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Morphology and transcriptome differences between the haploid and diploid drones of *Apis cerana*

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ABSTRACT

In general, drone honey bees are haploid and develop from unfertilized eggs. However, a diploid drone can arise in an inbred colony. In this study, the morphological characteristics and gene expression profile of the haploid and diploid drones of *Apis cerana* were analyzed to reveal the differences between them. The ploidy level of the drones was identified by Flow Cytometry (FCM). The characters of the fore wings, wet weight of reproductive organs and of newly emerged drones, were investigated. Then, a high throughput transcriptomic analysis was performed using RNA-seq in diploid and haploid drones. The results showed that the wet weight and reproductive organs of diploid drones were significantly lighter than those of haploid drones. About 201 million high-quality reads were generated from RNA-seq, and 75.99–78.12% of the data were mapped to *Apis cerana* genome. 360 genes were differentially expressed between diploid and haploid drone, with 152 up-regulated and 208 down-regulated in the diploid drones. Functional analysis identified that these genes were significantly enriched in 28 pathways. Comparative transcriptomic analysis detected several differentially expressed genes, which lay a foundation for future studies on molecular mechanisms underlying biology difference in drones in *Apis cerana*.

Introduction

Honeybees are economically-valuable insects and a key model for social insects. A normal bee colony consists of a queen, hundreds of drones and tens of thousands of workers. The queens and workers are both diploid females, while the drones usually are haploid, but they could be diploid. The sex of the bees is determined by a compensatory sex determinator (*csd*) that has multiple alleles in natural populations of bees (Beye et al., 2003; Gempe et al., 2009; Liu et al., 2011). When in general, honey bee males (drones) are hemizygous at the *csd* alleles, while the females are heterozygous at *csd* (Cho et al., 2006; Wang et al., 2012a). However, nonreproductive diploid males can exist when the csd alleles are homologous. In natural colonies, diploid male eggs rarely occur and usually are removed by workers during very early stage (Woyke, 1963a, 1963b). However some conditions, such as artificial inbreeding program can lead to a big proportion of diploid male eggs.

The biological characteristics of diploid drones have been well studied in *A. mellifera* (Woyke, 1963c, 1969, 1978), which are called "supermale" with a heavier body and larger body parts than the haploid drones (Woyke, 1974). The diploid drones produce diploid spermatozoa, while the process of spermatogenesis is similar to that in the haploids (Woyke and Skowronek, 1974).

Apis cerana is widely bred in the mountain areas in South China, mainly because of its resistance to the mite *Varroa destructor* (Peng et al., 1987; Fries et al., 1996) and wasps (Tan et al., 2007). The transcriptome analysis has suggested the differential gene expression is associated with the royal jelly yield (Liu et al., 2014) and foraging activity (Li et al., 2012) between *Apis cerana* and *Apis mellifera*. The transcriptomic difference between queens and workers of *Apis cerana cerana* (Wang et al., 2012b) and the recent release of *Apis cerana* genome (Park et al., 2015) have provided a foundational resource for comparative genomics and a better insight into the biology of the Asian honey bee. Until now, studies on diploid drones of *A. cerana* are limited. In our study, the drones that emerged from worker combs in inbreed colonies were collected, the cell ploidy of the drones was determined by FCM and the morphological indexes were measured. Then, the transcriptome differences between the diploid and haploid males were analyzed using high-throughput RNA-Seq.

Materials and methods

Sample collection

Honey bee of *Apis cerana cerana* (*A. c. cerana*) colonies were reared using standard beekeeping techniques at the Honeybee Research Institute, Jiangxi Agricultural University, Nanchang, China (28.46°N,

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115.49°E). The virgin queens (*A. c. cerana*) were treated with CO_2 twice to induce unfertilized eggs laying. Each treatment was about 5 min. Then, they were put back into the original colony where all drones were eliminated. The colony was rewarded with sugar water. At the same time, a queen-excluder was placed at the entrance for every colony to prevent the queen from mating flight. About 24 days, the unfertilized eggs from worker comb developed into adult drones and emerged. For haploid drones, 50 males that newly emerged from worker cells were collected in each colony before queen artificial insemination for morphology analysis and RNA extraction.

Other newly emerged drones were marked with a different color, and a super box was added to the bottom hive on a sunny afternoon for them to defecate and fly. When the drones reached sexual maturity, the virgin queens were inseminated with the semen of these drones. Then, the queens were introduced into another colony in which all of the drones were killed. Finally, the male offspring of these artificially inseminated queens were collected immediately after eclosion and their heads or thoraces and abdomens were detected for ploidy level analysis to confirm their diploid status. The residues of every two drones were assembled with one complete biological sample for transcriptome sequencing analysis after eliminating the intestines. Two biological replicates of the diploid and haploid males sampled from two colonies were prepared.

The RNA-seq samples were rinsed in the DEPC water and stored in liquid nitrogen for RNA extraction.

Morphology analysis

Before ploidy level analysis, the body weight, width and length of the fore wings, as well as the weight of productive organs of newly emerged drones, were investigated.

