



Transcriptome analysis reveals differentially expressed genes between the ovary and testis of the honey bee *Apis mellifera*

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Abstract – Honey bees (*Apis mellifera*) are an important social pollinator, crucial in maintaining ecological balance and biodiversity. The haploid-diploid sex determination system determines their sexes where males are haploids formed from unfertilized eggs while females are diploids formed from fertilized eggs. To better understand the gonad differentiation mechanism in honey bees, here the transcriptome difference between the honey bee ovary and testis were analyzed using the Illumina sequencing technology. A total of 4380 differentially expressed genes (DEGs) were identified, with 2255 up-regulated and 2125 down-regulated in the ovary compared to the testis. Among these, many candidate DEGs related to sex determination, spermatogenesis, and oogenesis were identified, such as *csd*, *fem*, and *dsx*. Moreover, 3092 differentially expressed alternative splicing events (ASEs) related to 1497 genes, such as *tra2*, *ovo*, and *squid*, were identified between the ovary and testis. The findings of this study provide valuable information on gene expression for understanding the underlying molecular mechanisms of gonad differentiation and gametogenesis in *A. mellifera*.

differentially expressed gene / ovary / testis / RNA-seq / sex differentiation

1. INTRODUCTION

As important social-economic insects, honey bees (*Apis mellifera* L.) are a source of several products for human consumption while serving as important pollinators in nature. In colonies, the queens and worker bees are females, while drones are males. The sex of honey bees is determined by the haploid-diploid system, where males are haploids formed from unfertilized eggs while females are diploids resulting from fertilized eggs. The queens and drones have fully

developed gonads, and their sexual maturity is asynchronous; after emergence, the queens need 5–10 days to reach sexual maturity (Chen, 2001), and the drones need 9–12 days (Bishop, 1920).

In animals, the sexual development process entails sex determination and differentiation, which not only leads to morphological differences in reproductive organs between male and female, but also affects many other aspects of the two sexes, such as behavior and physiology. In honey bees, sex is determined by the heterozygosity of a single complementary sex determiner (*csd*) which was identified by linkage analysis of genetic map (Beye et al., 2003). When the embryo is heterozygous at the *csd* locus, it develops into a fertile female; when the embryo is hemizygous at

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the *csd* locus, it develops into a fertile males; when the embryo contains two identical *csd* alleles, it develops into a diploid male (Beye et al., 2003). *csd* directs the female-specific splicing of *feminizer* (*fem*) gene, which then controls the female-specific splicing of *Amdoublesex* (*Amdsx*) gene, a conserved sex-determining gene at the end of the sex determination cascade in multicellular animals (Whiting, 1943). Therefore, the *csd*>*fem*>*Amdsx* genetic cascade controls the development of honey bee sexes, subsequently leading to sex-specific phenotypes (Beye et al., 2003; Cho et al., 2007; Hasselmann et al., 2008; Gempe et al., 2009).

Genes in the main sex determination cascade of honey bee have been identified (Beye et al., 2003; Hasselmann et al., 2008; Gempe et al., 2009; Cho et al., 2007). However, the molecular aspects of their reproductive activity and gonad development remain unknown. Therefore, in the present study, we performed comparative transcriptome sequencing analysis on the *A. mellifera* ovary and testis. The findings revealed major expression differences between the male and female gonads. These findings will increase the understanding of the underlying molecular mechanism in gonad differentiation, which provides valuable knowledge on gene expression for future research.

2. MATERIAL AND METHODS

2.1. Insects

The honey bees (*A. mellifera ligustica*) were reared under natural conditions at the Honeybee Research Institute of Jiangxi Agricultural University (28°46'N, 115°49'E), Nanchang, Jiangxi, China.

The queens used for collecting ovaries were bred from two *A. mellifera* colonies. Briefly, the queens of the two colonies were respectively restricted to a worker cell comb for 12 h to lay fertilized eggs, after which the comb was transferred to the super box of the same colony until the eggs hatched. The 1-day old larvae of similar size were grafted to the queen cells using a

grafting needle and then were placed in the super box. After emergence, the newly emerged queens were kept in queen cages and then transferred to their original colonies. The queen's ovaries were dissected under a stereomicroscope (Guiguang, GL-99TI, Guilin, China) after 12 days, then frozen immediately in liquid nitrogen. Ovaries from two queens in each colony were collected as a sample, and two biological replicates were sampled.

