Transcriptome comparison between newly emerged and sexually matured bees of *Apis mellifera*

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**ABSTRACT**

In order to understand the transcriptome characteristics of queens and drones of honeybee *Apis mellifera*, the transcriptome differences between newly emerged stage and sexually matured stage of queens and drones of *A. mellifera* L. were compared using high-throughput RNA-Seq. In drones, a total of 1618 DEGs were detected between the two stages. Out of these, 782 genes were up-regulated and 836 genes were down-regulated in sexually matured drones compared to newly emerged drones. In queens, the DEGs between the two stages were 1340, with 667 up-regulated and 673 down-regulated genes in matured queens compared to newly emerged queens. 411 genes showed the same expression trend in drones and queens during sexual maturing, with 233 (56.60%) up-regulated genes and 178 (43.31%) down-regulated at newly emerged stage. We found that genes encoding cuticular proteins (CP), cytochrome P450 (CYP), odorant binding proteins (OBP) and odorant receptor (OR), which are related to developments of bones, reproductive system and olfaction system, were differentially expressed between the sexually matured bees and the newly emerged bees. The results indicated that the expression levels of a large number of genes changed during sexual maturing of *A. mellifera* L. bees, which give us an insight into the characteristics of the gene expression during sexual maturing of adult queens and drones.

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Honeybee is a typical social insect. Usually, a honeybee colony comprises a queen, hundreds of drones and tens of thousands of workers. The drones are to mate with virgin queens and the mated queens are mainly responsible for reproduction. In order to improve the bee colony continuity and adaptability, honeybee has developed its special competitive mating mechanism, named mating flight. Queens and drones will choose the right opportunity for mating when they both reach sexual maturity which often accompanied with obvious physiological changes in the body of queens and drones. It has been confirmed at the physiological, biochemical and molecular levels that the physiological status of honeybee at different development stages of the sexual maturity are different (Kocher et al., 2008, 2010; Colonello-Frattini and Hartfelder, 2009; Behura and Whitfield, 2010; Chen et al., 2012; Fang et al., 2012; Zhang and Yuan, 2013).

Transcriptome changes during mating process have been conducted by several studies, a large number of genes showed expression changes during the mating flight (Wu et al., 2013a, 2013b, 2014). Through transcriptome comparison, 1615 DEGs were detected between ovaries of virgin and mated queens, moreover, a similar set of genes were found to be participated in the ovary activation of both queens and workers (Niu et al., 2014).

As two kinds of sex, the queens and drones have different physiological status during maturing. At present, the related regulation mechanism during sexual maturing of queens and drones has not yet been in-depth studied, especially the gene expression information of adult bees during sexual maturing are lacking. In this study, we used the high-throughput RNA sequencing technology to find out the difference of gene expression between newly emerged and sexually matured bees of drones and queens. All the DEGs during maturing were then subjected to Gene Ontology category (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. Our results showed that a large number of genes changed during sexual maturing, and a lot of transcript sequences with important function were acquired for future gene expression or regulation research about growth, development and reproduction of *A. mellifera*.

**Materials and method**

**Bee rearing and sample collection**

*Apis mellifera* were sampled from the Honey bee Research Institute, Jiangxi Agricultural University, China. Queens were artificially bred in normal conditions according to standard rearing practices (Zeng, 2009). After emergence, twelve young queens were sampled as newly emerged queens immediately. Others were caged and introduced into prepared individual nucleus colonies without other queens and
prevented from taking mating flights by a strip of ‘queen excluder’ material. 12 days later, these queens reached sexual maturity and were sampled. Drones of A. mellifera L were obtained from hives. The laying queens were only allowed to access to empty drone-combs and laid haploid eggs in drone cells. Then, the drone-combs were taken out of the hives and placed in an incubator before emerging. After emergence, twelve newly emerged drones were sampled, the remained drones were paint-marked and put in natural colonies, 16 days later, sexually matured drones were sampled.

The intestines of all the samples were removed for preventing contamination, and all these samples were stored at −80 °C until use.

**RNA extraction, library preparation and sequencing**

Total RNAs were isolated from each sample respectively and the quality and quantity of the RNA were determined by a Qubit fluorimeter and an Agilent 2100 Bioanalyzer. After DNase I treatment, mRNAs were isolated from total RNAs using Oligo (dT) magnetic beads and fragmented into short sequences in the fragmentation buffer. Then cDNA was synthesized using the mRNA fragments as templates. Short cDNA fragments were purified and resolved with EB buffer for end repair and single nucleotide A (adenine) addition. After that, the short cDNA fragments were connected with adapters. The suitable fragments were selected for PCR amplification as templates. During the QC steps, Agilent 2100 Bioanalyzer and ABI StepOnePlus Real-Time PCR System were used in quantification and qualification of the sample libraries.