Flow cytometry

The ploidy levels of the drones were analyzed by FCM, as described by Cournault and Aron (Cournault and Aron, 2008). The heads or thoraces and abdomens were cut off and crushed, then 1 mL of trypsin was added for 60 min of digestion in a water bath at 37 °C. After digestion, the cell suspension was filtered through a 300-mesh stainless steel wire mesh. The filtered cell suspension was centrifuged at 1, 500 r/min for 3 min. After discarding the supernatant, 200 μL of phosphate buffer saline (PBS) was added to prepare a single cell suspension. Before the test, 200 μL of propidium iodide (PI) dye was added, for staining, for 30 min at room temperature. The ploidy levels of all of the samples were determined using a flow cytometer (Cytomics 500MCL, BECKMAN COULTER). The test results of FCM were presented in a histogram of the DNA relative content. According to the position of the peak of the standard samples (diploid worker) in the histogram, the cell ploidy of the samples was confirmed. The peak of the standard samples was at position 200 and that of the haploid and diploid drones were at approximately 100 and 200, respectively.

RNA extraction, library preparation and Illumina sequencing

Total RNA was extracted from each sample using E.Z.N.A.TM Total RNA Kit II (OMEGA) according to the manufacturer's protocol and treated with RNase-free DNase I (Takara). For transcriptome analysis, the cDNA library was constructed. First, we enriched poly (A) mRNA using beads with Oligo (dT) and interrupted mRNA into short fragments as templates, then synthesized double-strand cDNA using SuperScript Double-Stranded cDNA Synthesis kit (Invitrogen) with random hexamer (N6) primers (Illumina). The short fragments were then purified using a QiaQuick PCR extraction kit and resolved with EB buffer to finish the end reparation, and were connected using sequencing adaptors. After resolution by agarose gel electrophoresis, the fragments suitable for PCR amplification were selected and were constructed two paired-end libraries which were sequenced using an Illumina HiSeq™2000 at Beijing Genomics Institute (BGI), Shenzhen, China.

Data filtering and mapping

The original image data were transferred into sequence data via base calling, which were defined as raw data or raw reads and saved as FASTQ files, which include the detailed read sequences and the read quality information. Low-quality reads were removed by a stringent filtering process, such as reads with adaptors, reads with >10% unknown reads, and low quality reads (the bases is over 50% in a read with a quality value ≤10). The data were deposited in the NCBI sequence read archive (SRX955729 for haploid and SRX955730 for diploid). The clean reads were compared to genome reference and gene reference of *Apis cerana* (http://mnbldb.snu.ac.kr/) using program BWA and Bowtie respectively. The tolerance parameters were the default settings, allowing mismatches of no more than two bases.

Analysis of sequencing results: differential expression

The level of gene expression was calculated as FPKM (expected fragments per kilobase of transcript per million fragments sequenced) (Trapnell et al., 2010). The FPKM method is able to eliminate the influence of different gene length and sequencing discrepancy on the calculation of gene expression. DEGs were identified based on the analysis method of the Poisson distribution. We use FDR ≤ 0.001(false discovery rate no greater than 0.001) (Eisen et al., 1998) and the absolute value of $Log_2Ratio \geq 1$ (two-fold change) as the threshold to judge the significance of gene expression difference. More stringent criteria with smaller FDR and bigger fold-change value can be used for subsequent analyses. And the correlation value between each two samples was calculated based on FPKM result.

Functional analysis of differentially expressed genes

Functional enrichment analyses including Gene Ontology (GO) and KEGG were performed to investigate functional enrichment in GO terms or metabolic pathways among DEGs. The expression pattern analysis was performed with Cluster and javaTreeview software. The calculated p-value goes through Bonferroni Correction (Kanehisa et al., 2008), taking corrected p-value ≤ 0.05 as a threshold. GO terms fulfilling this condition are defined as significantly enriched GO terms in DEGs. The GO annotations were functionally classified by WEGO software for gene function distributions.

Usually, genes interacting with each other play roles in certain biological functions. To further understand their biological functions, KEGG pathway analysis was conducted to identify significantly enriched metabolic pathways or signal transduction pathways in DEGs comparing with the whole genome background. Pathways with a Qvalue ≤ 0.05 are considered significantly enriched.

Results

Ploidy level analysis

As shown in Fig. 1, all of the samples detected by FCM formed an obvious peak, indicating that there were sufficient numbers of cells in each sample. In the speculated diploid samples, 41 out of 50 samples were confirmed to be diploid by FCM. Thus, >80% of them were diploid drones.

