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ORIGINAL ARTICLE

A comparison of honeybee (*Apis mellifera*) queen, worker and drone larvae by RNA-Seq

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Abstract Honeybees (*Apis mellifera*) have haplodiploid sex determination: males develop from unfertilized eggs and females develop from fertilized ones. The differences in larval food also determine the development of females. Here we compared the total somatic gene expression profiles of 2-day and 4-day-old drone, queen and worker larvae by RNA-Seq. The results from a co-expression network analysis on all expressed genes showed that 2-day-old drone and worker larvae were closer in gene expression profiles than 2-day-old queen larvae. This indicated that for young larvae (2-day-old) environmental factors such as larval diet have a greater effect on gene expression profiles than ploidy or sex determination. Drones had the most distinct gene expression profiles at the 4-day larval stage, suggesting that haploidy, or sex dramatically affects the gene expression of honeybee larvae. Drone larvae showed fewer differences in gene expression profiles at the 2-day and 4-day time points than the worker and queen larval comparisons (598 against 1190 and 1181), suggesting a different pattern of gene expression regulation during the larval development of haploid males compared to diploid females. This study indicates that early in development the queen caste has the most distinct gene expression profile, perhaps reflecting the very rapid growth and morphological specialization of this caste compared to workers and drones. Later in development the haploid male drones have the most distinct gene expression profile, perhaps reflecting the influence of ploidy or sex determination on gene expression.

Key words caste differentiation; environmental factors; gene expression; haploid and diploid; honeybees; larval development

Introduction

In the animal kingdom, approximatively 20% of species are haplodiploid animals such that haploid eggs develop into males and diploid eggs develop into females (Beye, 2004). Honeybees (*Apis mellifera*), a eusocial insect that reproduces by arrhenotokous parthenogenesis (Trivers & Hare, 1976), are a good model for studies of development of haplodiploid organisms. Queens and workers are females that develop from fertilized eggs, whereas drones are males from unfertilized eggs (Ratnieks & Keller, 1998). These three castes dramatically differ in gender, morphology, physiology and behaviour. Drones are highly specialized for mating, with larger compound eyes to detect virgin queens in flight, more wing sensilla, smaller mandibles, a large endophallus, no sting and hypopharyngeal glands. Queens are specialized for egg laying with large ovaries (Snodgrass, 1925). Several studies have investigated how the honeybee haplodiopoid genome is involved in sex determination and developmental regulation of honeybee males and females. The drone developmental pathway is considered to be primarily controlled by a genetic mechanism related to the ploidy of the embryo. Heterozygosity at the hypervariable complementary sex determiner gene (*csd*) locus determines that the

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embryo develops into a queen and worker, whereas homozygosity at the locus or (more commonly) one single copy determines drone development (Beye, 2004; Gempe *et al.*, 2009). Pires *et al.* (2016) compared gene expression between honeybee male and female embryos and observed certain messenger RNAs (mRNAs) and microRNAs (miRNAs) were expressed differently during haploid and diploid embryogenesis. Vleurinck *et al.* (2016) compared gene expression in brains of honeybee males and females and reported that both sex and caste signals are involved in the gene regulation in male and female brains. Similar results have been demonstrated in the fire ant (*Solenopsis invicta*): the gene expression profiles in haploid males are very different from those of diploid females and even diploid males at three developmental time points (Nipitwattanaphon *et al.*, 2014).

The effects of animal haploid genome on developmental regulation are far more complex as they have endopolyploidy. Diploidy is found in the muscles of male bumblebees (*Bombus terrestris* L.) (Aron *et al.*, 2005). In honeybees, Woyke and Paluch (1985) showed that haploid males reach endopolyploidy levels during their development and the endoreduplication is also observed in particularly active tissues, such as ventriculus, Malpighian tubules, fat body and silk gland. More interestingly, the endopolyploidy levels in drone larvae are much higher than in embryos and adults (Woyke & Paluch, 1985). However, the molecular mechanisms of regulating the larval development of drone, queen and worker remain unclear.

The honeybee queen and worker developmental pathways are controlled primarily by an environmental mechanism. Queens and workers are both females, but while queens have hundreds of ovarioles and hypertrophied ovaries, most workers normally have inactive ovaries and an underdeveloped spermatheca so that they cannot mate. Both workers and queens develop from diploid eggs, but queen larvae are supplied with an abundance of royal jelly over their whole larval stage, whereas workers are fed with worker jelly in the first 3 days and then are fed with a yellowish, pollen-containing food (Haydak, 1970). The royal jelly for queen larvae is dramatically different from jellies fed to either worker or drone larvae in terms of minerals and vitamin, sugar content, juvenile hormone and major royal jelly protein content (Haydak, 1970; Asencot & Lensky, 1984; Brouwers, 1984; Kamakura, 2011). This determines their caste differentiation.