At the same time, testis samples were collected from drones bred by the same two colonies. For this, the queen in each colony was restricted in a drone foundation to lay unfertilized eggs for 12 h. The queen was then released, and the comb was transferred to the super box in the same colony until the eggs hatched. A day before drone emergence, the comb was transferred to an incubator regulated at 34.5 °C and relative humidity of 70%. After emergence, the drones were marked by coloring the back of their thorax and returned to their original colony. The drones reached sexual maturity after 12 days (Graham, 2015), and they were captured. Their testes were dissected under a stereomicroscope and then were immediately frozen in liquid nitrogen. Testes collected from ten drones in each colony were pooled as a sample, and two biological replicates were sampled.

2.2. cDNA library construction and sequencing

Total RNAs were extracted from the ovary and testis samples using Trizol Reagent (US Patent No.5,346,994). The extracted total RNA purity (OD260/280), concentration, nucleic acid absorption peak, and RNA integrity were detected using NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE) and Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). The mRNAs were then enriched using magnetic beads with oligo (dT) while being randomly interrupted in fragmentation buffer to construct the sequencing libraries. The first cDNA strands were synthesized using random hexamers and M-MuLV reverse transcriptase,

while the second was synthesized using enzyme buffer, dNTPs, RNase H, and DNA polymerase I. The synthesized cDNAs were purified using AMPure XP beads (Beckman Coulter, USA) then subjected to end repairing, poly (A) tailing, and addition of sequencing adaptors. cDNA fragments of 240 bp in length were preferentially selected using AMPure XP beads, and the cDNA libraries were constructed by PCR amplification, enriched, and sequenced using the Illumina HiSeq 4000 platform (Illumina, CA, USA).

2.3. Mapping of sequencing data to the reference genome

Raw sequences were filtered using the FastQC software to remove reads containing just adaptors and low-quality reads (reads containing “N” more than 10%; reads containing bases with mass value $Q \leq 10$ accounted for more than 50% of the whole read). The clean reads were submitted to the NCBI SRA database under the accession numbers SRR15276287, SRR15276288, SRR15276289, and SRR15276290.

Cleaned reads were aligned to the *A. mellifera* genome version Amel_HAv3.1 (ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/003/254/395/GCA_003254395.2_Amel_HAv3.1/) using the HISAT2 software (Kim et al., 2015).

2.4. Gene expression level determination and standardization

The fragments per kilobase of script per million fragments mapped (FPKM) was used to measure gene expression using the cuffquant and cuffnorm components of the Cufflinks software (Trapnell et al., 2012). The FPKM was calculated as follows:

$$\frac{\text{cDNA fragments}}{\text{Mapped fragments (millions)} \times \text{Transcript length (kb)}}$$

where the cDNA fragments represent the number of reads mapped to a transcript, mapped fragments (Millions) represent the total number of reads mapped to the transcripts, and

transcript length (kb) represents the length of each transcript.

2.5. Screening of DEGs and ASEs

The DESeq software was used to identify differentially expressed genes between the ovary and testis. The Benjamini–Hochberg method was adopted to correct the p values, which is indicated as FDR (corrected p values). The $\log_2(\text{fold change}) \geq 1$ and $\text{FDR} < 0.01$ were used as the DEG screening criteria. Gene Ontology (GO) enrichment analysis of functional significance was then performed by mapping all DEGs to the GO database (Harris et al., 2004). Finally, pathway analysis was performed in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa and Goto, 2000).

The cleaned reads were assembled into longer transcripts using the StringTie software (Pertea et al., 2015). The ASprofile software (Florea et al., 2013) determined the alternative splicing types in each gene and their corresponding expression levels. Differentially expressed ASEs were identified using rMATS software (Shen et al., 2014) following the $|\Delta\psi| > c$ ($c = 0.0001$) and $\text{FDR} < 0.05$ criteria.