Morphology comparison

The morphological index comparison between the haploid and diploid drones is summarized in Table 1. The sample sizes of the

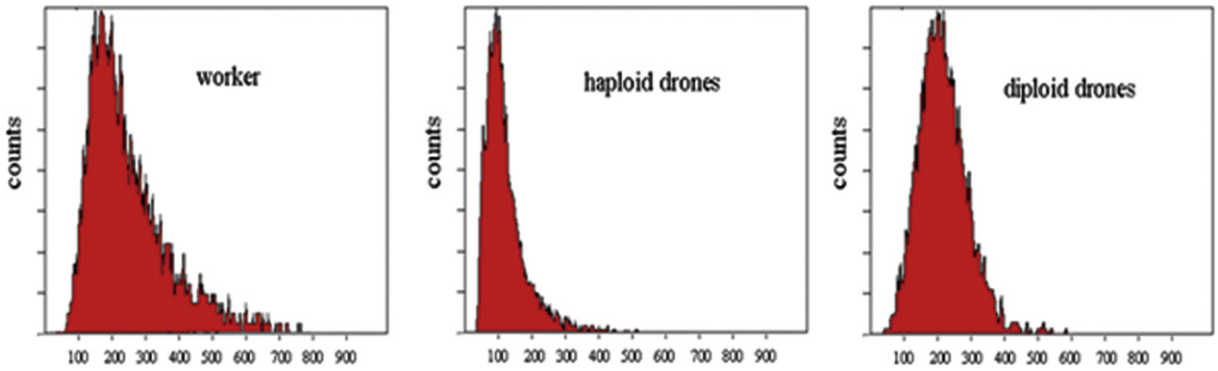

Fig. 1. The testing results of FCM.

Table 1
Morphological index comparison between haploid and diploid drones.

Drones type	Weight of newly emerged drones (mg)	Length of wings (mm)	Width of wings (mm)	No. of wing hooks	Weight of reproductive organs (mg)
Haploid	105.644 ± 7.234	9.005 ± 0.560	3.278 ± 0.154	16.838 ± 1.277	7.015 ± 0.035
Diploid	99.782 ± 5.069*	8.547 ± 0.548	3.177 ± 0.117	16.673 ± 1.438	6.045 ± 0.020*

* indicate significant difference ($P < 0.05$).

diploid drones in each colony varied slightly (N = 42–46) because they were confirmed by FCM. The data were analyzed by *t*-test using SPSS 16.0. In terms of the weight of newly emerged drones and reproductive organs, the diploid drones were significantly lighter than the haploid drones ($N = 50$, $p < 0.05$). For other characteristics (the fore wing length, fore wing width, number of hooks on the wing), the difference was not significant.

Illumina HiSeq mRNA sequencing

In total, 201 million clean reads were generated form the four libraries. The numbers and percentage of reads containing N, adaptor, tags with low quality and clean reads, are shown in Fig. 2. The reads from all the libraries were mapped to *Apis cerana* genome, about 75.99–78.12% of clean reads mapped to genome, 37.85–45.76% of

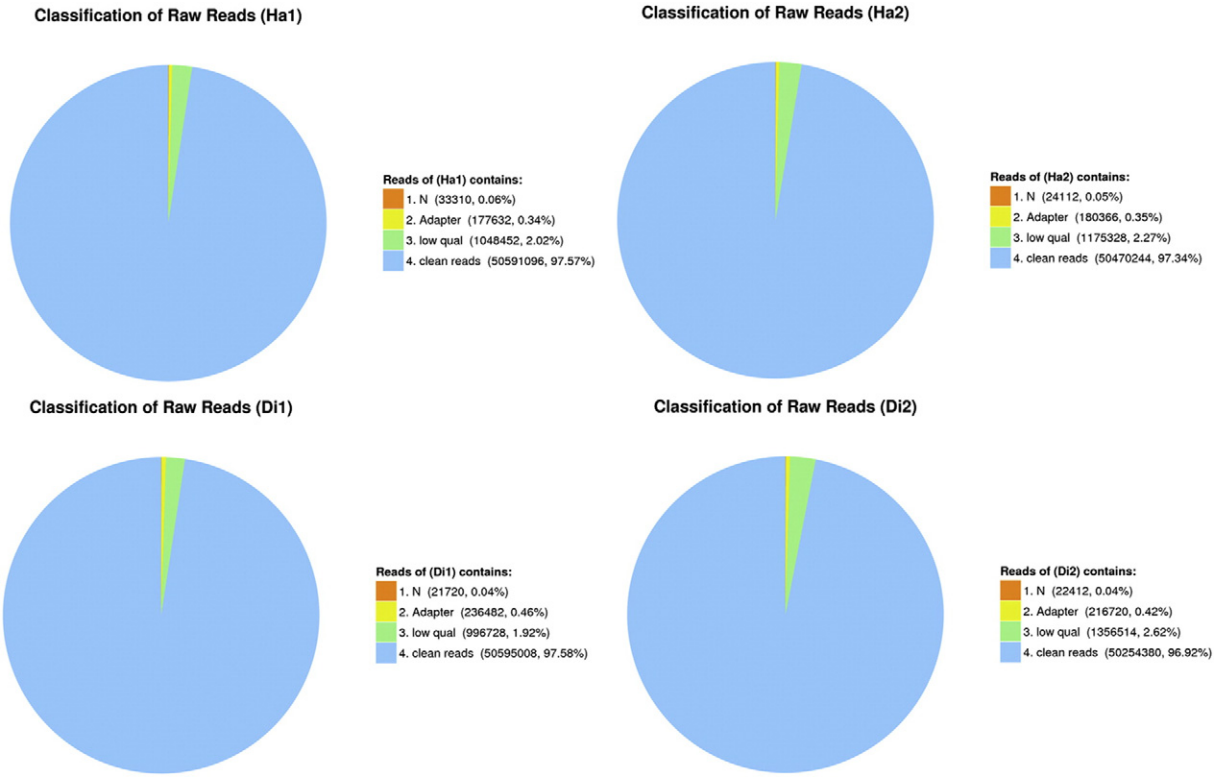

Fig. 2. Raw data filtering.