Thousands of genes are differentially expressed between queen and worker larvae, including the signaling molecules vitellogenin, juvenile hormone and the mammalian target of rapamycin (mTOR) pathway, which are involved in the caste differentiation process (Hepperle & Hartfelder, 2001; Guidugli *et al.*, 2005; Barchuk *et al.*, 2007; Patel *et al.*, 2007; Chen *et al.*, 2012). However, thus far no study has compared gene expression in developing honeybee male and female larvae. The objective of this study was to systematically compare gene expression in workers, queens and drones at two points in larval development by RNA-Seq. The larval stage is crucial for the development of honeybees, as the polyploidization is higher at this point than at any other point during their lifespan (Woyke & Paluch, 1985). This would allow a genomic comparison of how and when the sex and caste differentiation pathways of honeybees diverge, and also a comparison of the gene expression differences arising from genetic and environmentally regulated developmental pathways.

Materials and methods

Insects

Six hives of the standard Chinese commercial strain of western honeybee (*Apis mellifera*) with a mature egg-laying queen and eight frames were located at the Honeybee Research Institute of Jiangxi Agricultural University (28.46°N, 115.49°E).

RNA-Seq analysis of queen, worker and drone larvae

For RNA-Seq, the mated queen was controlled on an empty worker and a drone frame to lay diploid and haploid eggs for 6 h. Subsequently, 2- and 4-day worker and drone larvae (six biological replicates from six colonies) were sampled using a bee grafting pen to lift the larvae from the wax cells in which they were developing. Larvae were immediately flash-frozen in liquid nitrogen. For 2-day-old larval samples (36–42 h after hatching), approximately 30 larvae were collected for each RNA sequencing sample, while for 4-day-old larval samples (84–90 h after hatching) six were collected. Four-day-old larvae are much larger than 2-day larvae and hence fewer larvae were needed for adequate RNA yield (6 μg). For the queen larvae samples, a new queen rearing methodology developed by Pan *et al.* (2013) was employed to control the queen to lay eggs for 6 h. Afterwards, eggs were immediately removed from queen cells and returned back to their natal colonies. Two- and 4-day queen larvae samples were then collected from queen cells at 2- and 4-day time points. Each larval group had six biological replicates from three different honeybee colonies (each colony provided two biological replicates for each group, therefore totally 36 samples were collected).

Total RNA of each sample was extracted from honeybee larvae according to the standard protocol of the TRIzol Reagent (Life Technologies, Carlsbad, CA, USA). RNA integrity and concentration were checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA).

Messenger RNA was isolated from total RNA using a NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB, E7490). A complementary DNA (cDNA) paired-end library was constructed following the manufacturer's instructions for the NEBNext Ultra RNA Library Prep Kit (NEB, E7530) and the NEBNext Multiplex Oligos (NEB, E7500) from Illumina. In brief: enriched mRNA was fragmented into approximately 200nt RNA inserts, which were used as templates to synthesize the cDNA. End-repair/dA-tail and adaptor ligation were then performed on the double-stranded cDNA. Suitable fragments were isolated by AgencourtAMPure XP beads (Beckman Coulter, Inc., Brea, CA, USA), and enriched by polymerase chain reaction (PCR) amplification. Finally, the constructed cDNA libraries of the honeybee were sequenced on a flow cell using an IlluminaHiSeq^{TM} 2500 sequencing platform.

Low-quality reads, such as adaptor-only reads or reads with >5% unknown nucleotides were filtered from subsequent analyses. Reads with a sequencing error rate less than 1% (Q20 >98%) were retained. These remaining clean reads were mapped to the *Apis mellifera* official genes (OGSv3.2) using Tophat2 software (Kim *et al.*, 2013). The aligned records from the aligners in BAM/SAM format were further examined to remove potential duplicate molecules. Gene expression levels were estimated using FPKM values (fragments per kilobase of exon per million fragments mapped) by the Cufflinks software (Trapnell *et al.*, 2010).

Co-expression network analysis

To explore the correlations of gene expression among queen, worker and drone larvae, the read count of each gene from six larval groups were used for weighted correlation network analysis (WGCNA) in R package (3.1.1) according to the method developed by Langfelder and Horvath (2008), resulting in a gene clustering tree for each larval group shown in Figure 1.

Identification of differentially expressed genes

DESeq and Q-value were employed and used to evaluate differential gene expression among queen, worker and drone larvae by estimating the count data from high-throughput sequencing assays and testing for differential expression based on a model using the negative binomial distribution (Anders & Huber, 2010). The false discovery rate (FDR) control method was used to identify the threshold of the *P*-value in multiple tests in order to compute the significance of the differences by using the read count of each gene. Here, only genes with an absolute value of $\log_2$ ratio ≥1 and FDR significance score <0.01 were used for subsequent analysis. Gene abundance differences between sample groups were calculated based on the ratio of the FPKM values which were used for presenting the gene expression of each gene.

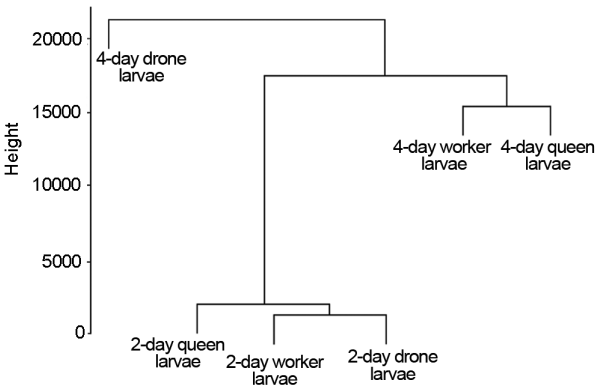

Fig. 1 The gene clustering tree of 2- and 4-day drone, worker and queen larvae. Read counts of all genes from six larval groups were analyzed by a weighted correlation network analysis (WGCNA) in R package (3.1.1) for the correlations of whole gene expression among drone, worker and queen larvae. The *y*-axis value is euclidean distance.