At last, the libraries were sequenced using Illumina HiSeq™ 2000.

**Raw data processing and statistical analysis**

The original image data produced by the sequencer were transformed into sequence data by base calling and the clean reads were obtained by discarding the dirty raw reads. The clean reads were mapped to reference gene sequences of Apis mellifera (ftp://ftp.ncbi.nih.gov/genomes/Apis_mellifera) using the SOAP2 alignment algorithm, with a tolerance of no more than two mismatches. The sequencing saturation, distribution and coverage of reads were used to assess sequencing quality. The clean reads were submitted to the Sequence Read Archive (SRA) database in NCBI under accession numbers SRR3569809, SRR3569811, SRR3569812, and SRR3569813.

**Identification and functional analysis of DGEs**

The level of gene expression was calculated as RPKM, the formula is shown below:

$$ RPKM = \frac{10^6 \times C}{NL/10^3} $$

Set RPKM to be the expression of unigene A, and C to be number of reads that uniquely aligned to unigene A, N to be total number of reads that uniquely aligned to all unigenes, and L to be the base number in the CDS of unigene A. The RPKM method is able to eliminate the influence of different gene length and sequencing level on the calculation of gene expression. Therefore the calculated gene expression can be directly used for comparing the difference of gene expression between samples (Mortazavi et al., 2008).

We can identify DEGs between two samples referring to “The significance of digital gene expression profiles” which has been cited hundreds of times (Audic and Claverie, 1997). Gene Ontology analysis, and pathway enrichment analysis were performed to investigate functional enrichment among up- or down-regulated genes using the DFCI Honey Bee Gene Index database (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=honeybee), Cluster (http://bonsai.hgc.jp/~mdehoon/software/cluster/), AmiGO (http://amigo.geneontology.org/amiigo), and the KEGG database (http://www.genome.jp/kegg/).

**Validation of RNA-Seq data by qRT-PCR**

To verify the data obtained by RNA-Seq, qRT-PCR was performed in triplicate and GAPDH was selected as the reference gene to correct for sample variation in qRT-PCR efficiency and errors in sample quantification. The qRT-PCR data were expressed relative to the expression of GAPDH using the 2−ΔΔCt method, an independent-sample t-test available in SPSS software. The primers were designed with Primer Premer5.0 software and synthesized by Generay Biotech (Generay, PRC) based on the mRNA sequences obtained from the GenBank database (Table 1).

**Results and discussion**

**Statistic of sequencing results**

Illumina HiSeq™ 2000 platform was used to identify DEGs among the four groups. More than 31,567,186 raw reads per library were obtained and over 87.80% of these reads were identified as clean reads before they were mapped to the reference database. More than 93.10% of the clean reads successfully matched to either unique or multiple locations of the honey bee genome. Of them, >98.01% of the mapped reads are matched to unique gene (Table 2).

**DEGs between the newly emerged drones and sexually matured drones**

Very few studies have investigated the molecular mechanism of sexual maturation in male insects. To identify changes in gene expression associated with sexual maturity, we used strict statistical criteria [false discovery rate (FDR) ≤ 0.01 and the absolute value of log2Ratio ≥ 1] to screen for genes that showed significant expression difference between the newly emerged drones and sexually matured drones. A total of 1618 DEGs were detected between the two stages of drones, with 782 up-regulated genes and 836 down-regulated genes in sexually matured drones compared to newly emerged drones (Document S1). GO analysis of these DEGs indicated that 139 GO items were significantly enriched (p < 0.05), Document S5A lists the top 47 significantly enriched terms.

KEGG pathway analysis indicated that 21 pathways were significantly enriched (p < 0.05), including “Parkinson’s disease”, “Oxidative phosphorylation”, “Alzheimer’s disease”, “Circadian rhythm – fly” and “Linoleic acid metabolism” and “NOD-like receptor signaling pathway”, and so on. Document S5B lists the top 10 significantly enriched pathways. The enrichment of the “Circadian rhythm” related genes might suggest that the mating behavior of drones is under control of circadian rhythm.