Table 2
Summary of reads numbers based on the RNA-sequencing data.

Reads number (Percentage)	Di1		Di2		Hi1		Hi2	
	Map to genome	Map to gene	Map to genome	Map to gene	Map to genome	Map to gene	Map to genome	Map to gene
Total reads	50595008 (100.00%)	50595008 (100.00%)	50254380 (100.00%)	50254380 (100.00%)	50591096 (100.00%)	50591096 (100.00%)	50470244 (100.00%)	50470244 (100.00%)
Total base pairs	4553550720 (100.00%)	4553550720 (100.00%)	4522894200 (100.00%)	4522894200 (100.00%)	4553198640 (100.00%)	4553198640 (100.00%)	4542321960 (100.00%)	4542321960 (100.00%)
Total mapped	39524508 (78.12%)	39524508 (78.12%)	39199123 (78.00%)	21714790 (43.21%)	38445574 (75.99%)	19150308 (37.85%)	39378850 (78.02%)	20226646 (40.08%)
Perfect match	29473928 (58.25%)	29473928 (58.25%)	27982903 (55.68%)	15472633 (30.79%)	27415385 (54.19%)	14646299 (28.95)	28665131 (56.80%)	15559619 (30.83%)
Mismatch	10050580 (19.86%)	10050580 (19.86%)	11216220 (22.32%)	6242157 (12.42%)	11030189 (21.80%)	4504009 (8.90%)	10713719 (21.23%)	4667027 (9.25%)
Unique match	37993866 (75.09%)	37993866 (75.09%)	36988454 (73.60%)	20252462 (40.30%)	36473650 (72.09%)	18364406 (36.30%)	37068978 (73.45%)	19503544 (38.64%)
Multi-position match	1530642 (3.03%)	1530642 (3.03%)	2210669 (4.40%)	1462328 (2.91%)	1971924 (3.90%)	785902 (1.55%)	2309872 (4.58%)	723102 (1.43%)
Total unmapped reads	11070500 (21.88%)	11070500 (21.88%)	11055257 (22.00%)	28539588 (56.79%)	12145522 (24.01%)	31440786 (62.15%)	11091394 (21.98%)	30243596 (59.92%)

clean reads mapped to genes, and 9485 genes were identified (Table 2).

Gene expression differences between the diploid and haploid drones

Differentially expressed genes (DEGs) between the haploid and diploid drones were identified. A total of 360 DEGs (Supplementary Table S1) were detected, including 152 up-regulated genes and 208 down-regulated genes in haploid drones (Fig. 3).

Gene ontology analysis of DEGs

To further understand the functions of the 360 DEGs, they were mapped to terms in the GO database (http://www.geneontology.org/). Significant enrichment of eighteen Go terms within differentially expressed genes between haploid and diploid drones was calculated (Supplementary Table S2). The results showed two functional categories in regard to biological process and molecular function. However, there were no Go nodes with a p value <0.05 for the differentially expressed genes related to cellular component. The terms of "oxidoreductase activity" in molecular function category, "carbohydrate metabolic process", "catabolic process" and single-organism metabolic process" in biological process were dominant items. It indicated that these differentially expressed genes may play a critical role in the energy metabolism in drones.

Pathway enrichment analysis of DEGs

To identify biochemical pathways these DEGs involved, all the DEGs were mapped to terms in the KEGG database. It indicated that 28 pathways were significantly enriched (Q-value < 0.05), including "Metabolic pathway", "Carbon metabolism", "Biosynthesis of amino acids", "Glycolysis/Gluconeogenesis" and "Huntington's disease", and so on (Supplementary Table S3).