2.6. Quantitative real-time PCR (qRT-PCR)

To perform quantitative PCR experiment, we bred many queens and drones. Then, ovary and testis samples were collected from 12-day-old queens and drones, respectively. Their total RNAs were extracted, and the RNA integrity was detected by agarose gel electrophoresis. In addition, the RNA concentration and purity (OD260/280 value) were detected using an ultraviolet spectrophotometer. The RNA samples (1 $\mu\text{g}/\mu\text{L}$) were reverse transcribed into cDNA using the MLV reverse transcriptase kit (Invitrogen, CA, USA). Nine DEGs (*squid*, *rbp1*, *tra2*, *vgr*, *vasa*, *dicer1*, *fem*, *stat92e*, *dpp*) were selected for qRT-PCR analysis using *A. mellifera* *gapdh* gene as an internal reference. The primer

sequences (Table S1) were designed based on the mRNA sequences from the GenBank database using the Prime Primer 5.0 software. The qRT-PCR reaction system consisted of 5 µL of SYBR®Premix Ex Taq™ II, 0.2 µL of ROX correction fluid, 0.4 µL each of forward and reverse primers, 1 µL of cDNA, and 3 µL of H₂O. The PCR conditions were as follows: 95 °C, 5 min; 94 °C, 2 min; 40 cycles (95 °C, 10 s, T_m, 15 s, 72 °C, 15 s); 72 °C, 10 min. To establish the melting curve of the qRT-PCR product, the primers were heated slowly with a gradual increase of 1 °C every 5 s from 72 to 99 °C. The specificity of the PCR products was analyzed by dissolution curve. For each gene, eight biological replicates were adopted, with four technical repeats each. The data were analyzed by 2^{-ΔΔCT} (Schmittgen and Livak, 2008). Significant differences were analyzed by *t* test with a cutoff value of 0.05 using the SPSS17.0 software (SPSS Inc., 2008).

3. RESULTS AND DISCUSSION

3.1. Transcriptome sequencing of ovary and testis

Four RNA-seq libraries of *A. mellifera* ovary and testis were generated from two biological replicates, each. A total of 35,310,578–40,246,018 high-quality reads were obtained after removing the sequencing adaptors and low-quality reads (Table I). The total length of reads in each sample was 4,443,325,050–5,062,141,958nt, with a percentage of Q30 bases more than 94.29%, implying

that the base sequencing and identification were reliable and accurate. Approximately 30,660,123–34,952,594 (82.58–89.47%) of the cleaned reads mapped to the *A. mellifera* genome version Amel_HAv3.1.

3.2. Repeated correlation assessment

The correlation heat map between gene expression of the paired biological replicates of ovary and testis revealed that the *r*² values between two different biological replicates were more than 0.88 and between ovary and testis were less than 0.1, suggesting high reliability and repeatability of the RNA-seq data (Figure S1).

3.3. Differentially expressed genes between the ovary and testis

A total of 4380 DEGs between the ovary and testis, including 2255 up-regulated genes in the ovary and 2125 in the testis, were identified (Figure 1). The top three up-regulated genes in the ovary were odorant-binding protein 7 (LOC677668), two-pore potassium channel protein sup-9 (LOC413020), and cilia- and flagella-associated protein 58 (LOC412109). In the testis, hypothetical protein WN51_08187 (LOC113219380), carboxypeptidase B-like (LOC551327), and galactose-1-phosphate uridylyltransferase (LOC724540) were the top three up-regulated genes (Table S2).

In addition, GO enrichment of the DEGs showed that eight terms were significantly

Table I
Summary of the sequencing data

Samples	Total raw reads	Total clean reads	Total clean bases	% ≥ Q30	GC content	UniqMapped reads	UniqMapped ratio
Ovary-1	35,733,426	35,310,578	4,443,325,050	94.29%	40.14%	31,561,769	89.38%
Ovary-2	37,092,766	36,662,242	4,612,969,330	94.45%	40.60%	32,802,017	89.47%
Testis-1	40,739,626	40,246,018	5,062,141,958	94.50%	41.15%	34,952,594	86.85%
Testis-2	40,380,252	37,127,078	4,669,243,028	94.72%	42.26%	30,660,123	82.58%