Of the 1618 DEGs, many olfactory related genes showed expression difference between newly emerged drones and sexually matured drones, including 12 OBP genes, 26 OR genes and the chemosensory protein 5 (CSP5) gene. Of the 12 OBPs, 10 of them were up-regulated in the sexually matured drones and 2 down-regulated. For the 26 ORs, 16 of them were up-regulated in the sexually matured drones and 10 down-regulated. The chemosensory protein 5 gene was up-regulated in the sexually matured drones. Odorant binding proteins are a kind of

**Table 1**

<table>
<thead>
<tr>
<th>Primer sequence.</th>
<th>Official Symbol</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
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<tbody>
<tr>
<td>0bp13</td>
<td>CTCGTGTGTTATCTGCG</td>
<td>CTTCTTAACCTGTCCTGCTT</td>
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</tr>
<tr>
<td>0bp14</td>
<td>CAAAATAGCCTTATGGAGGC</td>
<td>TCCATGACTCTGTTATGCCC</td>
<td></td>
</tr>
<tr>
<td>0bp15</td>
<td>AAATTTAACCAGATTGCTTACG</td>
<td>CTCGCTGGCTTATCTGATGTCT</td>
<td></td>
</tr>
<tr>
<td>0bp17</td>
<td>AGTCGCTGCTGATGATTCGCAA</td>
<td>GAAATATACGGCAAGATCCCG</td>
<td></td>
</tr>
<tr>
<td>0bp21</td>
<td>TTAGGAAATGCGGCCTACG</td>
<td>TTCTCATACATGATGGCTT</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>TCTATGCTGTTACGTCATAC</td>
<td>GAAGGCTACCACTACCATCTAA</td>
<td></td>
</tr>
</tbody>
</table>

- mdehoon/software/cluster/; AmiGO (http://amigo.geneontology.org/amiigo), and the KEGG database (http://www.genome.jp/kegg/).
also coordinate the sexual maturation of honeybee drones. Sensory proteins provided by the gland in the corpora allata can synthesize or decompose the hormone (Li et al., 2004). The higher expression of these substances compared to newly emerged bees will lead to a better resistance of adult bees to some toxic and harmful substances associated with pheromone synthesis, such as 9-ODA. In addition, as a class of detoxifying enzyme, higher expression of cytochrome P450s may be associated with the fact that queens need to feel the stimulation from the environment together with chitin as a barrier which forms the stratum corneum (Liu et al., 2010). The higher expression of cuticle protein genes in the newly emerged drones might be because the newly emerged bees are weak and their physique is soft, they need to synthesize chemosensory protein to feel the changes of the environment. Their main function is feeling environment with small molecular weight widely distributed in lymph in a variety of insect chemoreceptors. Their main function is feeling environment.

Of the DEGs, there are 19 cuticular protein coding genes, most of them were expressed higher at the newly emerged stage. Insect cuticle proteins are a kind of structure proteins which can resist the external environment together with chitin as a barrier which forms the stratum corneum (Liu et al., 2010). The higher expression of cuticle protein genes in the newly emerged drones might be because the newly emerged bees are weak and their physique is soft, they need to synthesize more cuticle proteins to be transported to the epithelial cells of integument to build the exoskeleton.

We found the gene JH (GB15327) and a yolk protein Vitellogenin (GB13999) were up-regulated in matured drones. Cytochrome P450 genes are widely distributed in all aerobic organisms, such as insects, plants, bacteria, etc. They not only participate in the biological metabolism, but also in synthesis or decomposition the hormone (Li et al., 2004). The higher expression of most cytochrome P450s at sexually matured stage may be associated with pheromone synthesis, such as 9-ODA. In addition, as a class of detoxifying enzyme, higher expression of cytochrome P450s will lead to a better resistance of adult bees to some toxic and harmful substances compared to newly emerged bees.

Three chemosensory proteins (CSP) differentially expressed between these two stages. Of them, CSP1 (GB17875) was up-regulated in the sexually matured queens, while CSP2 (GB18819) and CSP3 (GB19453) were opposite. CSPs are a kind of water-soluble proteins with small molecular weight widely distributed in lymph in a variety of insect chemoreceptors. Their main function is feeling environment chemical stimulation, carrying and protecting non-volatile odor molecules reaches the corresponding receptors through lymph in chemoreceptor. The up-regulation of CSP1 indicates that sexual mature queens need to synthesize chemo-sensory protein to feel the changes of the environment.

A total of 1340 DEGs were detected between newly emerged queens and sexually matured queens, with 667 up-regulated genes and 673 down-regulated genes in emerged queens (Document S2).

GO analysis of these DEGs indicated significantly enrichment of 114 GO terms (p < 0.05), Document S5C lists the top 46 significantly enriched terms. The "metabolic process" and "cellular process" in biological process, "cell" and "cell part" in cellular component, "binding" and "catalytic activity" in molecular function were dominant items in each category respectively.

KEGG pathway analysis indicated that 19 pathways were significantly enriched (p < 0.05), including "Parkinson's disease", "Oxidative phosphorylation", "Alzheimer's disease", "ECM-receptor interaction", "Citrate cycle (TCA cycle)", and so on. Document S5D lists the top 9 significantly enriched pathways.

