Integration of lncRNA–miRNA–mRNA reveals novel insights into oviposition regulation in honey bees

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Background

The honey bee (*Apis mellifera*) is a highly diverse species commonly used for honey production and pollination services. The oviposition of honey bee queen affects the development and overall performance of the colony. To investigate the ovary activation and oviposition processes on a molecular level, a genome-wide analysis of lncRNAs, miRNAs and mRNAs expression in ovaries of the queens was performed to screen for differentially expressed coding and noncoding RNAs . Further analysis identified relevant candidate genes or RNAs.

Results

The analysis of the RNA profiles in different oviposition phase of the queens revealed that 740 lncRNAs, 81 miRNAs and 5481 mRNAs were differently expressed during the ovary activation; 88 lncRNAs, 13 miRNAs and 338 mRNAs were differently expressed during the oviposition inhibition process; and finally, 100 lncRNAs, 4 miRNAs and 497 mRNAs were differently expressed during the oviposition recovery process. In addition, functional annotation of differentially expressed RNAs revealed several pathways that are closely related to oviposition, including hippo, MAPK, notch, Wnt, mTOR, TGF-beta and FoxO signaling pathways. Furthermore, in the QTL region for ovary size, 73 differentially expressed genes and 14 differentially expressed lncRNAs were located, which are considered as candidate genes affecting ovary size and oviposition. Moreover, a core set of genes served as bridges among different miRNAs were identified through the integrated analysis of lncRNA-miRNA-mRNA network.

Conclusion

The observed dramatic expression changes of coding and noncoding RNAs suggest that they may play a critical role in honey bee queens' oviposition. The identified candidate genes for oviposition activation and regulation could serve as a resource for further studies of genetic markers of oviposition in honey bees.

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Abstract

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Introduction

The honey bee (*Apis mellifera*) is a highly diverse species commonly used for honey production and pollination services. The oviposition of honey bee queen is a complex behavior and it is crucial for the reproductive success and affects the development of the colony (Woodward 2010). However, most reproductive traits are complex in terms of their genetic architecture, present low heritability and are sex-limited (Manfredini et al. 2015; Mello et al. 2014). Thus, it is hard to be improved by using traditional selection methods, *eg.* selective breeding. With the development of molecular technologies, new approaches applied to improve reproductive traits and other complex traits, such as marker-assisted selection (MAS) and genomic selection (Kramarenko et al. 2014; Spötter et al. 2012). These methods have been used widely in domestic animals for years. However, in honey bees these strategies became popular only in recent years (Spötter et al. 2012). A better understanding of the genetic architecture of honey bee will help scientists develop a better strategy for acceleration of the genetic improvement of the reproductive traits.

Honey bees provide an excellent model for oviposition molecular studies. The fact that queens specialize in oviposition, leaving other tasks, for example brood caring, to sterile female workers (Koeniger 2008), potentially reduces the complexity of studying reproductive traits. The process of queens' ovary activation is so fast that queens start to lay eggs around 3 days after the mating (Gary 1992). In addition, the activity of queens' oviposition is constantly adjusted throughout the year in order to change the colony's strength according to the environmental conditions (Schneider 1992). Such adjustments can be accomplished within a short period (Koeniger 2008), which guarantees colonies' survival and development in the context of dramatic changes of internal and external conditions. Molecular studies have shown that these changes and regulations are associated with profound differences in coding gene expressions (Lago et al. 2016; Pandey & Bloch 2015) such as ecdysone receptor (*EcR*), mushroom body large-type Kenyon cell-specific protein-1 (*MBLK-1*), ecdysone-induced protein 74 (*E74*) and ultraspiracle (*Usp*) (Pandey & Bloch 2015).

Recently, the characterization of non-coding RNAs, microRNAs (miRNAs) and long non-coding

RNAs (lncRNAs) has become a fruitful area of animals and plants researches. In previous works, several miRNAs, such as bantam, miR-184 and miR-315, have been reported to play important roles in modulating tissue patterns, cell differentiation, ovary development and caste determination in honey bees (Ashby et al. 2016; Macedo et al. 2016). Additionally, miR-14 and miR-8 have been suggested to be associated with juvenile hormones (*JH*) and ecdysteroids (*Ec*), which play key roles in ovary development and other reproductive behaviors in honey bees (Boecking et al. 2000; Flatt et al. 2005; Goodman & Cusson 2012; Hartfelder & Emlen 2005; Hoover et al. 2003; Riddiford 1994; Wyatt & Davey 1996). The other highly expressed non-coding RNAs, lncRNAs, also has a great influence in biological processes, such as cell differentiation, development, immune responses and tumourigenesis (Okazaki et al. 2002; Ota et al. 2004; Wilusz et al. 2009). Moreover, Necsulea *et al.* found lncRNAs that were preferentially expressed in animals' ovary (Necsulea et al. 2014), and lincRNAs (long intergenic non-coding RNAs) were observed by Jayakodi *et al.* (2015) in *Apis mellifera* to be expressed preferentially in ovary tissue. Furthermore, lncRNAs can be targeted by miRNAs and thus regulate the expression of mRNAs (Fan et al. 2015; Gong et al. 2016). Therefore, it is valuable to investigate the critical role of lncRNAs, miRNAs and lncRNA-miRNA-mRNA network in honey bee queens' oviposition.

In order to identify differentially expressed RNAs in ovary activation and oviposition regulation process, we first examined the lncRNA, miRNA and mRNA expression profiles in ovaries of virgin queens, egg-laying queens, egg-laying inhibited queens and egg-laying recovered queens using high throughput sequencing method, then compared the RNA expression patterns to help identify candidate genes and/or RNAs that contribute to oviposition activation and regulation. Next, we selected candidate genes or RNAs which may have high effects in regulating ovary size and oviposition by assign the differently expressed RNAs into a QTL for ovary size. Furthermore, the lncRNA-miRNA-mRNA network was constructed to explore the interaction among different RNAs.

Materials and methods

92 **Ethics statement**

93 The apiaries for honey bee sample collection were maintained by Institute of Apicultural
94 Research, Chinese Academy of Agricultural Sciences (IAR, CAAS), Beijing, China. No specific
95 permits were required for the described studies.