Sequences differentially expressed between sample groups were identified by comparison against various protein databases by Basic Local Alignment Search Tool X (BLASTX), including the National Center for Biotechnology Information (NCBI) non-redundant protein (Nr) database, and Swiss-Prot database with a cut-off E-value of 10^{-5}. Furthermore, genes were searched against the NCBI non-redundant nucleotide sequence (Nt) database using BLAST by a cut-off E-value of 10^{-5}. Genes were retrieved based on the best BLAST hit (highest score) along with their protein functional annotation. All significantly differentially expressed genes (DEGs) were mapped to terms in the Gene Ontology (GO) database. The GO enrichment analysis of functional significance used a hypergeometric test (*P*-value < 0.05 indicates the significance) (He *et al.*, 2014; Qin *et al.*, 2014) to identify significantly enriched GO terms in DEGs compared to the complete genome. DEGs from queen versus drone larvae and worker versus drone larvae comparisons were selected for the GO enrichment analysis. DEGs between 2- and 4-day

larvae in three larval castes were also employed in the GO enrichment analysis.

DEGs were mapped to the Kyoto Encylcopedia of Genes and Genomes (KEGG) protein database (KEGG database: http://www.genome.jp/kegg/kegg1.html) by BLAST (E-value < 1e-5). KOBAS 2.0 software was used to test the statistical enrichment of DEGs in KEGG pathways using a hypergeometric test (Q-value < 0.05) (Xie *et al.*, 2011).

Statistics of raw data and saturation analysis of sequencing

In RNA-Seq, six libraries were generated from our experimental groups, and summaries of RNA sequencing analyses are shown in Table S1. In each library, more than 97% clean reads were unique mapped reads of which more than 86% of reads were paired reads. Very few clean reads (<2.3%) were multiple mapped reads. Each library had a sufficient coverage of the expected number of distinct genes (stabilized at 3M reads). The Pearson correlation coefficient among three biological replicates of each experimental group were all >0.80 (Table S2), which is a conventionally accepted threshold for valid replicates (Trapnell *et al.*, 2010) indicating that there was acceptable sequencing quality and repeatability among the biological replicates of each group.

Results

Number of total genes detected in all samples

We compared gene expression measured with RNA-Seq in queen, worker and drone larvae sampled when 2 and 4 days old. There was no significant difference ($P > 0.05$, analysis of variance followed by Fisher's protected least significant difference test) among the six larval groups in terms of total gene number detected in the RNA-Seq (Fig. 2A). Most genes were expressed in all these three castes, very few (<1.6%) were uniquely expressed in only one group (Fig. 2B).

Results of co-expression network analysis

A weighted correlation network analysis (WGCNA, Fig. 1) showed that all the 2-day-old samples formed quite a tight cluster. Within that cluster, 2-day-old worker and drone larvae were more similar than 2-day-old queen larvae. The 4-day-old larval samples were more

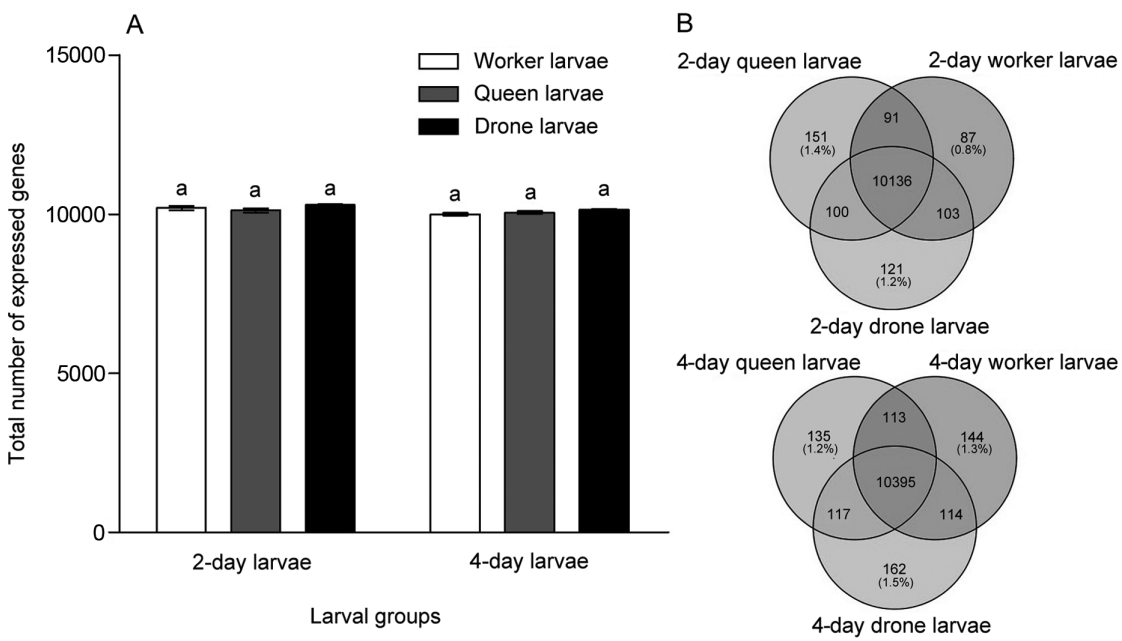

Fig. 2 Total number of expressed genes in six larval groups (A) and Venn diagram of expressed genes among six larval groups (B). Open, grey and black bars represent worker, queen and drone larvae groups, respectively. Each group has six biological replicates. Same letters 'a' on top of bars indicate no significant difference ($P < 0.05$, analysis of variance followed by Fisher's protected least significant difference test in Statview 6.0).