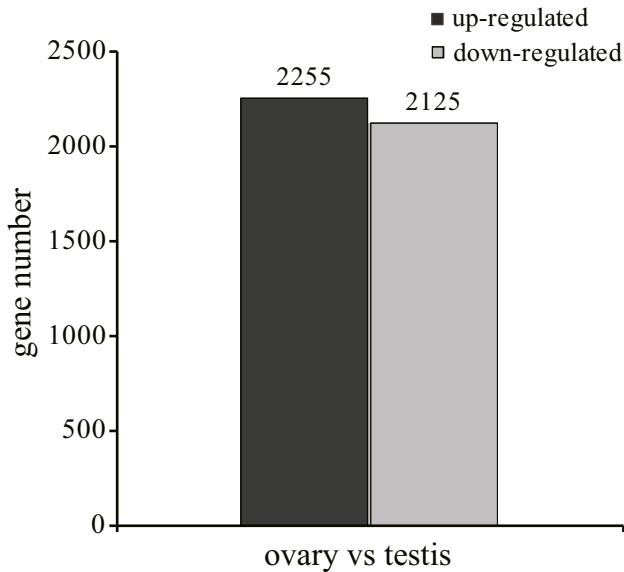


Figure 1. Up- and down-regulated DEGs in the ovary compared to the testis in 12-day-old honey bees (*Apis mellifera*). Both ovary and testis contain two biological replicates

enriched, including transmembrane transport (GO:0,055,085), oxidation–reduction process (GO:0,055,114), potassium ion transmembrane transport (GO:0,071,805), regulation of transcription, DNA-templated (GO:0,006,355), integral component of membrane (GO:0,016,021), and transcription factor activity, sequence-specific DNA binding (GO:0,003,700) ($q < 0.05$; Table S3). The KEGG pathway analysis revealed that three signaling pathways, namely ECM-receptor interaction (KO04512), lysosome (KO04142), and glycosaminoglycan degradation (KO00531), were significantly enriched ($q < 0.05$; Table S4). The ECM-receptor interaction pathway is implicated in cell migration, proliferation, follicle growth, and oocyte maturation (Berkholtz et al., 2006).

Among the up-regulated genes in the ovary, ten GO terms and two KEGG pathways were significantly enriched ($q < 0.05$; Tables S3 and S4). However, the testis up-regulated genes significantly enriched eight GO terms and 17 KEGG pathways ($q < 0.05$; Tables S3 and S4); of them, the ascorbate and aldarate metabolism (ko00053) is the most relevant pathway to the

caput epididymis and may play important roles in sperm maturation (Hu et al., 2018).

3.4. DEGs involved in sex determination

Five DEGs related to sex determination in honey bee or *Drosophila* were identified, including *csd* ($\log_2(\text{testis/ovary}) = 4.74$), *fem* ($\log_2(\text{testis/ovary}) = 4.92$), *dsx* ($\log_2(\text{testis/ovary}) = 2.44$), *tra2* ($\log_2(\text{testis/ovary}) = -2.07$), and *rbp1* ($\log_2(\text{testis/ovary}) = -1.06$), with GenBank accession numbers LOC406074, LOC724970, LOC725126, LOC725195, and LOC413835. The *csd*, *fem*, and *dsx* were up-regulated in the testis, while *tra2* and *rbp1* were up-regulated in the ovary (Figure 2).

The *csd* gene is the initial signal for honey bee sex determination (Beye et al., 2003), which is realized through heterozygosity of *csd* gene. When *csd* is heterozygous, the embryo develops into a female; when *csd* is homozygous or hemizygous, the embryo develops into a male (Beye et al., 2003). The *csd* gene has many alleles among the bee populations hence subjected to

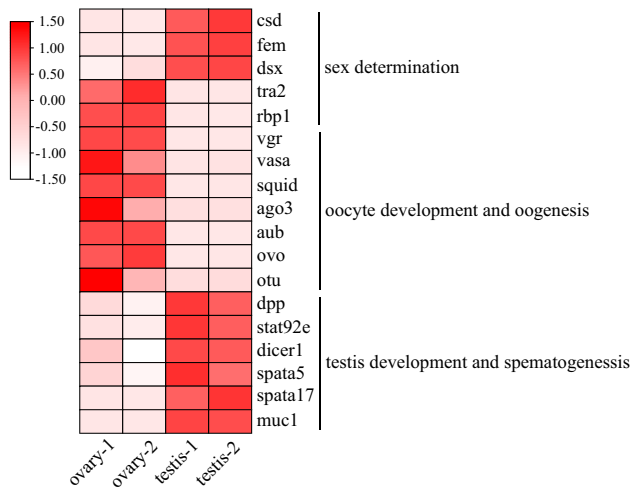


Figure 2. Heatmap of eighteen candidate DEGs involved in sex determination and development of gonads and germ cells. X-axis indicates the names of the samples. Y-axis shows the names of the 18 genes and their functional classifications

balance selection (Hasselmann and Beye, 2004; Liu et al., 2011, 2012; Wang et al., 2012). Coming second in the honey bee sex determination cascade is *fem*, which maintains and stabilizes the female-specific development of embryos while regulating the female-specific splicing of its downstream gene *dsx* (Hasselmann et al., 2008; Gempe et al., 2009). The *fem* expression is also necessary for female gonad differentiation (Gempe et al., 2009). However, in this study these two genes are up-regulated in the testis; it is unknown whether they have function in honey bee testis.