96 **Sampling**

97 All samples were obtained from *Apis mellifera ligustica* honeybee colonies. In June 2015, 20
98 sister queens from a single source colony were reared using standard beekeeping techniques
99 (Harbo 1986). Five days before the emergence, the queens were transferred to an incubator at 36℃
100 and kept individually in plastic vials. One day old, the queens were marked and each was
101 introduced to her own nucleus colony. The strength of each colony was similar. The entrance of
102 each hive was covered with a queen excluder that confined the queen within the hive but allowed
103 workers to exit and enter.
104 Six day old queens were randomly assigned to one of the four groups representing different
105 treatments: (1) virgin queens (n=5); (2) egg-laying queens (n=5) that successfully laid eggs after
106 instrumental insemination; (3) egg-laying inhibited queens (n=5) consisting of egg-laying queens
107 caged in a small cage and kept inside the original hive for 7 days; (4) egg-laying recovery queens
108 (n=5), which were first caged in a small cage inside the original hive for 7 days to prevent them
109 from egg-laying and then released into their individual colonies for 24 hours. All egg-laying
110 recovery queens were able to lay eggs within the 24 hours after their release from the small cages.
111 Ovaries of all queens in the four groups were extirpated and stored at -80 ℃ at the end of the
112 treatment. For instrumental insemination, the source and quantity of the semen was the same for
113 all mated queens. Each sample consisted of the ovary from a single queen. Three samples per
114 treatment group were used for RNAseq (total = 12 samples).

115 **RNA extraction and library preparation for sequencing**

116 Total RNA was extracted from ovary samples using Trizol reagent (Invitrogen, Carlsbad, CA,

USA) according to the manufacturer's instructions. The purity of RNA was checked using the NanoPhotometer spectrophotometer (IMPLEN, CA, USA), and the concentration was measured using Qubit RNA Assay Kit in Qubit 2.0 Flurometer (Life Technologies, CA, USA). The integrity of RNA was assessed using the RNA Nano 600Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

LncRNA and mRNA library preparation was carried out using NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations. Paired-end reads of 150bp were generated using the Illumina Hiseq 4000 platform. After quality control, paired-end clean reads were aligned to the reference genome (*Amel_4.5*) using TopHat v2.0.9. Transcripts were assembled and annotated using Cufflinks (http://cufflinks.cbcb.umd.edu/). The known mRNAs and lncRNAs were identified according to the annotation of *Apis mellifera* genome sequence (*Amel_4.5*). The remaining transcripts were used to screen for putative lncRNAs using the following criteria: (1) length ≥ 200bp; (2) exon number ≥ 2; (3) sequencing coverage ≥ 3; (4) identified in at least two samples. The transcripts meeting the above criteria were further filtered by removing known non-lncRNA transcripts. Then, the transcripts that passed the filters were evaluated for coding potential using CPC (0.9-r2) (Kong et al. 2007) and Pfam-scan (v1.3) (Punta et al. 2012). Only those without coding potential were categorized as novel lncRNAs.

Small RNA library preparation was carried out using NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (NEB, USA) following manufacturer's recommendations. Single-end reads of 50bp were generated using the Illumina Hiseq 2500 platform. After quality control, the clean reads were mapped to reference sequence (*Amel_4.5*) applying Bowtie (Langmead et al. 2009). Mapped reads were used to identify known miRNAs using miRBase 20.0 (Griffiths-Jones 2010). Novel miRNAs were predicted with miREvo (Ming et al. 2012) and mirdeep2 (Friedländer et al. 2012) through exploring the characteristic hairpin structure, Dicer cleavage sites and minimum free energy.

All the sequencing data are available through the GEO database with accession number GSE93028.

Differentially expressed lncRNAs, miRNAs and mRNAs identification and clustering analysis

Differentially expressed (DE) lncRNAs, miRNAs and mRNAs (Benjamini & Hochber method corrected p-value < 0.05) were identified using DESeq R package (1.8.3) for each of the following comparisons: (1) egg-laying queens *vs.* virgin queens (ovary activation process); (2) egg-laying inhibited queens *vs.* egg-laying queens (oviposition inhibition process); (3) egg-laying recovery queens *vs.* egg-laying inhibited queens (oviposition recovery process). Furthermore, the expression of each RNA type was analyzed with unsupervised hierarchical clustering with the R package of "pheatmap". To do unsupervised hierarchical clustering, firstly, the expression of RNA was normalized. For normalization of lncRNA and mRNA, the following formula was used: $\mathrm{FPKm} = \log_{10}\mathrm{FPKM} + 1$. For normalization of miRNA, the following formula was applied: $\mathrm{TPm} = \log_{10}\mathrm{TPM} + 1$ (TPM, transcripts per kilobase million). Then the euclidean distance was used to measure the degree of similarity between the expression profiles of samples. The method in the package to cluster distance is "complete".

Prediction of lncRNA and miRNA target genes

The potential trans role of lncRNAs (acting on non-neighboring genes) can be assessed by correlating expression levels between lncRNAs and mRNAs. The trans role of lncRNAs in coding genes was examined based on the expression correlation coefficient (Pearson correlation ≥ 0.95 or ≤ -0.95). To predict miRNAs targets, we searched for the targets in the 3'UTR of genes models. For genes lacking a predicted 3'UTR, the region 1000bp downstream of the stop codon were included. The prediction was performed by Miranda with the following parameter: free energy < -10 kcal/mol and score > 140 (Enright et al. 2003).

Functional enrichment analysis

Apis mellifera gene set was annotated based on the corresponding *Drosophila melanogaster* orthologues and catergorized by their biological functions. Gene annotation was done by a homology-based method. *Apis mellifera* CDS sequences were blasted against the *Drosophila melanogaster* peptide sequences (Ensembl database *Release 74*) using the comment "-p blastx -m8 -e 1e-5-F F". The minimum peptide alignment must be more than 50 aa. The correspondence relationship of *Apis mellifera* genes and ontology categories was decided by the hit with the best alignment score. Gene ontology (GO) enrichment analysis with *Drosophila melanogaster* reference gene set was implemented by GOseq R package (Young et al. 2012). KEGG pathways analysis was performed using KOBAS to determine the involvement of genes in different biological pathways (Mao et al. 2005).