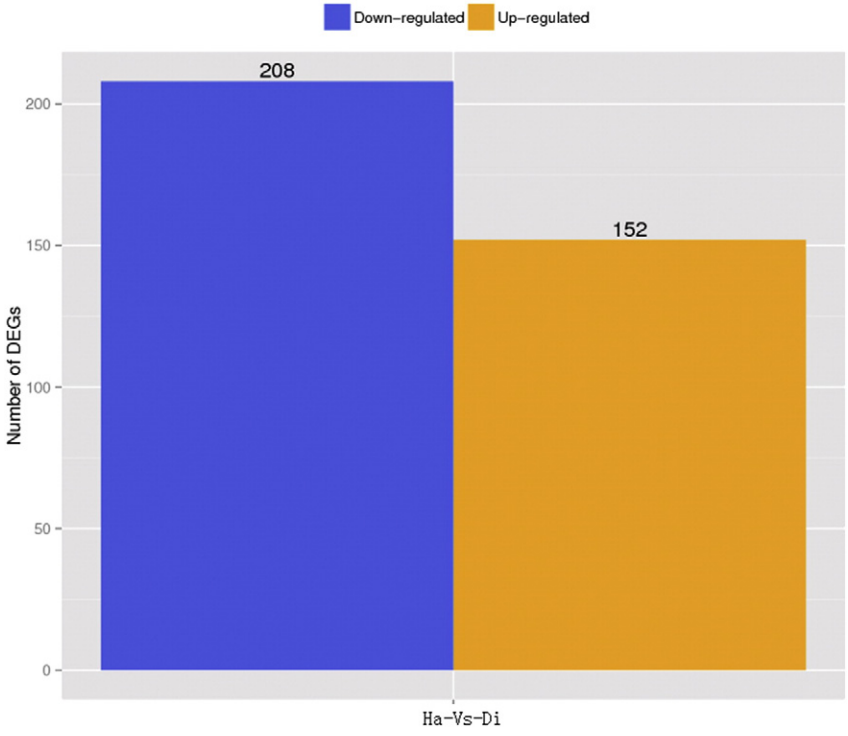

Fig. 3. Differentially expressed genes between Ha and Di.

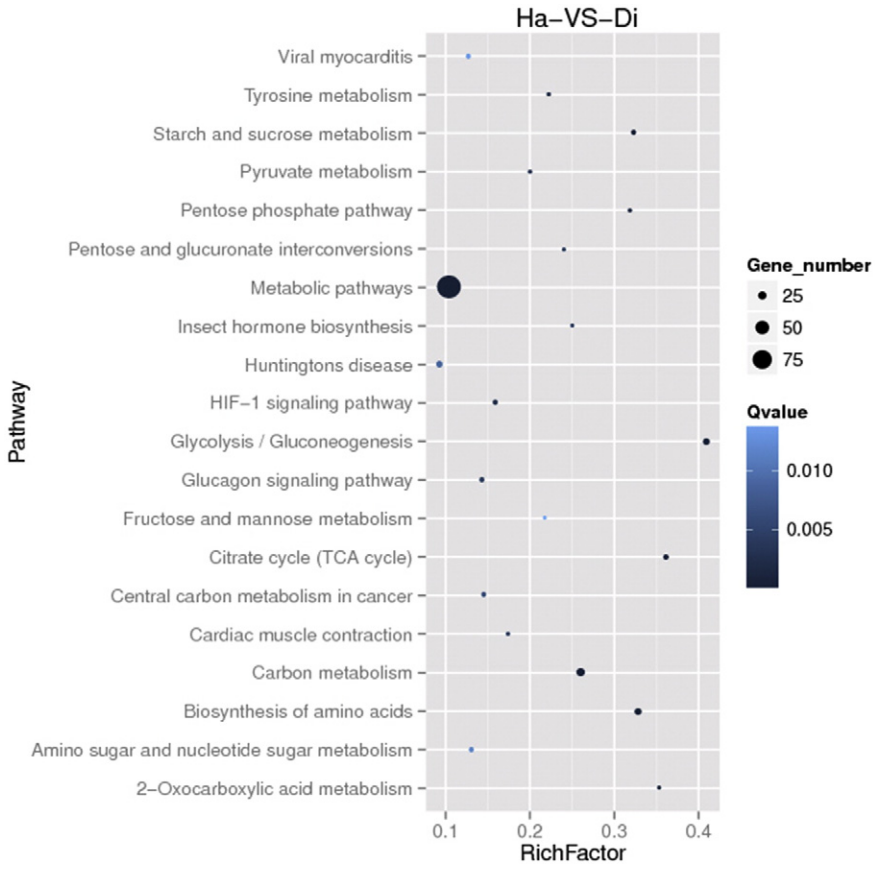

Fig. 4. Ha–VS–Di path enrichment.

Top 20 statistic enrichment pathway between haploid and diploid drones was showed in Fig. 4.

Discussion

In *Apis mellifera*, adult diploid drones are not produced in the colony, but the so-called 'lethal' eggs are viable and hatch, while all of these larvae are eaten alive by worker bees within 6 h after hatching (Woyke, 1962, 1963a). In contrast, some of the diploid drone larvae can reach the adult stage under favorable conditions in *Apis cerana indica* (Woyke, 1979). When a queen is inseminated by her many sons, she produces approximately 50% heterozygous eggs that develop into female larvae and approximately 50% homozygous eggs that develop into diploid drone larvae. An experiment showed that inseminated queens produce fertilized eggs in most worker cells, but only some of the brood cells are sealed with a domed capping, indicating that they contain drone pupae. Additionally, the comb had a scattered brood, implying that many diploid larvae disappeared during the larvae stage. The drone brood in worker cells was easily distinguished using the cells with a closed pore in the conical cocoon cap in *Apis cerana cerana*. After the remaining drones emerged, we found that, according to FCM, most were diploid. In addition to *Apis cerana indica*, some of the diploid drones could survive until adulthood in the natural colony in *Apis cerana cerana*. It indicated that *A. mellifera* can more effectively conserve energy by preventing of the rearing unnecessary larvae by cleaning diploid drones at their first development stage, which may enhance *A. mellifera* survival.