dsx is a conservative gene expressed at the end of the sex determination cascade in insects. It controls the somatic cells' sex identity and the germ cells' development (Steinmann-Zwicky, 1994). Besides, in *A. mellifera*, *dsx* produces sex-specific proteins (DSX-F and DSX-M) through alternative splicing to regulate sex development (Cho et al., 2007). In addition, *tra2* and *rbp1* are important splicing factors regulating alternative splicing of *dsx* pre-mRNA in the sex determination pathway in *Drosophila melanogaster* (Burtis and Baker, 1989; Hoshijima et al., 1991). Moreover, the *tra2* homologs have been successively isolated and linked to sex determination in other insects, such as *A. mellifera* (Nissen et al.,

2012), *Musca domestica* (Burghardt et al., 2005), *Ceratitidis capitata* (Marco et al., 2009), *Bombyx mori* (Suzuki et al., 2012), *Tribolium castaneum* (Shukla and Palli, 2013), and *Sclerotia aquatilis* (Nguantad et al., 2020). *tra2* also regulates ovariole number and ovary size in *Aedes albopictus* (Li et al., 2019). In the present study *tra2* and *rbp1* were significantly expressed higher in ovary than in testis, implying that they might be involved in ovary development or oogenesis in honey bees.

3.5. DEGs involved in oocyte development and oogenesis

Several genes involved in ovary development or oogenesis were up-regulated in the ovary, such as *vgr*, *vasa*, *squid*, *ago3*, *aub*, *ovo*, and *otu* with GenBank accession numbers LOC725920, LOC410692, LOC408936, LOC725111, LOC412427, LOC552100, and LOC100576989, respectively (Figure 2).

In insects, *vgr* codes for an ovary-specific protein that mediates the absorption of vitellogenin (vg) from the hemolymph by the developing oocytes in the ovaries (Tufail and Takeda, 2009). *vgr* expression is essential for reproductive development. For example, knocking down the

vgr expression by RNAi in *Nilaparvata lugens* (Lu et al., 2015) and *Haemaphysalis longicornis* Neumann (Boldbaatar et al., 2008) led to abnormal development of oocytes and abnormal eggs. The *vasa* gene is essential for polar cell formation where the VASA protein binds to mRNAs of target genes involved in germ cell establishment and oogenesis, promoting their translation (Gavis et al., 1996; Tomancak et al., 1998; Styhler et al., 1998). For example, RNAi-based knockdown of *vasa* in *Schistosoma mansoni* led to a reduction in the ovary volume and a lower number of dividing cells in immature ovary (Skinner et al., 2020). The *squid* gene functions in nuclear export, cytoplasmic transport, and translational control of the *gurken* mRNA during oogenesis (Norvell et al., 1999). The *aub* and *ago3* are germ cell PIWI-interacting RNA (piRNA) pathway components, essential in germline development, stem cell self-renewal, epigenetic regulation, and transposon silencing (Kennerdell et al., 2002; Malone and Hannon, 2009; Thomson and Lin, 2009). They form a “ping-pong” feed-forward piRNA amplification loop with PIWI in the *Drosophila* ovary, in which *ago3* binds to a sense piRNA and guides cleavage of antisense transposon mRNAs to produce antisense piRNAs and in turn *aub* binds to an antisense piRNA and guides cleavage of sense transposon mRNAs to produce a new sense piRNA (Brennecke et al., 2007; Gunawardane et al., 2007). Therefore, the up-regulation of these genes in the ovary implied that they might be involved in ovary development or oogenesis, but it needs further experimental verification.

In *Drosophila*, *ovo* and *otu* genes are crucial in oogenesis’ early and late stages (Kennerdell et al., 2002; Oliver et al., 1987; Steinhauer and Kalfayan, 1992; Tirronen et al., 1995; Hinson et al., 1999). The *ovo* mutation leads to the early arrest of oogenesis and complete absence of germline (Oliver et al., 1987), while *out* mutation causes abnormal ovarian development (Steinhauer and Kalfayan, 1992; Tirronen et al., 1995). A higher expression of both in the ovary suggests a common function in honey bees and *Drosophila*.