Chromosomal localization of DE lncRNAs and mRNAs in quantitative trait locus (QTL) for ovary Size

The localization of the DE lncRNAs and DE mRNAs on *Apis mellifera* chromosomes was accessed from NCBI database (*Amel_4.5*). Each RNA location was estimated in centimorgans and was compared with the location of a significant QTL previously identified for ovary size. This QTL locates on chromosome 11 between the position 8.9 Mb and 12.2 Mb (Graham et al. 2011; Linksvayer et al. 2009). Genes or RNAs which locate within the QTL confidence intervals were accepted as candidate genes for ovary size and potential candidate genes for oviposition.

Construction of lncRNA-miRNA-mRNA network

To construct lncRNA-miRNA-mRNA network, we first selected lncRNAs which were predicted to act as miRNA targets or decoys by Fan's methods (Fan et al. 2015). Next, to define the miRNA-mRNA relationships, the Pearson correlation coefficient value between a miRNA and its target mRNA was calculated, and strongly correlated miRNA-mRNA pairs (the absolute value of greater 0.8) were selected (either positive or negative). To construct the network, each DE RNA node must be either in a lncRNA-miRNA pair or in a miRNA-mRNA pair. The nodes in the network consisted of miRNAs, lncRNAs acting as miRNA targets, lncRNAs acting as miRNA

decoys, mRNAs acting as miRNA targets. The network was visualized using Cytoscape (version 3.4.0) (Smoot et al. 2011).

Real time PCR

In order to confirm sequencing results, the expression of 5 lncRNAs, 5 mRNAs and 5 miRNAs were validated by real time PCR using the same 12 ovary samples used for sequencing. Following total RNA extraction, ovarian samples were reversely transcribed to generate cDNA. For cDNA synthesis of lncRNA and mRNA, an M-MLV FIRST STRAND KIT (Invitrogen, Shanghai, China) and an oligo (dT)18 primer were used in a reverse transcription reaction of 20 µl, following the supplier's instructions. For miRNA cDNA synthesis, a miRcute miRNA cDNA synthesis kit (Tiangen biotech (Beijing) Co.,LTD) was used. In brief, *E.coli* Poly(A) Polymerase was used to add poly(A) tail at 3' end and then Oligo(dT)-Universal tag was used in a reverse transcription reaction following the supplier's instructions. Two microliters of each cDNA was subjected to PCR amplification using specific primers (Supplemental Table S1). PCR efficiency of each gene was estimated by standard curve calculation using four points of cDNA serial dilutions. Cycle threshold (*Ct*) values were transformed to quantities using the comparative *Ct* method, setting the relative quantities of virgin queens group for each gene to 1 (quantity=10-ΔCt/slope). Data normalization of lncRNA and mRNA were carried out using the Actin reference gene. Data normalization of miRNA was carried out using the U6 reference gene. The correlation between the results of sequencing and PCR was calculated using correlation test.

Results

Genome-wide identification of DE lncRNAs, mRNAs and miRNAs from honey bee queens

Sequencing of all lncRNA and mRNA libraries generated 1,243,644,174 raw paired-end reads with a length of 150 bases, resulting in a total of 16.7 gigabases. Sequencing of all miRNA libraries generated 152,659,565 raw single-end reads with a length of 50 bases, resulting in a total of 7.631 gigabases. The whole expression profiles of lncRNAs, miRNAs and mRNAs of ovaries at four different conditions are presented in Fig. 1. From the expression profiles, DE

lncRNAs, mRNAs and miRNAs were discriminated between different groups (Table 1 and Supplemental Table S2). 740 lncRNAs, 5481 mRNAs and 81 miRNAs were differentially expressed in ovary activation process (egg-laying queens *vs.* virgin queens). 88 lncRNAs, 338 mRNAs and 13 miRNAs were differentially expressed in oviposition inhibition process (egg-laying inhibited queens *vs.* egg-laying queens). 100 lncRNAs, 497 mRNAs and 4 miRNAs were differentially expressed in oviposition recovery process (egg-laying recovery queens *vs.* egg-laying inhibited queens). A summary of the up-/down-regulated information is shown in Table 1.

GO and Pathway enrichment analysis

Functional annotation analysis of target genes of the DE lncRNAs, miRNA and mRNA was performed to identify GO terms and KEGG pathways with higher confidence (Supplemental Table S3, S4 and S5). Because GO terms and pathways enriched with the DE lncRNAs, miRNA and mRNAs were similar to each other, here we only describe the enrichment results of DE mRNAs. In the ovary activation process, most of the enriched GO_BP terms of DE mRNAs were involved in tissue development, energy producing and hormone biosynthesis and metabolism, such as oocyte microtubule cytoskeleton polarization, fatty acid oxidation, neurotrophin signaling pathway, ecdysteroid catabolic process (Supplemental Table S3). In the oviposition inhibition process, contrary to the ovary activation process, several GO terms were not enriched, but enrichment occurred again when oviposition recovered, such as cellular response to transforming growth factor beta stimulus, positive regulation of cyclase activity, post-embryonic hemopoiesis, larval lymph gland hemopoiesis, eye pigment biosynthetic process, and compound eye cone cell fate commitment (Supplemental Table S3).

DE mRNAs enrichment ($p < 0.05$) was seen in KEGG pathways (Supplemental Table S3). Several pathways were both enriched in ovary activation and oviposition regulation process, namely glycerolipid metabolism, glycerophospholipid metabolism, hippo signaling pathway – fly, inositol phosphate metabolism, MAPK signaling pathway – fly, neuroactive ligand-receptor interaction, notch signaling pathway, phosphatidylinositol signaling system and Wnt signaling

246 pathway (Table 2).

247 **Chromosomal localization of DE lncRNAs and mRNAs in QTL region for ovary size**

248 If the differentially expressed lncRNAs and mRNAs were found located within the confidence
249 interval of the QTL for ovary size, they could be regarded as candidate genes for ovary size and
250 potential candidate genes for oviposition. In this way, 73 candidate genes and 14 lncRNAs
251 (Supplemental Table S6) were identified.