In contrast to our results, Woyke (1969, 1978) reported that diploid drones are heavier than the haploids and that many of their body parts are larger, showing a super-male character comparing to haploid drones in *Apis mellifera*. In that experiment, the diploid drones were reared in queen cells in the first 2 or 3 days (Woyke, 1974a), the diet and cell size differences may have resulted in the difference in the body weight of bees. The studies in queen-worker differentiation of honey bees indicate that cell size plays critical role in caste development (Jung-Hoffman, 1966; Shi et al., 2012). Studies have shown that cell size affect the quality and quantity of larval food the workers deposit into the cells (Kamakura, 2011; Barchuk et al., 2007). Herrmann showed that the body weight of the diploid larvae that were obtained from rearing in vitro for three days and were then grafted into drone brood cells was not different from the haploid drones reared from the colony in *Apis mellifera* (Herrmann et al., 2005). Therefore, the various conditions for rearing the diploid drones may also result in different wet weight. In this study, the drones were all emerged from worker cells. The different environmental and nutrient effects can be eliminated, while the size of cells may also restrict the development of drones. Additionally, the weights of the reproductive organs of the diploid drones were lighter than those of the haploid drones in *A. c. cerana*. The result was similar to Woyke's description (Woyke, 1978). However, the wing hook number was not different between diploid and haploid drones in this study, which was also the case for the length and width of the wings.

Our RNA-seq data showed that cytochrome *c* oxidase (ACSNU06766T0, ACSNU08873T0) were down-regulated in the haploids. Cytochrome *c* oxidase, composed of multiple subunits, is a telomerase that is located in the mitochondrial inner membrane respiratory chains (Li and Huang, 2001). It is the only enzyme that can directly make contact with oxygen generated from the respiratory chain complexes, indicating its leading role and importance in oxidative metabolism and ATP synthesis. The enzymes are also responsible for detoxification and xenobiotic breakdown (Chan et al., 2011). Amino acid changes and structure transformations of cytochrome *c* oxidase can cause mitochondrial diseases. It is not confirm whether the up-regulation expression of cytochrome *c* oxidase in diploid drones means the higher activity of the mitochondria compare to haploid drones.

We found that the hexamerin (ACSNU05738T0) and hexamerin 110 ACSNU03003T0) were down-regulated in the haploid drones. Hexamerins were hemolymph-specific storage protein, which have a widespread presence in the insects (Ma et al., 2002). They are considered to be an amino acid repositories and play important roles in metamorphosis and skin formation. The high levels of Hex 110 transcript level were related to ovary activation in honeybee workers in *A. mellifera* (Bitondi et al., 2006). The transcripts of the subunit of Hex 110, Hex70a and Hex70b were detected in developing ovaries and tests in honey bee, suggested that in addition to their primary role in supplying amino acids for metamorphosis, hexamerins serve as storage proteins for gonad development and egg production (Martins et al., 2011). Therefore, the differential expression of hexamerin may suggest the possibility of distinct spermiogenesis between the diploid and diploid drones. However, previous studies have indicated that in *A. mellifera*, the spermatogenesis in diploids was very similar to that in the haploids without reducing the number of chromosomes (Woyke and Skowronek, 1974). Further investigation is still needed to determine whether the transcript level of hexamerins in drones contributes to the reproductive performance.

Ribosomal proteins (RPs), a family of RNA-binding proteins, play an important role in intracellular protein synthesis (Bhavsa et al., 2010). Previous studies have indicated that RPs have critical biological functions in the regulation of development, sexual differentiation, brain maturation and extraribosomal transcription and translation (Byrne, 2009; Duncan et al., 2009; Meng et al., 2012; Lindström, 2009). In the present study, 6 ribosomal protein genes (ACSNU00930T0, ACSNU05322T0, ACSNU06379T0, ACSNU01559T0, ACSNU08093T0, ACSNU06853T0), composed of a large (60S) and a small (40S) subunit, were up-regulated in haploid drones. Coincidentally, the wet weight of newly emerged drones and reproductive organs in haploid drones were heavier than that in the diploids. The up-regulation of ribosome biogenesis may promote the physiological development, thus resulting in the increased weight in haploid drones.

Additionally, the transcripts of six myosin genes (ACSNU09552T0, ACSNU05677T0, ACSNU05678T0, ACSNU05676T0, ACSNU10576T0, ACSNU09553T0) were significantly up-regulated in haploid drones. Myosins comprise a family of ATP-dependent motor proteins, participate in transporting all kinds of organelles and vesicles (Mallik and Gross, 2004) and were believed to associate with neuron function (Calábria et al., 2011). The higher transcripts may play their role in muscle contraction and a wide range of other motility processes.

In addition to those functionally annotated genes, there were also many differentially expressed genes with unknown functions, which may be involved in the physiological differentiation between haploid and diploid drones.

In honeybees, although genomic and transcriptome studies have achieved considerable advances (The Honey Bee Genome Sequencing Consortium, 2006; Woodard et al., 2011; Liu et al., 2014), there have not been any studies on the transcript differences between the haploid and diploid drones. Our results provide the first step to understand the morphology and patterns of gene expression in the emerged diploids, and give further insight into the difference in biology and physiology between the haploid and diploid drones in *Apis cerana*.

Supplementary data to this article can be found online at doi:10.1016/j.aspen.2016.10.010.