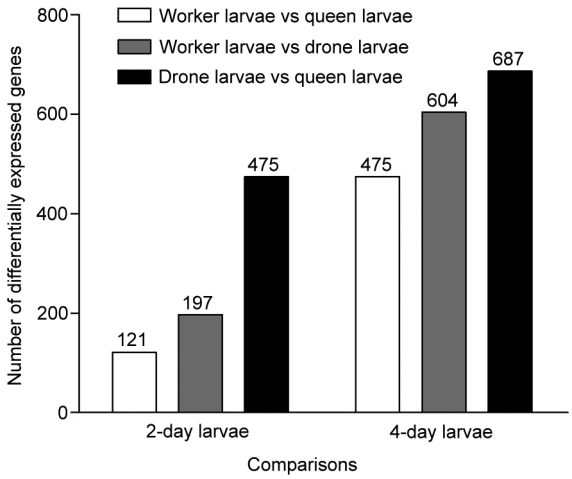

Fig. 3 Number of differentially expressed genes (DEGs) among queen, worker and drone larvae at 2- and 4-day stages. Open, grey and black bars represent comparisons of worker larvae versus queen larvae, worker larvae versus drone larvae and queen larvae versus drone larvae, respectively.

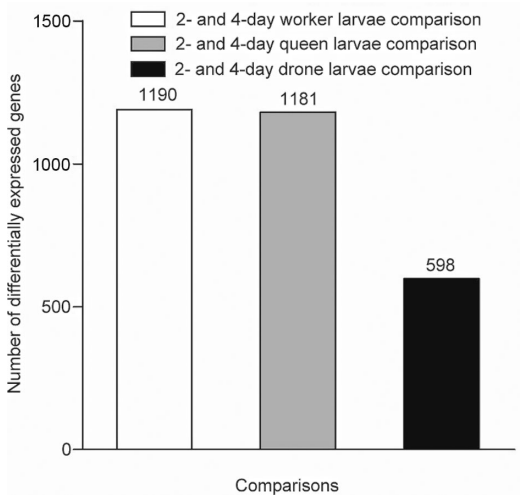

Fig. 4 Number of differentially expressed genes (DEGs) between 2- and 4-day larvae in the same larval types. Open, grey and black bars represent comparisons of 2-day versus 4-day worker larvae, 2-day versus 4-day drone larvae and 2-day versus 4-day queen larvae, respectively.

divergent. Four-day drone larvae were the most distinct group, whereas 4-day-old queen and worker larvae (the two female castes) remained quite closely clustered.

Differentially expressed genes among queen, worker and drone larvae

When considering the numbers of DEGs between groups (Fig. 3, Table S3–S8), for both 2- and 4-day larvae, the fewest number of DEGs were between worker and queen larvae (the two female castes), and the greatest number of DEGs were between queen and drone larvae (the fertile male and female sexes).

Further, comparisons among 4-day-old larvae had higher numbers of DEGs compared to 2-day-old larvae comparisons (Fig. 3). While comparing the DEGs between 2- and 4-day larvae of the same larval type, queen and worker larvae showed more DEGs than drones (1181 and 1190 against 598, Fig. 4, Tables S9–S11).

Interestingly, 33 genes of the DEGs between males and females were involved in hormone biosynthesis, oocyte maturation, venom, eye development, sex determination, wnt signaling pathway and notch signaling pathway (Fig. 5).

GO enrichment analysis

Figure 6 showed that DEGs between 2-day female and male larvae were enriched in 11 categories such as membrane, catalytic activity and metabolic process, whereas there were more DEGs and categories (29) in the 4-day comparisons. Furthermore, Figure 7 showed that categories and percentages of DEGs in 2- and 4-day queen, worker and drone larval comparisons were similar, although drone larvae had fewer DEGs in each category compared to worker and queen larvae. However, percentages of DEGs in four GO enrichment categories including protein binding transcription factor activity, translation regulator activity, antioxidant activity and morphogen activity were different in drones compared to female queen and workers.