252 **Construction of the lncRNA-miRNA-mRNA network**

253 The bioinformatic analysis predicted that 469 lncRNAs were targeted by 69 miRNAs and 117
254 lncRNAs acted as decoys to 31 miRNAs. The transcriptome network was constructed based on
255 the lncRNA-miRNA and the miRNA-mRNA relationship pairs. The resulting network consists
256 of 229 lncRNA-miRNA pairs and 225 miRNA-mRNA pairs (Supplemental Fig.S1 and Table S7).

257 To further investigate the potential candidate genes and RNAs for ovary activation and
258 oviposition, a reproductive associated network was constructed containing the DE miRNAs and
259 mRNAs which played specific or suspected roles in reproduction, and the DE lncRNAs and
260 mRNAs located in the QTL region for ovary size. The network was constructed with 105
261 lncRNA-miRNA pairs and 83 miRNA-mRNA pairs, consisted of 105 lncRNAs, 25 miRNAs and
262 74 mRNAs (Fig. 2 and Supplemental Table S8).

263 **Validation of RNA-Seq data by real time PCR**

264 In order to validate the sequencing results, the expression of 5 lncRNAs, 5 mRNAs and 5
265 miRNAs were tested by using real time PCR with the same RNA samples used for sequencing
266 (Supplemental Table S1). The expression profiles of these genes/RNAs detected by real time
267 PCR were consistent with those obtained by sequencing, which confirmed the reliability of our
268 sequencing results.

269 **Discussion**

In the present study, dynamical lncRNAs, mRNAs and miRNAs expression profiles in ovary activation and oviposition processes in honey bees were identified. However, the complex molecular mechanism behind the oviposition activation and regulation still needs to be illustrated.

Representative enriched pathways

The gene function analysis showed that DE RNAs enrichment was seen in a number of pathways in ovary activation and/or oviposition regulation process. Some of the pathways are particularly interesting, such as Wnt, hippo, TGF-beta, notch, MAPK, FoxO and mTOR signaling pathways (Fig.3). More than 50% of the genes in those pathways were differently expressed according to our results. Some of the pathways have known or suspected roles in honey bees. For example, Wnt, hippo, notch, MAPK and TOR pathways were reported to be involved in caste determination in honey bees (Ashby et al. 2016; Wheeler et al. 2014). Caste determination is inseparably linked with the ovary development status. Although, so far, studies on the effect of these pathways on oviposition are not available, some insights can be drawn from other species.

The Wnt signaling pathway was found to be involved in the development of reproductive system such as the development of ovarian follicules, ovulation and luteinization (Sun & Wang 2003). The hippo signaling pathway was also reported to be related to the regulation of mouse ovarian functional remodeling (Ye et al. 2017). Moreover, the hippo signaling pathway can coordinate with Wnt, TGF-beta and notch signaling pathways affecting organ size in *Drosophila* (Barry & Camargo 2013). Because after queen mating, the size of ovary will become bigger than the virgin's (Rinderer 1987), we also observed many genes in Wnt, TGF-beta, hippo and notch signaling pathways that were differentially expressed in mated queens compared with virgin queens. It indicated that those pathways may participate in ovarian function remodeling after mating to prepare for oviposition in honey bees. The oocyte growth and development is crucial to successful oviposition, particularly during the height of the brood-rearing season when a good queen can lay up to 1, 500 eggs per day (Koeniger 2008). Studies in mammal found that TGF-beta, MAPK and FoxO signaling pathways regulate oocytes growth and development (Edmonds

et al. 2010; Kretzschmar et al. 1997; Zhang et al. 2011). Also, there were studies showing that the TGF-beta signaling pathway was essential for oogenesis in *Drosophila* (Twombly et al. 1996). TGF-beta, MAPK and FoxO signaling pathways demonstrated enrichment in DE RNAs in our results, which indicated that these pathways may involve in oocyte growth and development in honey bees.

The queen is the only fertile female in a honey bee colony, and it constrains the reproduction of worker bees. A recent study reported that notch signaling facilitated the queen to repress ovary activity and maintain reproductive sterility in the worker bees (Duncan et al. 2016). Also, TOR pathway was found to be associated with the reproductive status in workers (Patel et al. 2007). DE RNAs enrichment was observed in the present study in both notch and TOR signaling pathways in mated queens which demonstrated that notch and TOR pathways possessed signaling functions in strengthening the reproductive constraint after queen mating.

Further, the studied pathway maps were looked up in KEGG database to assess whether there is a relationship among them. The results showed that they were closely interacting with each other as shown in Fig.3, whereby for example TGF-beta signaling pathway was part of hippo and Wnt signaling pathways. These pathways were enriched both in oviposition activation and oviposition process. Considering roles of these pathways in ovarian function remodeling, oocyte growth and development and other related processes, they are critical for a successful oviposition by complex fine-tuning relationships.

Among the DE genes in those pathways, several genes were found to participate in more than one pathway. The gene nejire (*Nej*, also known as CREB-binding protein (*CBP*)) participated in three pathways, namely notch, FoxO and TGF-beta signaling pathways. Additionally, *Nej* was significantly up-regulated in the egg-laying queens compared to virgin queens. Also studies in *Drosophlia melanogaster* found that *Nej* was involved in regulation of many pathways during embryo development, through hedgehog, wingless and TGF-beta signaling pathways (Fernandez-Nicolas & Belles 2016). Taken together, we could conclude that *Nej* may participate

in embryonic development in honey bees through notch, FoxO and TGF-beta signaling pathways, and can be considered as the potential candidate genes for oviposition.

Genes and lncRNAs co-localized in QTL region for ovary size

We compared the location of the DE genes and DE lncRNAs on the honey bee genome available at the NCBI database (*Amel_4.5*) with one QTL for ovary size. 73 genes and 14 lncRNAs were identified, and some of them together with their key function will be explained further.

Among the 73 genes, G2/mitotic-specific cyclin-B3 (*CycB3*) is the one we paid special attention. It was shown for example that *CycB3* controlled oocyte maturation and early embryo development in mouse (Polański et al. 2012), but studies of *CycB3* in reproduction in honey bees are scarce. Fig.3 showed that *CycB3* was significantly up-regulated in ovary activation process and participated in the FoxO signaling pathway, which implies that *CycB3* may play important roles in oviposition and affect oocyte maturation in honey bees through FoxO signaling pathway.