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References

Barchuk, A.R., dos Santos Cristino, A., Kucharski, R., Simoes, Z.L.P., Maleszka, R., 2007. Molecular determinants of caste differentiation in the highly eusocial honeybee *Apis mellifera*. BMC Dev. Biol. 7, 70.

Beye, M., Hasselmann, M., Fondrk, M.K., Page, R.E., Omholt, S.W., 2003. The gene csd is the primary signal for sexual development in the honeybee and encodes an SR-type protein. Cell 114, 419–429.

Bhavsa, R.B., Makley, L.N., Tsonis, P.A., 2010. The other lives of ribosomal proteins. Hum. Genomics 4 (5), 327–344.

Bitondi, M.M.G., Nascimento, A.M., Cunha, A.D., Guidugli, K.R., Nunes, F.M.F., Simoes, Z.L.P., 2006. Characterization and expression of the Hex 110 gene encoding a glutamine-rich hexamerin in the honey bee, *Apis mellifera*. Arch. Insect Biochem. Physiol. 63, 57–72.

Byrne, M.E., 2009. A role for the ribosome in development. Trends Plant Sci. 14, 512–519.

Calábria, L.K., Peixoto, P.M.V., Lima, A.B.P., Peixoto, L.G.P., Moraes, V.R.A., Teixeira, R.R., Santos, C.T., Silva, L.O., Silca, M.F.R., Santos, A.A.D., Cairasco, N.G., Martins, A.R., Espreafico, E.M., Espindola, F.S., 2011. Myosins and DYNLL1/LC8 in the honey bee (*Apis mellifera* L.) brain. J. Insect Physiol. 57, 1300–1311.

Chan, Q.W.T., Mutti, N.S., Foster, L.J., Kocher, S.D., Amdam, G.V., Wolschin, F., 2011. The worker honeybee fat body proteome is extensively remodeled preceding a major life-history transition. PLoS One 6, e24794.

Cho, S., Huang, Z.Y., Green, D.R., Smith, D.R., Zhang, J., 2006. Evolution of the complementary sex-determination gene of honeybees: balancing selection and trans-species polymorphisms. Genome Res. 16, 1366–1375.

Cournault, L., Aron, S., 2008. Rapid determination of sperm number in ant queens by flow cytometry. Insect. Soc. 5, 283–287.

Duncan, K.A., Jimenez, P., Carruth, L.L., 2009. The selective estrogen receptor-alpha coactivator, RPL7, and sexual differentiation of the songbird brain. Psychoneuroendocrinology 34S, S30–S38.

Eisen, M.B., Spellman, P.T., Brown, P.O., Botstein, D., 1998. Cluster analysis and display of genome-wide expression patterns. Proc. Natl. Acad. Sci. U. S. A. 95 (25), 14863–14868.

Fries, I., Wei, H.Z., Shi, W., Chen, S.J., 1996. Grooming behavior and damaged mites (*Varroa jacobsoni*) in *Apis cerana cerana* and *Apis mellifera ligustica*. Apidologie 27, 3–11.

Gempe, T., Hasselmann, M., SchiØtt, M., Hause, G., Otte, M., Beye, M., 2009. Sex determination in honeybees: two separate mechanisms induce and maintain the female pathway. PLoS Biol. 7 (10), e1000222. http://dx.doi.org/10.1371/journal.pbio.1000222.

Herrmann, M., Trenzcek, T., Fahrenhorst, H., Engels, W., 2005. Characters that differ between diploid and haploid honey bee (*Apis mellifera*) drones. Genet. Mol. Res. 4, 624–641.

Jung-Hoffman, I., 1966. Die determination von Köningin und Arbeiterin der Honigbiene. Zoologie Bienenforsh. 8 pp. 296–322.

Kamakura, M., 2011. Royalactin induces queen differentiation in honeybees. Nature 473, 478–483.

Kanehisa, M., Araki, M., Goto, S., Hattori, M., Hirakawa, M., Itoh, M., Katayama, T., Kawashima, S., Okuda, S., Tokimatsu, T., Yamanishi, Y., 2008. KEGG for linking genomes to life and the environment. Nucleic Acids Res. 36 (Database issue): D480–D484. http://dx.doi.org/10.1093/nar/gkm882.

Li, L.Z., Huang, Z.X., 2001. Progresses in cytochrome *c* oxidase studies. Chinese J. Inorg. Chem. 17, 761–774.

Li, Z.G., Liu, F., Li, W.F., Zhang, S.W., Niu, D., Xu, H.S., Hong, Q.H., Chen, S.L., Su, S.K., 2012. Differential transcriptome profiles of heads from foragers: comparison between *Apis mellifera ligustica* and *Apis cerana cerana*. Apidologie 43, 487–500.

Lindström, M.S., 2009. Emerging functions of ribosomal proteins in gene-specific transcription and translation. Biochem. Biophys. Res. Commun. 379, 167–170.

Liu, Z.Y., Wang, Z.L., Wu, X.B., Yan, W.Y., Zeng, Z.J., 2011. *csd* alleles in the red dwarf honey bee (*Apis florea*, Apidae, Hymenoptera) show exceptionally high nucleotide diversity. Insect Sci. 18, 645–652.