Discussion

Here we used genomic analyses to compare the divergence of the three honey bee castes during larval development: queen, workers and drones. Our results indicated that in early larval development (day 2) queens had the most distinct gene expression profiles when compared to workers and drones, but by day 4 of larval development male drones had the most distinct gene expression profiles when compared to the queen and worker castes (Fig. 1). This suggests that developmental pathways segregate most strongly by sex and a genetically controlled developmental system, but these differences only manifest in the later stages of larval development. Previous studies showed that cell nuclei in 1st-instar male larvae are haploid but become polyploidy in later instar larvae

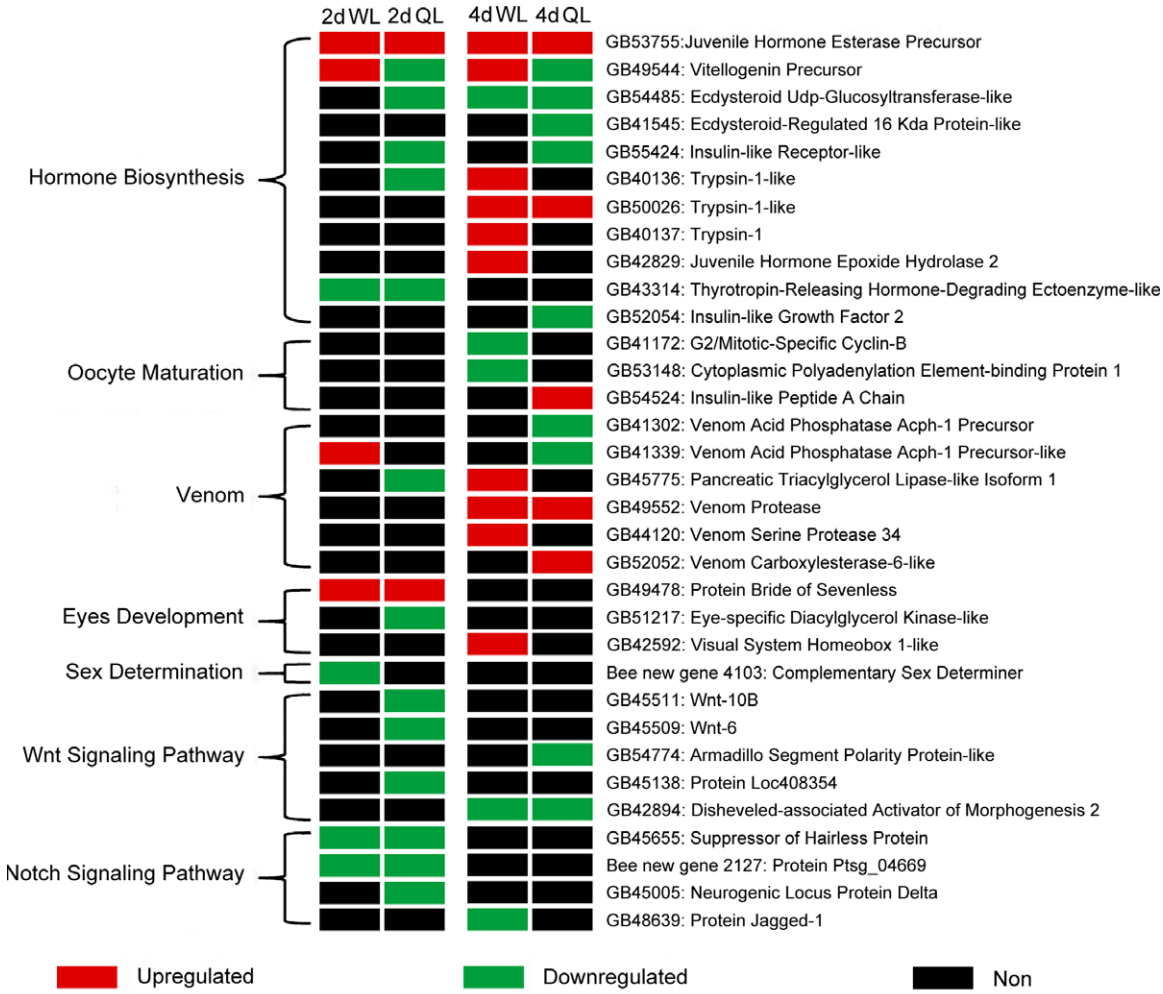

Fig. 5 Gene expression of 33 genes among six larval groups. Differentially expressed genes (DEGs) were measured as their read counts under a statistic value of false discovery rate <0.01 and the absolute value of $\log_2$ FC >1. Genes significantly upregulated in worker or queen larvae compared to drone larvae are colored red. Genes significantly downregulated in the same comparisons are colored green. Black represents no significant difference in expression ratio. WL and QL represent worker larvae and queen larvae, respectively.

(Risler, 1954; Woyke & Paluch, 1985). Perhaps the polyploidization plays a very important role in the regulation of gene expression in older male larvae.

Larvae sampled when 2 days old are quite similar in terms of gene expression profiles regardless of caste allocation. For 2-day-old larvae, queens were the most divergent group, suggesting that in the early stages of larval development the influence of the rearing environment and royal jelly diet has a greater impact on gene expression profiles than the sex determination system. Royal jelly dramatically differs in terms of minerals and vitamins, sugar content, juvenile hormones and major royal jelly protein content compared to worker and drone jelly (Haydak, 1970; Asencot & Lensky, 1984; Brouwers, 1984; Kamakura, 2011), and these nutritional differences can induce thousands of DEGs between queen and worker larvae (Chen *et al.*, 2012). Vleurinck *et al.* (2016) showed that more DEGs and differentially spliced genes (DSGs) were detected in worker and queen pupae brains than worker and drone brains. Our findings indicate that these nutritional differences have a greater impact on gene expression than haplodioploidy in early larval development.