Two lncRNAs, XLOC_073978 and XLOC_081294 (sequence information noted in Supplemental Table S2) are of particular interest. The predicted targets of XLOC_073978 included myophilin-like, yellow-f and cytochrome P450 9Q1 (*CYP9Q1*). The predicted targets of XLOC_081294 included yellow-b, odorant binding protein 10 (*Obp10*), myosin regulatory light chain 2 (*Mlc2*), *CYP9Q1, CYP9Q2* and *CYP9Q3*. Myophilin (also known as *CHD64*) was previously identified as *JH* response gens (Rewitz et al. 2006), which regulated many aspects of physiology and development of insects (Flatt et al. 2005), including reproduction (Flatt et al. 2005; Goodman & Cusson 2012; Hartfelder & Emlen 2005; Riddiford 1994; Wyatt & Davey 1996). *Mlc2* was previously detected changing expression during the ovary activation process (Manfredini et al. 2015). Concerning yellow-b, yellow-f and *Obp10*, they had been reported to relate to ovary activation and response with *Ec*, one of the most critical hormones affecting reproduction in honey bees and other insects (Amdam et al. 2010; Bloch et al. 2000; Hagedorn 1985; Pandey & Bloch 2015). Furthermore, it is notable that three target genes from CYP450 family (*CYP9Q1, CYP9Q2* and *CYP9Q3*), some of which were previously detected to interact

with *Ec* (Mello et al. 2014; Rewitz et al. 2006), showed changes of expressions in our results.

Therefore, all the predicted targets of XLOC_073978 and XLOC_081294 were associated with reproduction in honey bees. This highlights their roles in oviposition. Because the genes elsewhere in the genome might share pathways with genes in the QTL region and reflect downstream effects of the QTL (Fernandezrodriguez et al. 2011), they will be useful for identifying candidate genes and/or RNAs for ovary activation and oviposition by combining the information obtained from expression analysis with the QTL location analyses. Further studies will involve in studying the genes that interacted with the QTL genes.

Analysis of DE RNAs with known or suspected roles in reproduction

Table 3 and Table 4 show that 31 mRNAs and 36 miRNAs were significantly regulated in caste determination or other reproductive related process, which indicated that they have known or suspected roles of in ovary activation. The oviposition status is positively correlated with two genes (heat shock protein 90 (*Hsp90*) and *Usp*) and negatively correlated with ten genes. *Hsp90* has been reported as a candidate marker gene for caste-specific ovary development (Lago et al. 2016). According to our results, *Hsp90* can also be a candidate marker for the oviposition status of the honey bee queen. Among the negatively correlated genes, four are CYP450 family genes. Several genes of CYP450 family were reported to act as response genes of *Ec* and 20-hydroxyecdysone (*20E*) which is the active *Ec* in most insects (Buszczak & Segraves 1998) including honey bees (Yamazaki et al. 2011). Importantly, some CYP450 genes were identified as targets of lncRNAs, which are located in the QTL region of ovary size in our results. This highlights their roles in oviposition.

The other six miRNAs that are negatively correlated with the oviposition status are bantam, miR-12, miR-279a-3p, miR-31a, miR-993 and miR-996. Bantam plays an important role in embryonic development and was identified as a crucial target of the signaling pathways of hippo and EGFR/MAPK in *Drosophila* (Herranz et al. 2012; Nolo et al. 2006; Thompson & Cohen 2006). The DE mRNAs enrichment was seen in hippo and EGFR/MAPK signaling pathways in

our study, suggesting that bantam may affect ovary activation or oviposition by the hippo and/or EGFR/MAPK signaling pathway. Also four miRNAs (miR-1, miR-133, miR-184 and miR-190) were down-regulated during oviposition activation and recovery, but the suspension of oviposition did not affect their expression. MiR-184, which is highly conserved and widely studied in insects, was reported to affect caste determination of honey bees (Guo et al. 2013; Macedo et al. 2016; Mello et al. 2014; Shi et al. 2015). Furthermore, studies in *Dorsophlia* found that loss of miRNA-184 induced loss of egg production (Iovino et al. 2009). In our trial, miRNA-184 was down-regulated in mated queens but not in virgin queens. Thus, it can be speculated that miRNA-184 could be a candidate marker for oviposition of the honey bee queen.

In addition, a positive correlation was observed between a set of three miRNAs (miR-263a, miR-2944-3p and miR-92b) and the oviposition status. MiR-263a and miR-92b were found to be involved in neuronal development and affected caste determination in honey bees (Ashby et al. 2016), which played important roles in reproductive activities (Heifetz et al. 2014). When queen's oviposition are activated or regulated, the neuronal activity and excitability increases. Therefore, we deduced that miR-263a, miR-2944-3p and miR-92b might be associated with the neuronal development and they further affect oviposition activation and regulation.

Furthermore, as shown in table 4, many miRNAs, which respond to *Ec*, *JH* and vitellogenin (*Vg*), showed significant changes in their expressions. *Ec*, *Vg* and *JH* are among the most important hormones in regulating reproductive activities in honey bees (Lago et al. 2016; Nunes et al. 2013; Oxley & Oldroyd 2010). Particularly, *Vg* serves as a yolk precursor in egg development and affects oviposition in almost all oviparous species (Stephen M. Downs 2009). Changes of expressions of miRNAs in our study may regulate or be regulated by *Ec*, *Vg* and *JH*, and further affect ovary activation and/or oviposition.

Key roles in the lncRNA-miRNA-mRNA network

In the network constructed with miRNAs, mRNAs and lncRNAs, we found that some genes served as bridges linking different miRNAs, four of which (gene id: 408284, 408609, 409587,

and 409152) acting as miRNA targets linked let-7, miR-100, miR-12, miR-14, miR-316 and miR-996. Two of them are worthy of noting here. One was coiled-coil domain-containing protein 93 (*CCDC93*, id: 408609). Oh et al. (2011) found that *CCDC93* regulated the expression level of cyclin B1 (*CycB1*), a cyclin gene in human cells. Our results showed that *CycB3*, another cyclin gene, was localized in the QTL region for ovary size, which indicated that *CCDC93* may interact with cyclin genes and further affect oviposition. The other was heat shock 70 kDa protein cognate (*Hsc70-3*, id: 409587). The interaction between *Hsc70* and *Hsp90* was reported previously (King et al. 2001). More importantly, the Hsc70/Hsp90 chaperone machinery is responsible for loading small RNA duplexes into Argonaute proteins, which are critical to small silencing RNAs—small interfering RNAs (siRNAs) or microRNAs (miRNAs)—direct posttranscriptional gene silencing of their mRNA targets (Iwasaki et al. 2010). Therefore, *Hsc70* is essential for miRNAs to implement their impact on the expression of target mRNAs. Our results confirm similar findings showing that *Hsc70-3* acted as a target of miRNA and served as a bridge linking different miRNAs in the lncRNA-miRNA-mRNA network, which highlight its role in the interaction among different RNAs in oviposition. Taken together, we can conclude that both *CCDC93* and *Hsc70-3* play important roles in the network and further affect gene expressions in oviposition.