3.6. DEGs involved in testis development and spermatogenesis

Several genes involved in testis development or spermatogenesis were up-regulated in the testis, including *dpp*, *stat92e*, *dicer1*, *spata5*, *spata17*, and *mucl1* with GenBank accession numbers LOC727101, LOC413742, LOC726766, LOC411003, LOC107964258, and LOC551924, respectively (Figure 2).

The *dpp* signaling regulates germline stem cell (GSC) self-renewal and differentiation by interacting with Birt-Hogg-Dubé syndrome (BHD) in fruit fly testis (Singh et al., 2006). The *dicer1* enzyme is required for germ cell development and spermatogenesis in mammals (Björkgren and Sipilä, 2015). For example, in male mice, the absence of *dicer1* results in infertility prolongs the time required for germ cells to enter the first prophase during meiosis from interphase and increases the apoptosis of primary spermatocytes (Yannick et al., 2011). *stat92e* activation promotes the male GSC establishment and maintenance in adult testes (Kiger et al., 2001; Tulina and Matunis, 2001) and male germ cell sex determination during gonad coalescence (Wawersik et al., 2005). SPATA5 and SPATA17 are members of the AAA-protein family, which consists of ATPase associated with diverse activities. *spata5* is expressed at the early stages of spermatogenesis in mice (Liu et al., 2000). *spata5* knockdown in male *Nilaparvata lugens* decreases the male accessory gland protein content and causes the malformation of vas deferens and seminal vesicle (Ge et al., 2016). Besides, SPATA17 is important in testis development and testis-specific apoptosis (Nie et al., 2011, 2013). MUC1 is a nonglobular transmembrane glycoprotein expressed in male germ cell line and ejaculated sperms (Martínez-Conejero et al., 2008), facilitating the separation of delayed cell maturation and splitting of spermatozoa into the lumen of the seminiferous tubules (Franke et al., 2001). Therefore, the up-regulation of these genes in the testis implied that they are involved in testis development or spermatogenesis in honey bees.

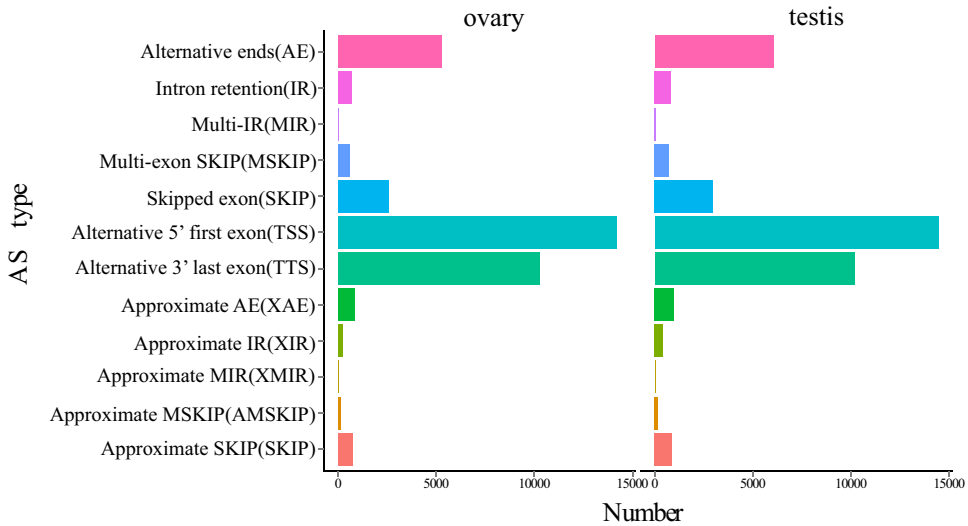


Figure 3. Statistical bar chart of ASEs detected in the ovary and testis. X-axis indicates the number of ASEs. Y-axis shows the 12 types of ASEs

3.7. Differentially expressed alternative splicing events (ASEs)

Alternative splicing is essential in the sex determination of many insects, such as *Drosophila* where a regulatory cascade of X:A>*sxl*>*tra*>*dsx* determines sex through alternative splicing (Sánchez, 2008). This study analyzed 12 types of ASEs present in the ovary and testis. A total of 35,486 and 37,774 ASEs were detected in the ovary and testis, respectively. The alternative 5' first exon (transcription start site) and alternative 3' last exons (transcription terminal site) were the two most abundant ASE types in the ovary and testis (Figure 3). Additionally, 3092 differentially

expressed ASEs between the ovary and testis related to 1497 genes were identified (Tables II and S5). We found that multiple DEGs mentioned above contain ASEs, such as *tra2*, *ovo*, *squid*, *aub*, *mucl1*, and *dicer1*.