Liu, H., Wang, Z.L., Tian, L.Q., Wu, X.B., Yan, W.Y., Zeng, Z.J., 2014. Transcriptome differences in the hypopharyngeal gland between western honeybees (*Apis mellifera*) and eastern honeybees (*Apis cerana*). BMC Genomics 15:744. http://dx.doi.org/10.1186/1471-2164-15-744.

Ma, C.X., Liu, H.X., Sha, Z.L., Li, Y.P., 2002. Progress in research on insect storage proteins. Entomol. Knowl. 39, 416–420.

Mallik, R., Gross, S.P., 2004. Molecular motors: strategies to get along. Curr. Biol. 14, 971–982.

Martins, J.R., Anhezini, L., Dallacqua, R.P., Simões, Z.L.P., Bitondi, M.M.G., 2011. A honey bee hexamerin, HEX 70a, is likely to play an intranuclear role in developing and mature ovarioles and testioles. PLoS One 6 (12), e29006. http://dx.doi.org/10.1371/journal.pone.0029006.

Meng, F., Lu, W.J., Yu, F.F., Kang, M.J., Guo, X.Q., Xu, B.H., 2012. Ribosomal protein L11 is related to brain maturation during the adult phase in *Apis cerana cerana* (Hymenoptera, Apidae). Naturwissenschaften 99, 343–352.

Park, D., Jung, J.W., Choi, B.S., Jayakodi, M., Lee, J., Lim, J., Yu, Y., Choi, Y.S., Lee, M.L., Park, Y., Choi, I.Y., Yang, T.J., Edwards, O.R., Nah, G., Kwon, H.W., 2015. Uncovering the novel characteristics of Asian honey bee, *Apis cerana*, by whole genome sequencing. BMC Genomics 16:1. http://dx.doi.org/10.1186/1471-2164-16-1.

Peng, Y.S., Fang, Z.Y., Xu, S.Y., Ge, L.S., 1987. The resistance mechanism of the Asian honey bee, *Apis cerana cerana* Fabr., to ectoparasitic mite, *Varroa jacobsoni* Oudemans. J. Invertebr. Pathog. 47, 54–60.

Shi, Y.Y., Yan, W.Y., Huang, Z.Y., Wang, Z.L., Wu, X.B., Zeng, Z.J., 2012. Genome-wide analysis indicates that queen larvae have lower methylation levels in the honey bee (*Apis mellifera*). Epigenetics 18, 645–652.

Tan, K., Radloff, S.E., Li, J.J., Hepburn, H.R., Yang, M.X., Zhang, L.J., Neumann, P., 2007. Bee-hawking by the wasp, *Vespa velutina*, on the honeybees *Apis cerana* and *A. mellifera*. Naturwissenschaften 94 (6), 469–472.

The Honey Bee Genome Sequencing Consortium, 2006. Insights in social insects from the genome of the honeybee *Apis mellifera*. Nature 443, 931–994.

Trapnell, C., Williams, B.A., Pertea, G., Mortazavi, A., Kwan, G., Baren, M.J., Salzberg, S.L., Wold, B.J., Pachter, L., 2010. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat. Biotechnol. 28 (5), 511–515.

Wang, Z.L., Liu, Z.Y., Wu, X.B., Yan, W.Y., Zeng, Z.J., 2012a. Polymorphism analysis of csd gene in six *Apis mellifera* subspecies. Mol. Biol. Rep. 39, 3067–3071.

Wang, Z.L., Liu, T.T., Huang, Z.Y., Wu, X.B., Yan, W.Y., Zeng, Z.J., 2012b. Transcriptome analysis of the Asian honey bee *Apis cerana cerana*. PLoS One (10):7, e47954, http://dx.doi.org/10.137/journal.pone.0047954.

Woodard, S.H., Fischman, B.J., Venkat, A., Hudson, M.E., Varala, K., Cameron, S.A., Clark, A.G., Robinson, G.E., 2011. Genes involved in convergent evolution of eusociality in bees. Proc. Natl. Acad. Sci. 108, 7472–7747.

Woyke, J., 1962. The hatchability of 'lethal' eggs in a two sex-allele fraternity of honeybees. J. Apic. Res. 1, 6–13.

Woyke, J., 1963a. Drone larvae from fertilized eggs of the honeybee. J. Apic. Res. 2, 19–24.

Woyke, J., 1963b. What happens to diploid drone larvae in a honeybee colony. J. Apic. Res. 2, 73–75.

Woyke, J., 1963c. Rearing and viability of diploid drone larvae. J. Apic. Res. 2, 77–78.

Woyke, J., 1969. A method of rearing diploid drones in a honeybee colony. J. Apic. Res. 8, 65–74.

Woyke, J., 1974. The story of diploid drones in the honeybee (supplement to bee world). History of the Bee Research Association. 55, pp. 151–153.

Woyke, J., 1978. Comparative biometrical investigation on diploid drones of the honeybee III. The abdomen, and weight. J. Apic. Res. 17, 206–217.

Woyke, J., 1979. Sex determination in *Apis cerana indica*. J. Apic. Res. 18, 122–127.

Woyke, J., Skowronek, W., 1974. Spermatogenesis in diploid drones of the honeybee. J. Apic. Res. 13, 183–190.