Although the total number of expressed genes in the three castes was not significantly different (Fig. 2), hundreds of genes were significantly differentially expressed among them (Fig. 3). The drone larvae versus queen larvae comparison had the greatest number of DEGs, indicating that both genomic and nutritional differences expand

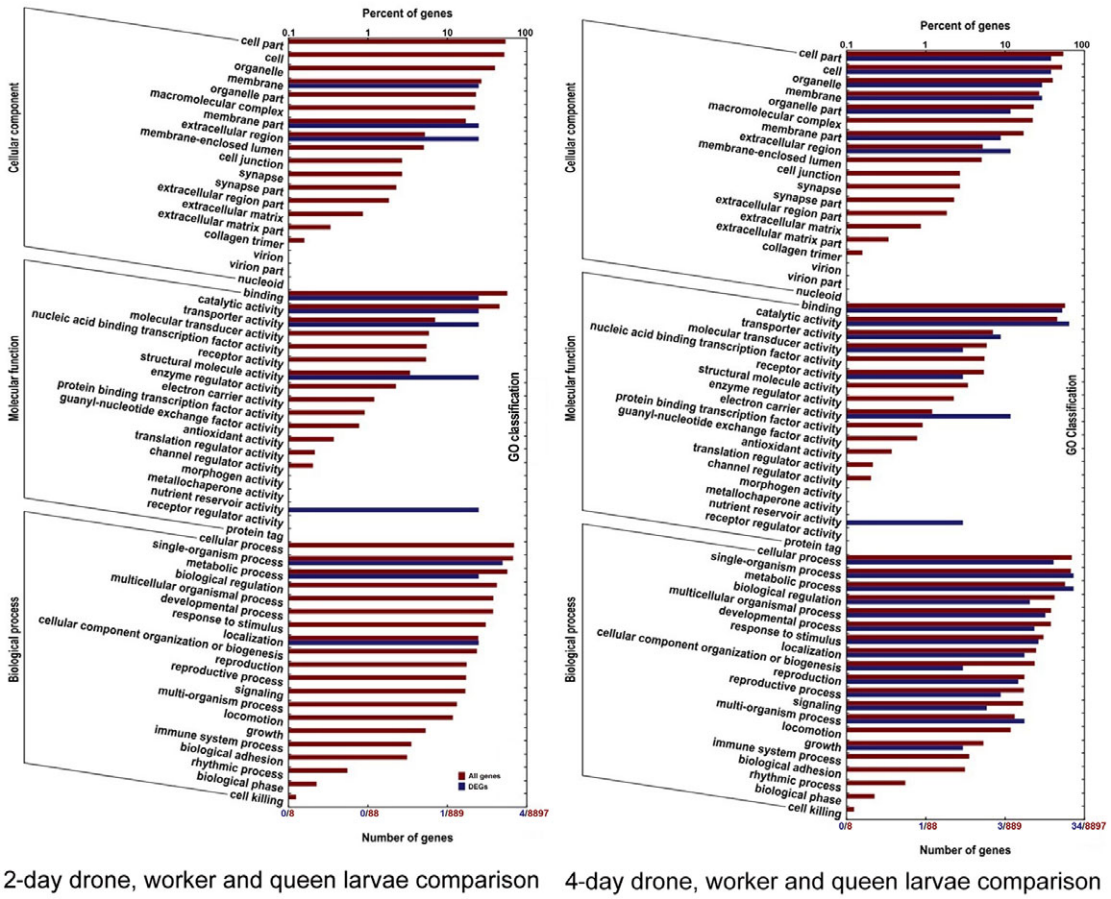

Fig. 6 Gene Ontology classification of same differentially expressed genes (DEGs) from queen versus drone larvae and worker versus drone larvae comparisons. The results are summarized in three main categories: biological process, cellular component and molecular function. *y*-axis indicates category, *x*-axis indicates the percentage of DEGs. Red bars are all genes, blue bars are DEGs.

the extent of developmental differentiation of drones and queens. When comparing DEGs between 2- and 4-day larvae of the same larval type, queen and worker larvae showed more DEGs than drones (Fig. 4), further emphasizing the clear difference between the male and female developmental trajectories. Comparisons among 4-day-old larvae had higher numbers of DEGs compared to 2-day-old larvae comparisons (Fig. 3), and GO enrichment categories were higher in 4-day male and female larvae compared to 2-day comparisons, suggesting that the extent of differentiation among drone, queen and worker larvae increased with their age.

