly specialized nurse bees in a colony, that specialize on feeding royal jelly to queen larvae only.

By mapping the significantly regulated genes into KEGG, over 80% expressed genes were involved in amino acid metabolism and biosynthesis, protein biosynthesis, vitamins biosynthesis and RNA regulation processes. In particular most up-regulated genes in AQL compared to those in AWL involved in amino acid biosynthesis such as lysine biosynthesis, whereas the down-regulated genes involved in valine, leucine and isoleucine degradation, glycine, serine and threonine metabolism, cysteine and methionine metabolism This indicated that gene regulation controls AQL to secrete more proteins into royal jelly than AWL, which is consistent with the evidences showed in previous studies that RJ and WJ were significantly different in protein components (Haydak, 1970; Kamakura, 2011).

However, the results of our behavioral experiments provided no evidence for a small group of nurses that exclusively attended queen larvae. Most nurse bees attended both worker and queen larvae (Fig. 2). Although the proportion of nurse bee who only attended queen larvae was infinitesimally smaller, it still cannot be considered as a sub-caste group that specialized on attending queen larvae. This result might be influenced by a possible recording bias. The recording period was only 3 h per day, thus those nurse bees only attending queen larvae might attend worker larvae out of the observation period as well. Queen larvae attending behavior of AQL were consistent throughout the whole period of observation, and there was no significant difference among four age groups (Fig. 3). This result indicated that there was no evidence for queen attendance being more preferred at a specific worker age. However, workers who attended queen larvae at least once were more active in larval attendance than bees that were never observed attending

Table 3
Twenty most differentially expressed and highest copied genes in gland samples.

	Gene number	RawIntensity -AWL	RawIntensity -AQL	log2 Ratio (AQL/AWL)	p-Value	FDR	Description
Up-regulated (AQL vs AWL)	gi|58585141	1246	55,288	5.468976	3.53E-12	3.34E-11	Major royal jelly protein 3 precursor [*Apis mellifera*]
	gi|328794346	616	18,646	4.917308	5.96E-13	6.08E-12	PREDICTED: major royal jelly protein 3-like, partial [*Apis mellifera*]
	gi|62198226	2823	48,250	4.092652	0	0	Major royal jelly protein 7 precursor [*Apis mellifera*]
	gi|58585163	2140	7164	1.740559	0	0	Alpha-glucosidase precursor [*Apis mellifera*]
	gi|58585137	30,484	77,051	1.335186	2.36E-10	1.94E-09	Major royal jelly protein 5 precursor [*Apis mellifera*]
Down-regulated (AQL vs AWL)	gi|328779412	23,745	3758	−2.66217	0	0	PREDICTED: 40S ribosomal protein SA [*Apis mellifera*]
	gi|328783412	21,229	6198	−1.77872	0	0	Hypothetical protein LOC726323 [*Apis mellifera*]
	gi|328783951	12,596	3722	−1.76141	0	0	PREDICTED: nuclear protein 1-like [*Bombus terrestris*]
	gi|328784405	8239	2685	−1.62014	0	0	PREDICTED: hypothetical protein LOC725594 [*Apis mellifera*]
	gi|328776443	10,104	3414	−1.56797	0	0	Hypothetical protein LOC409854 [*Apis mellifera*]
	gi|328787337	9661	3333	−1.5379	0	0	Vacuolar protein sorting-associated protein 13C-like [*Apis mellifera*]
	gi|328793111	7198	2545	−1.50249	0	0	PREDICTED: 40S ribosomal protein S13 [*Apis mellifera*]
	gi|328793132	7359	2603	−1.50192	0	0	PREDICTED: 60S ribosomal protein L10 isoform 1 [*Apis mellifera*]
	gi|328778920	8543	3118	−1.45671	0	0	Ribosomal protein S15 [*Camponotus floridanus*]
	gi|328777595	6594	2414	−1.45226	0	0	PREDICTED: 60S ribosomal protein L7 [*Apis mellifera*]
	gi|328788590	6780	2722	−1.31915	0	0	PREDICTED: 40S ribosomal protein S15Aa-like isoform 3 [*Apis mellifera*]
	gi|328783444	9879	3978	−1.31488	0	0	PREDICTED: 60S ribosomal protein L26 isoform 1 [Apis mellifera]
	gi|229892247	26,142	11,450	−1.19359	0	0	Heat shock protein 90 [*Apis mellifera*]
	gi|110768146	20,571	9306	−1.14695	0	0	PREDICTED: ubiquitin-40S ribosomal protein S27a-like [*Megachile rotundata*]
	gi|328792038	38,736	17,857	−1.11976	0	0	PREDICTED: 40S ribosomal protein S23-like [*Apis mellifera*]

queens (Fig. 4). We also observed that AQL paused in activities for a couple of minutes before attending queen larvae, whereas AWL attended worker larvae without any such breaks. It may be that nurses were stimulated by some environmental factors in bee hive and delivered a greater amount of food to queen larvae, and paused in activity to synthesise more food.