The GO analysis of the differentially expressed ASEs related genes revealed that 341 GO terms were significantly enriched ($q < 0.05$, Table S6). Among them, oocyte anterior/posterior axis specification (GO:0,007,314) and oocyte microtubule cytoskeleton organization (GO:0,016,325) were related to oogenesis. However, only the WNT signaling pathway (ko04310) was significantly enriched ($q < 0.05$, Table S7) in the KEGG analysis. The WNT signaling pathway

Table II
Differentially expressed ASEs and related genes between the ovary and testis

Variable splicing type	ASE numbers	Relative genes
Alternative 3' splice sites (A3SS)	408	346
Alternative 5' splice sites (A5SS)	376	300
Mutually exclusive (MXE)	406	193
Retained intron (RI)	64	62
Skipped exon (SE)	1838	1051

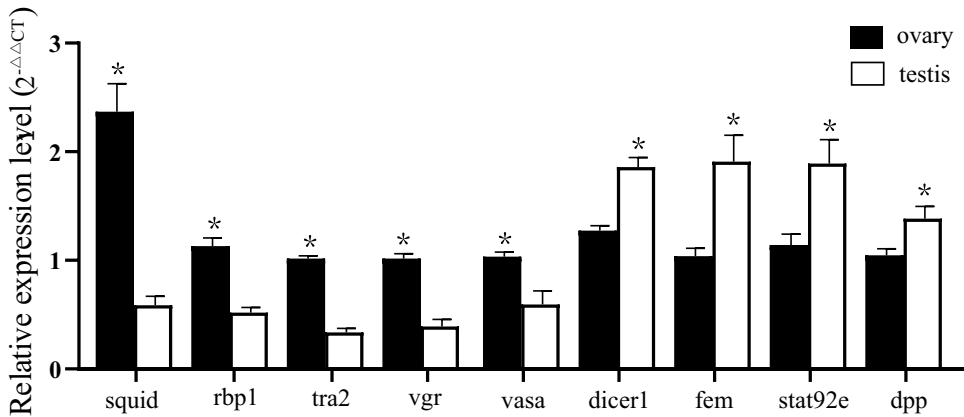


Figure 4. Quantitative RT-PCR verification of nine DEGs between the ovary and testis. X-axis indicates the gene names. Y-axis shows the relative expression levels of genes. *represents significant difference

is implicated in gonad differentiation and development (Harwood et al., 2008; Golestaneh et al., 2009; Nicol and Guigen 2011). These findings imply that alternative splicing is important in *A. mellifera* gonad and germ cell development.

3.8. Validation of DEGs by qRT-PCR analysis

Nine DEGs were selected for the validation of the reliability of the RNA-seq results using qRT-PCR. All the nine genes showed significant expression differences between the ovary and testis (t test, $p < 0.05$, Table S8), with five being up-regulated in the ovary and four in the testis (Figure 4). This is consistent with the RNA-seq results, indicating that the transcriptome analysis findings were highly reliable.

In summary, this study analyzed the transcriptome difference between the *A. mellifera* ovary and testis using RNA-seq. A large amount of DEGs and ASEs were identified, and many of them are related to sex determination, oogenesis, and spermatogenesis. These findings provide valuable information for understanding reproductive biology of honey bees.

SUPPLEMENTARY INFORMATION

The online version contains supplementary material available at <https://doi.org/10.1007/s13592-022-00926-5>.

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AUTHOR CONTRIBUTION

ZLW conceived and designed the experiments. LXP, WWH, FPC, and XFH performed the experiments and analyzed the data. LXP and ZLW wrote the paper.

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DATA AVAILABILITY

The clean reads for this study are available from the NCBI SRA database. Other data that support the results of this study are available from the corresponding author upon request.

DECLARATIONS

Ethics approval Not applicable.

Consent for publication All authors have read and approved the final manuscript for publication.

Conflict of interest The authors declare no competing interests.

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