Conclusions

In the present study, lncRNAs, mRNAs and miRNAs expression profiles were evaluated and compared during ovary activation and dynamical oviposition process in honey bees. Bioinformatic analyses suggest that some lncRNAs, miRNA and genes are involved in important biological processes associated with oviposition activation and regulation. Additionally, lncRNA-miRNA-mRNA network revealed the potential interactions among different RNAs. Moreover, candidate genes or RNAs for oviposition were identified, which are particularly attractive for further in-depth studies.

Acknowledgements

The authors thank Wei Feng, who is the beekeeper in the apiary, for his assistance in beekeeping.

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Sheet6 Enriched KEGG pathways for DE lncRNAs in oviposition recovery process

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465 miRNA pair or a miRNA-mRNA pair.

References

Amdam GV, Page RE, Fondrk MK, and Brent CS. 2010. Hormone response to bidirectional selection on social behavior. *Evolution & development* 12:428-436.

Ashby R, Forêt S, Searle I, and Maleszka R. 2016. MicroRNAs in Honey Bee Caste Determination. *Sci Rep* 6.

Barry ER, and Camargo FD. 2013. The Hippo superhighway: signaling crossroads converging on the Hippo/Yap pathway in stem cells and development. *Curr Opin Cell Biol* 25:247-253. 10.1016/j.ceb.2012.12.006

Bloch G, Hefetz A, and Hartfelder K. 2000. Ecdysteroid titer, ovary status, and dominance in adult worker and queen bumble bees (Bombus terrestris). *Journal of Insect Physiology* 46:1033-1040.

Boecking O, Bienefeld K, and Drescher W. 2000. Heritability of the Varroa-specific hygienic behaviour in honey bees (Hymenoptera : Apidae). *Journal of Animal Breeding and Genetics-Zeitschrift Fur Tierzuchtung Und Zuchtungsbiologie* 117:417-424. DOI 10.1046/j.1439-0388.2000.00271.x

Buszczak M, and Segraves WA. 1998. Drosophila metamorphosis: The only way is USP?: Current Biology. *Current Biology* 8:879-882.

Duncan EJ, Hyink O, and Dearden PK. 2016. Notch signalling mediates reproductive constraint in the adult worker honeybee. *Nature Communications* 7.

Edmonds JW, Prasain JK, Dorand D, Yang Y, Hoang HD, Vibbert J, Kubagawa HM, and Miller MA. 2010. Insulin/FOXO signaling regulates ovarian prostaglandins critical for reproduction. *Developmental Cell* 19:858-871.

Enright AJ, John B, Gaul U, Tuschl T, Sander C, and Marks DS. 2003. MicroRNA targets in Drosophila. *Genome Biol* 5:: R1.

Fan C, Hao Z, Yan J, and Li G. 2015. Genome-wide identification and functional analysis of lincRNAs acting as miRNA targets or decoys in maize. *BMC Genomics* 16:1-19.

Fernandez-Nicolas A, and Belles X. 2016. CREB-binding protein contributes to the regulation of endocrine and developmental pathways in insect hemimetabolan pre-metamorphosis. *Biochim Biophys Acta* 1860:508.

Fernandezrodriguez A, Munoz M, Fernandez A, Pena RN, Tomas A, Noguera JL, Ovilo C, and Fernandez AI. 2011. Differential Gene Expression in Ovaries of Pregnant Pigs with High and Low Prolificacy Levels and Identification of Candidate Genes for Litter Size1. *Biol Reprod* 84:299-307.

Flatt T, Tu MP, and Tatar M. 2005. Hormonal pleiotropy and the juvenile hormone regulation of Drosophila development and life history. *Bioessays* 27:999–1010.

Friedländer MR, Mackowiak SD, Li N, Chen W, and Rajewsky N. 2012. miRDeep2 accurately identifies known and hundreds of novel microRNA genes in seven animal clades. *Nucleic Acids Res* 40:37-52.

Gary N. 1992. The Hive and the Honey bee. In: Graham JM, ed. *The Hive and the Honey bee*: Dadant & Sons, Inc, 271-307.

Gong Z, Qian Y, Zeng Z, Zhang W, Li X, Zu X, Hao D, Pan C, Liao Q, and Bo X. 2016. An integrative transcriptomic analysis reveals p53 regulated miRNA, mRNA, and lncRNA networks in nasopharyngeal carcinoma. *Tumor Biology* 37:1-13.

Goodman WG, and Cusson M. 2012. *8–The Juvenile Hormones*: Plenum Press.

Graham AM, Munday MD, Kaftanoglu O, Page RE, Amdam GV, and Rueppell O. 2011. Support for the reproductive ground plan hypothesis of social evolution and major QTL for ovary traits of Africanized worker honey bees (Apis mellifera L.). *BMC Evol Biol* 11:95.