To explain the morphological, physiological and behavioral differences among female and male honeybees, we noted 33 interesting DEGs which are involved in hormone biosynthesis, oocyte maturation, venom, eye development, sex determination, wnt signaling pathway and notch signaling pathway (Fig. 5). Both 2- and 4-day drone larvae differed from female honeybees in terms of the expression of genes involved in hormone biosynthesis, oocyte maturation and the mTOR pathway (Fig. 5, Fig. S1–S3). Many previous studies have shown that genes involved in hormone biosynthesis such as *juvenile hormone esterase precursor*, *vitellogenin precursor* and *ecdysteroids* that are involved in the caste differentiation of the queen and workers (Wirtz & Beetsma, 1972; Rachinsky *et al.*, 1990; Chen *et al.*, 2012; Cameron *et al.*, 2013). In this study, these hormone-related genes were also significantly differentially expressed between male drones and female queen and workers (Fig. 5), suggesting that these genes may be involved in sex determination also. *Insulin-like receptor-like*, *Insulin-like peptide A chain*, *cytoplasmic polyadenylation element-binding protein 1* and *G2/mitotic-specific cyclin-B (LOC551860)* that are involved in oocyte maturation and the mTOR pathway [a pathway that determines ovary development in

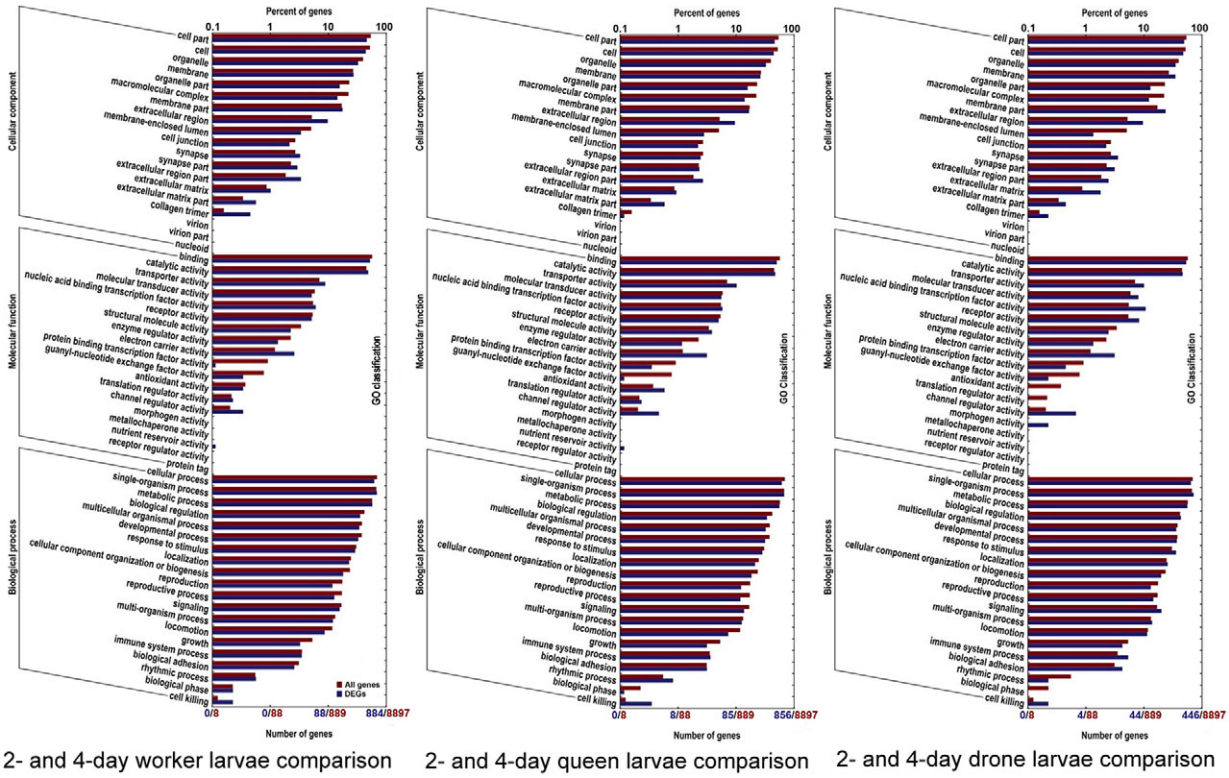

Fig. 7 Gene Ontology classification of differentially expressed genes (DEGs) between 2- and 4-day larvae in three larval castes. The results are summarized in three main categories: biological process, cellular component and molecular function. *y*-axis indicates category, *x*-axis indicates the percentage of DEGs. Red bars are all genes, blue bars are DEGs.

queen and workers (Wheeler *et al.*, 2006; de Azevedo & Hartfelder, 2008)], were differentially expressed among drone, queen and worker larvae (Fig. 5). This may reflect an early genomic signal of the key reproductive differences between the three castes.

Honeybee drones have much larger compound eyes than workers and queens, and females produce venom but not in males (Trivers & Hare, 1976). Here we showed that 2-day worker and queen larvae had one and two DEGs involved in the development of eyes compared to 2-day drone larvae, and one DEG was found in 4-day worker larvae (Fig. 5). Marco and Hartfelder (2016) reported that honeybee workers and drones have already started their eye development process from the 3rd instar stage, and a few genes involved in this process are differentially expressed between the sexes. Roat and Cruz-Landim (2011) also showed the antennal lobes of workers, queens and drones are already different at larval stage and drones have bigger antennal lobes than that of workers and queens. Our results are consistent with their findings, reflecting that the differentiation of eye development between honeybee males and females is from a very early larval stage. Furthermore, male and female honeybee larvae differed in venom gene expression, with four and three DEGs in 2-day queen versus drone and worker versus drone comparisons, respectively, and one in 4-day comparisons (Fig. 5). Although it is unclear when honeybee females start the developmental process of their venom glands, these significantly differentially expressed venom-gland related genes might play an important role in regulating in the development of venom glands in female castes. This still requires further investigation.