Further, the interesting DGE and behavioral results in this study provide another example of how environmental factors influence the honey bee. While we could detect significant differences in gene expression between nurses attending queen and worker larvae, our behavioral studies did not identify these as two separate populations. Rather, it seemed most likely that the gene expression differences reflected qualitative differences in nursing activity (queen nurses were behaviourally and metabolically more active than worker nurses) instead of a fully distinct cryptic subcaste. Our findings highlight the flexibility of the relationships between physiology as determined by DGE and behavioral performance.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.aspen.2014.10.006.

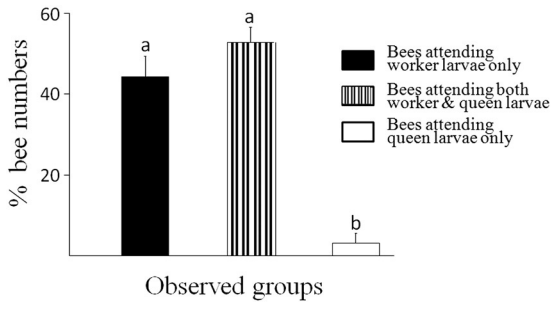

Fig. 2. Marked nurse workers were classified into each of three groups: attending worker larvae only, attending queen larvae only and attending both worker and queen larvae. Total number of nurse workers of each group were calculated and tested with ANOVA test statistics. Nurse workers attending both queen and worker larvae had the highest proportion, whereas significantly less nurse worker exclusively attended queen larvae. This indicates that there was no of a specialized group of workers that exclusively attended queen larvae. Different letters on top of bars indicate significant difference ($P < 0.05$) among the treatments. Raw data was test with ANOVA test statistics, while proportion of those three groups and their standard errors were presented.

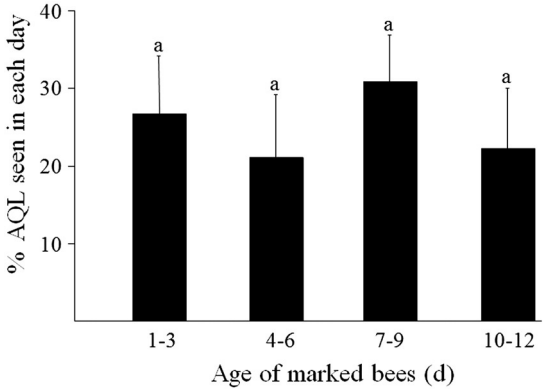

Fig. 3. Proportion of AQL seen in each day. Percentage of bees who attended queen larvae at least once in each day were separated into four groups: days 1–3, days 4–6, days 7–9, and days 10–12. Total numbers of AQL seen everyday in each group were pairwisely compared with a Mann–Whitney *U*-test in statview 5.0 package. There was no significant difference among these four age groups, suggesting that bee colonies have no specific age group of nurse workers that preferred queen larvae attendance. Different letters on top of bars indicate significant difference ($P < 0.05$). Proportion of AQL and standard errors were presented.

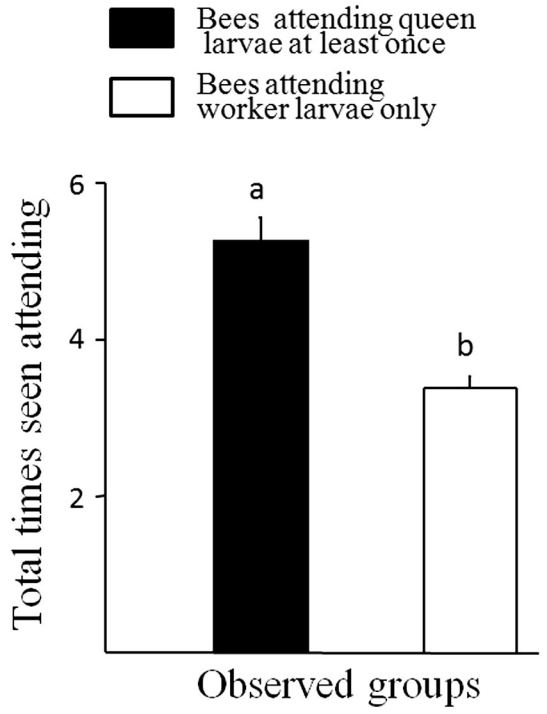

Fig. 4. Total numbers of attending times of AQL and AWL seen over the whole observation period in three replicate colonies. Black and white bars show values for AQL (defined as nurse workers seen attending queen larvae at least once) and AWL (never attended queen larvae). Total nursing activity of AQL and AWL were compared with a Mann–Whitney *U*-test in statview 5.0 package. The AQL had significantly more larval attendance events overall than AWL, suggesting a higher level of activity in the AQL group. Different letters on top of bars indicate significant difference ($P < 0.05$). Standard errors were presented on tops of the bars.

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Data accessibility

HPG gland samples: NCBI SRA: SRX286369 (AQL); NCBI SRA: SRX286370 (AWL).

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