506 Griffiths-Jones S. 2010. miRBase: microRNA sequences and annotation. Curr Protoc Bioinformatics Chapter
507 12:Unit 12 19 11-10. 10.1002/0471250953.bi1209s29
508 Guo X, Su S, Skogerboe G, Dai S, Li W, Li Z, Liu F, Ni R, Guo Y, and Chen S. 2013. Recipe for a busy bee:
509 microRNAs in Honey Bee caste determination. *PLoS One* 8:e81661.
510 Hagedorn HH. 1985. 7 – The Role of Ecdysteroids in Reproduction. *Endocrinology II*:205-262.
511 Harbo JR. 1986. Propagation and Instrumental Insemination - Bee Genetics and Breeding - CHAPTER 15. *Bee*
512 *Genetics & Breeding* 01:361–389.
513 Hartfelder K, and Emlen DJ. 2005. 3.13–Endocrine Control of Insect Polyphenism. *Comprehensive Molecular*
514 *Insect Science*:651-703.
515 Heifetz Y, Lindner M, Garini Y, and Wolfner M. 2014. Mating Regulates Neuromodulator Ensembles at Nerve
516 Termini Innervating the Drosophila Reproductive Tract. *Current Biology Cb* 24:731-737.
517 Herranz H, Hong X, and Cohen S. 2012. Mutual Repression by Bantam miRNA and Capicua Links the
518 EGFR/MAPK and Hippo Pathways in Growth Control. *Current Biology Cb* 22:651-657.
519 Hoover SER, Keeling CI, Winston ML, and Slessor KN. 2003. The effect of queen pheromones on worker honey
520 bee ovary development. *Naturwissenschaften* 90:477-480.
521 Humann FC, and Hartfelder K. 2011. Representational Difference Analysis (RDA) reveals differential expression of
522 conserved as well as novel genes during caste-specific development of the honey bee (Apis mellifera L.)
523 ovary. Insect Biochemistry & Molecular Biology 41:602-612.
524 Iovino N, Pane A, and Gaul U. 2009. miR-184 has multiple roles in Drosophila female germline development. *Dev*
525 *Cell* 17:123-133. 10.1016/j.devcel.2009.06.008
526 Iwasaki S, Kobayashi M, Yoda M, Sakaguchi Y, Katsuma S, Suzuki T, and Tomari Y. 2010. Hsc70/Hsp90
527 Chaperone Machinery Mediates ATP-Dependent RISC Loading of Small RNA Duplexes. *Mol Cell* 39:292.
528 Jayakodi M, Jung JW, Park D, Ahn YJ, Lee SC, Shin SY, Shin C, Yang TJ, and Kwon HW. 2015. Genome-wide
529 characterization of long intergenic non-coding RNAs (lincRNAs) provides new insight into viral diseases
530 in honey bees Apis cerana and Apis mellifera. *BMC Genomics* 16:680. 10.1186/s12864-015-1868-7
531 King FW, Wawrzynow A, Höhfeld J, and Zylicz M. 2001. Co-chaperones Bag-1, Hop and Hsp40 regulate Hsc70
532 and Hsp90 interactions with wild-type or mutant p53. *Embo Journal* 20:6297-6305.
533 Koeniger G. 2008. Bee Genetics and Breeding. In: Rinderer TE, ed. *Bee Genetics & Breeding*: Academic Press
534 (London), 255-275.
535 Kramarenko AS, Lopukchov AA, Gladyr EA, Singina GN, Ermilov AN, Yanchukov IN, Brem G, and Zinovieva
536 NA. 2014. 206 genome-wide associations for reproductive traits in Russian holstein population.
537 *Reproduction Fertility & Development* 27:194.
538 Kretzschmar M, Doody J, and Massagué J. 1997. Opposing BMP and EGF signalling pathways converge on the
539 TGF-beta family mediator Smad1. *Nature* 389:618-622.
540 Lago DC, Humann FC, Barchuk AR, Abraham KJ, and Hartfelder K. 2016. Differential gene expression underlying
541 ovarian phenotype determination in honey bee, Apis mellifera L, caste development. *Insect Biochemistry &*
542 *Molecular Biology* 79:1-12.
543 Langmead B, Trapnell C, Pop M, Salzberg SL, and Quals P. 2009. Ultrafast and memory-efficient alignment of
544 short reads to the human genome. 10.
545 Linksvayer TA, Rueppell O, Siegel A, Kaftanoglu O, Jr PR, and Amdam GV. 2009. The genetic basis of
546 transgressive ovary size in honeybee workers. *Genetics* 183:693.

547 Macedo LMF, Nunes FMF, Freitas FCP, Pires CV, Tanaka ED, Martins JR, Piulachs MD, Cristino AS, Pinheiro DG,
548 and Simões ZLP. 2016. MicroRNA signatures characterizing caste-independent ovarian activity in queen
549 and worker honeybees (A pis mellifera L.). *Insect Mol Biol* 25:216-226.
550 Manfredini F, Brown MJF, Vergoz V, and Oldroyd BP. 2015. RNA-sequencing elucidates the regulation of
551 behavioural transitions associated with the mating process in honey bee queens. *BMC Genomics* 16:563.
552 Mao X, Cai T, Olyarchuk JG, and Wei L. 2005. Automated genome annotation and pathway identification using the
553 KEGG Orthology (KO) as a controlled vocabulary. *Bioinformatics* 21:3787-3793.
554 10.1093/bioinformatics/bti430
555 Mello TRP, Aleixo AC, Pinheiro DG, Nunes FMF, Bitondi MMG, Hartfelder K, Barchuk AR, and Simões ZLP.
556 2014. Developmental regulation of ecdysone receptor (EcR) and EcR-controlled gene expression during
557 pharate-adult development of honeybees (Apis mellifera). *Front Genet* 5:445.
558 Ming W, Yang S, Shi S, and Tian T. 2012. miREvo: an integrative microRNA evolutionary analysis platform for
559 next-generation sequencing experiments. *Bmc Bioinformatics* 13:1-10.
560 Necsulea A, Soumillon M, Warnefors M, Liechti A, Daish T, Zeller U, Baker JC, Grützner F, and Kaessmann H.
561 2014. The evolution of lncRNA repertoires and expression patterns in tetrapods. *Nature* 505:635-640.
562 Nolo, Riitta, Morrison, Clayton M, Tao, Chunyao, Zhang, Xinwei, Halder, and Georg. 2006. The bantam
563 MicroRNA Is a Target of the Hippo Tumor-Suppressor Pathway. *Current Biology* 16:1895-1904.
564 Nunes FM, Ihle KE, Mutti NS, Simões ZL, and Amdam GV. 2013. The gene vitellogenin affects microRNA
565 regulation in honey bee (Apis mellifera) fat body and brain. *Journal of Experimental Biology* 216:3724.
566 Oh YJ, Lee EH, Lee IK, Kim K-S, and Kim H. 2011. Coiled-Coil Domain-Containing Protein 98 (CCDC98)
567 Regulates Cyclin B1 Expression by Affecting WTAP Protein Stability. *Journal of Life Science* 21:1067-
568 1075.
569 Okazaki Y, Furuno M, Kasukawa T, Adachi J, Bono H, Kondo S, Nikaido I, Osato N, Saito R, and Suzuki H. 2002.
570 Analysis of the mouse transcriptome based on functional annotation of 60,770 full-length cDNAs. *Nature*
571 420:563-573.
572 Ota T, Suzuki Y, Nishikawa T, Otsuki T, Sugiyama T, Irie R, Wakamatsu A, Hayashi K, Sato H, and Nagai K. 2004.
573 Complete sequencing and characterization of 21,243 full-length human cDNAs. *Nature Genetics* 36:40-45.
574 Oxley PR, and Oldroyd BP. 2010. *Chapter 3 - The Genetic Architecture of Honeybee Breeding*: Elsevier Science &
575 Technology.
576 Pandey A, and Bloch G. 2015. Juvenile hormone and ecdysteroids as major regulators of brain and behavior in bees.
577 *Current Opinion in Insect Science* 12:26-37.
578 Patel A, Fondrk MK, Kaftanoglu O, Emore C, Hunt G, Frederick K, and Amdam GV. 2007. The making of a queen:
579 TOR pathway is a key player in diphenic caste development. *PLoS One* 2:e509.
580 10.1371/journal.pone.0000509
581 Polański Z, Homer HA, and Kubiak JZ. 2012. Cyclin B in mouse oocytes and embryos: importance for human
582 reproduction and aneuploidy. *Results & Problems in Cell Differentiation* 55:69.
583 Rewitz KF, Rybczynski RWarren JT, and Gilbert LI. 2006. Developmental expression of Manduca shade, the P450
584 mediating the final step in molting hormone synthesis. *Molecular & Cellular Endocrinology* 247:166-174.
585 Riddiford LM. 1994. Cellular and Molecular Actions of Juvenile Hormone I. General Considerations and
586 Premetamorphic Actions. *Advances in Insect Physiology* 24:213-274.
587 Rinderer TE. 1987. Bee genetics and breeding. *The Quarterly Review of Biology*.