Honeybee sex is determined by the *csd* gene and interacts with *feminizer* (*fem*) and *doublesex* (*dsx*) genes (Charlesworth, 2003; Gempe *et al.*, 2009). Here we did not find *fem* and *dsx* were differentially expressed between male and female larvae. Only the *csd* gene was downregulated in 2-day worker larvae compared to 2-day drone larvae (Fig. 5). A previous study showed that *csd* and *fem* genes are required only to initiate sex-specific differentiation in early embryogenesis (Gempe *et al.*, 2009). Therefore, it still remains unclear how sex-determining genes play a role in larval development and this needs further investigation.

Many genes from wnt and notch signalling pathways were significantly downregulated in female larvae compared to male larvae. In detail, three and two genes from the wnt signalling pathway in 2- and 4-day queen larvae were downregulated compared with drone larvae, respectively, and one was downregulated in 4-day worker larvae compared to drone larvae (Fig. 5 and Fig. S4). Three genes involved in the notch signalling pathway were downregulated in 2-day queen and drone comparisons, whereas two and one were downregulated in 2- and 4-day worker and drone comparisons (Fig. 5 and Fig. S5). Wnt and notch signalling pathways are two conserved pathways playing an important role in embryogenesis, morphogenesis and imaginal disc development in insects and other animals (Dearden *et al.*, 2006; Bolos *et al.*, 2007; Komiya & Habas, 2008; Wilson *et al.*, 2011). Therefore these DEGs may play an important role in the body development and formation of morphological traits of male and female honeybees, which requires further investigation.

Conclusion

Consequently, our data show the complexity of honeybee caste differentiation, and that clear differences between castes are established even very early in larval development, long before the formation of adult or reproductive structures. Honeybee ploidy, sex determination and environmental factors may all influence the larval gene expression profiles and developmental trajectory. Our findings contribute information on how genetic and environmental factors regulate male and female development in haplodiploid insects.

Acknowledgement

ZJZ and XJH conceived and designed the experiments. XJH and WJJ performed the experiments. XJH, XCZ and ABB analyzed the data. XJH, ZJZ and ABB wrote the paper. All authors read and approved the final manuscript. We thank Dr. Lianne Meah for revising the paper. This work was supported by the Science and Technology Project of Colleges and Universities of Jiangxi Province (KJLD13028), the Earmarked Fund for China Agriculture Research System (CARS-44-KXJ15) and China Scholarship Council (No. 201408360073).

Disclosure

The authors declare that they have no competing interests.

Additional information

RNA-Seq data of 2-day honeybee worker larvae: NCBI SRA: SRS1249139;

RNA-Seq data of 2-day honeybee queen larvae: NCBI SRA: SRS1263242;

RNA-Seq data of 2-day honeybee drone larvae: NCBI SRA: SRS1263244;

RNA-Seq data of 4-day honeybee worker larvae: NCBI SRA: SRS1263211;

RNA-Seq data of 4-day honeybee queen larvae: NCBI SRA: SRS1263243;

RNA-Seq data of 4-day honeybee drone larvae: NCBI SRA: SRS1263256.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 The insect hormone biosynthesis pathway from KEGG database and differentially expressed genes between male and female honeybee larvae. Significantly differentially expressed genes are marked with red color.

Fig. S2 The mediated oocyte maturation pathway from KEGG database and differentially expressed genes between male and female honeybee larvae. Significantly differentially expressed genes are marked with red color.

Fig. S3 The mTOR pathway from KEGG database and differentially expressed genes between male and female honeybee larvae. Significantly differentially expressed genes are marked with red color.

Fig. S4 The wnt signalling pathway from KEGG database and differentially expressed genes between male and female honeybee larvae. Significantly differentially expressed genes are marked with red color.

Fig. S5 The notch signalling pathway from KEGG database and differentially expressed genes between male and female honeybee larvae. Significantly differentially expressed genes are marked with red color.

Table S1. Statistics of RNA sequencing.

Table S2. Pearson correlation coefficient among three biological replicates of each larval group.

Table S3. DEGs in 2-day worker larvae against 2-day queen larvae. Genes were identified as DEGs if both FDR < 0.01 and the absolute value of $\log_2$ FC > 1. Gene ID, gene read counts, FPKM values, FDR, log2 FC, Regulation, COG class, COG class annotation, GO annotation, KEGG annotation, Swissprot annotation and gene annotation are shown in the table. Same in Tables S4–S11.

Table S4. DEGs in 2-day drone larvae against 2-day worker larvae.

Table S5. DEGs in 2-day drone larvae against 2-day queen larvae.

Table S6. DEGs in 4-day worker larvae against 4-day queen larvae.

Table S7. DEGs in 4-day drone larvae against 4-day worker larvae.

Table S8. DEGs in 4-day drone larvae against 4-day queen larvae.

Table S9. DEGs in 2-day worker larvae against 4-day worker larvae.

Table S10. DEGs in 2-day queen larvae against 4-day queen larvae.

Table S11. DEGs in 2-day drone larvae against 4-day drone larvae.