Of the 1340 DEGs, 5 OBP genes and 40 OR genes showed expression difference between the two stages of queens. Of the 5 OBP, 3 were up-regulated and 2 were down-regulated in the sexually matured queens. Most of the ORs were up-regulated at the sexually matured stage. The up-regulation of OBP and OR in sexually matured queens might be associated with the fact that queens need to feel the stimulation from the environment.
We found 21 cuticular protein genes, most of them were expressed higher at the newly emerged queens which might also need to synthesize more cuticle proteins to build the exoskeleton.

Besides, two JH related genes, juvenile hormone esterase (GB15327) and juvenile hormone acid methyltransferase (GB10517), were significantly up-regulated in matured honeybee queens compared with newly emerged queens. It was reported that reproductive maturation in female insects normally correlates with their ovarian maturity and egg production, and many hormonal genes such as JH and Vg dominate the reproductive maturation in female insects (Teal et al., 2000; Ringo et al., 1991; Soller et al., 1999; Yano et al., 1994). The up-regulation of JH related genes in matured queens suggests that JH might regulate the reproductive maturation of honeybee queens.

Comparison of sexual maturity related DEGs between queens and drones

597 genes were expressed differently between the newly emerged stage and sexually mature stage both in drones and queens, of them, 411 showed the same expression trend between drones and queens (Document S3), while 186 showed opposite expression trend (Document S4). Of the 411 co-expressed genes, 178 (43.31%) were up-regulated and 233 (56.69%) were down-regulated at the sexually matured stage (Fig. 1A and B). Gene expression clustering of all the up-regulated and down-regulated co-expression DEGs showed that samples from the same sex were clustered together (Document SSE and F).

We found that both matured drones and queens had significantly higher expression of JH gene compared to the newly-emerged bees. Moreover, 7 serine protease RpsSs which involve in the development, immunity and digestion in insects (Krem and Di Cera, 2002; Rawlings and Barrett, 1993; Zou et al., 2006; Wang et al., 2007), as well as other 34 DEGs (6 cytochrome P450s, 4 OBP, 10 ORs and 14 cuticular proteins), were expressed differentially between the two developmental stages of both drones and queens. It revealed that honeybee sexual maturation, as in other insect species (Soller et al., 1999; Teal et al., 2000), may undergo a hormonally regulated period and accelerate the development of honeybee body, olfactory, immunity and secondary sexual characters.

It is interesting that 37 ribosomal proteins were highly expressed at the newly emerged stage of drones, while just 3 ribosomal proteins showed expression difference between the two stages in queens. The reason might be that the newly emerged drones need to synthesize large amounts of proteins for individual development, while the matured drones no longer need. Thus in drones, these ribosomal proteins showed a higher expression at the newly emerged stage. However, in queens, not only the newly emerged queens need to synthesize a large amount of protein, the sexually matured queens also need a lot of protein for the development of eggs, therefore, in queens the expression level of most ribosomal protein genes showed no difference between the two stages.

qRT-PCR validation

To confirm the results of the deep sequencing data, five differentially expressed OBP genes were chosen for qRT-PCR verification. The reason for choosing OBPs is that they are important proteins in the chemical information exchange between insects and the environment, moreover, the five OBPs genes were significantly differentially expressed between newly emerged and sexually matured bees in both queen and drones. The results showed as in Tables 3 and 4. The variation trend of genes expressed between newly emerged- and sexually matured bees were similar with the result of RNA-Seq. These data demonstrate the reliability of the RNA-Seq results.

Conclusions

This study described the gene expression pattern during honeybee sexual maturation. Thousands of genes, including hormonal genes, serine proteases, cytochrome P450s, OBP, ORs and cuticular proteins, were significantly differentially expressed during this process, indicating that honeybee sexual maturation are accompanied with huge physiology and gene transcription changes. These results provide valuable information for identifying key genes involved in regulating honeybee sexual maturation.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.aspen.2016.08.002.

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Table 3

<table>
<thead>
<tr>
<th>Gene</th>
<th>Newly emerged drone</th>
<th>Sexually matured drone</th>
<th>p-Value</th>
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</thead>
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<td>OBP13</td>
<td>1</td>
<td>0.0066</td>
<td>p &gt; 0.05</td>
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<td>2.2227</td>
<td>p &gt; 0.05</td>
</tr>
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<td>1</td>
<td>5.1696</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>OBP17</td>
<td>1</td>
<td>1.7118</td>
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<td>OBP21</td>
<td>1</td>
<td>7.2986</td>
<td>p &lt; 0.05</td>
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Table 4

<table>
<thead>
<tr>
<th>Gene</th>
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<th>Sexually matured queen</th>
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References