588 Schneider SS. 1992. The Hive and the Honey bee. In: Graham JM, ed. *The Hive and the Honey bee*: Dadant & Sons,
589 Inc, 73-100.
590 Shi YY, Zheng HJ, Pan QZ, Wang ZL, and Zeng ZJ. 2015. Differentially expressed microRNAs between queen and
591 worker larvae of the honey bee (Apis mellifera). *Apidologie* 46:35-45.
592 Smoot ME, Ono K, Ruscheinski J, Wang PL, and Ideker T. 2011. Cytoscape 2.8: new features for data integration
593 and network visualization. *Bioinformatics* 27:431-432. 10.1093/bioinformatics/btq675
594 Spötter A, Gupta P, Nurnberg G, Reinsch N, and Bienefeld K. 2012. Development of a 44K SNP assay focussing on
595 the analysis of a varroa-specific defence behaviour in honey bees (Apis mellifera carnica). *Molecular*
596 *Ecology Resources* 12:323-332. 10.1111/j.1755-0998.2011.03106.x
597 Stephen M. Downs JO, John Klinger. 2009. Fatty acid oxidation and meiotic resumption in mouse oocytes.
598 *Molecular Reproduction & Development* 76:844-853.
599 Sun X, and Wang Y. 2003. Wnt signaling pathways in mammalian reproduction. *Progress in Biochemistry &*
600 *Biophysics* 30:180-184.
601 Thompson BJ, and Cohen SM. 2006. The Hippo pathway regulates the bantam microRNA to control cell
602 proliferation and apoptosis in Drosophila. *Cell* 126:767-774.
603 Wheeler DE, Buck NA, and Evans JD. 2014. Expression of insulin/insulin-like signalling and TOR pathway genes
604 in honey bee caste determination. *Insect Mol Biol* 23:113-121. 10.1111/imb.12065
605 Wilusz JE, Sunwoo H, and Spector DL. 2009. Long noncoding RNAs: functional surprises from the RNA world.
606 *Genes Dev* 23:1494-1504. 10.1101/gad.1800909
607 Woodward DR. 2010. *Queen bee : biology, rearing and breeding*: Northern Bee Books.
608 Wyatt GR, and Davey KG. 1996. Cellular and Molecular Actions of Juvenile Hormone. II. Roles of Juvenile
609 Hormone in Adult Insects. *Advances in Insect Physiology* 26:1-155.
610 Yamazaki Y, Kiuchi M, Takeuchi H, and Kubo T. 2011. Ecdysteroid biosynthesis in workers of the European
611 honeybee Apis mellifera L. *Insect Biochemistry & Molecular Biology* 41:283-293.
612 Ye H, Li X, Zheng T, Hu C, Pan Z, Huang J, Li J, Li W, and Zheng Y. 2017. The Hippo Signaling Pathway
613 Regulates Ovarian Function via the Proliferation of Ovarian Germline Stem Cells. *Cellular Physiology &*
614 *Biochemistry International Journal of Experimental Cellular Physiology Biochemistry & Pharmacology*
615 41:1051.
616 Young MD, Wakefield MJ, Smyth GK, and Oshlack A. 2012. goseq: Gene Ontology testing for RNA-seq datasets.
617 Zhang DX, Park WJ, Sun SC, Xu YN, Li YH, Cui XS, and Kim NH. 2011. Regulation of maternal gene expression
618 by MEK/MAPK and MPF signaling in porcine oocytes during in vitro meiotic maturation. *Journal of*
619 *Reproduction & Development* 57:49.
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Figure 1(on next page)

The cluster heat map of expression profiles of lncRNAs, mRNAs and miRNAs at different status during ovary activation and oviposition regulation.

A. The cluster heat map of expression profiles of lncRNAs; B. The cluster heat map of expression profiles of mRNAs; C. The cluster heat map of expression profiles of miRNAs.

V, group of virgin queens (n=3); Q, group of egg-laying queens (n=3); C, group of egg-laying inhibited queens (n=3); R, group of egg-laying recovery queens (n=3).

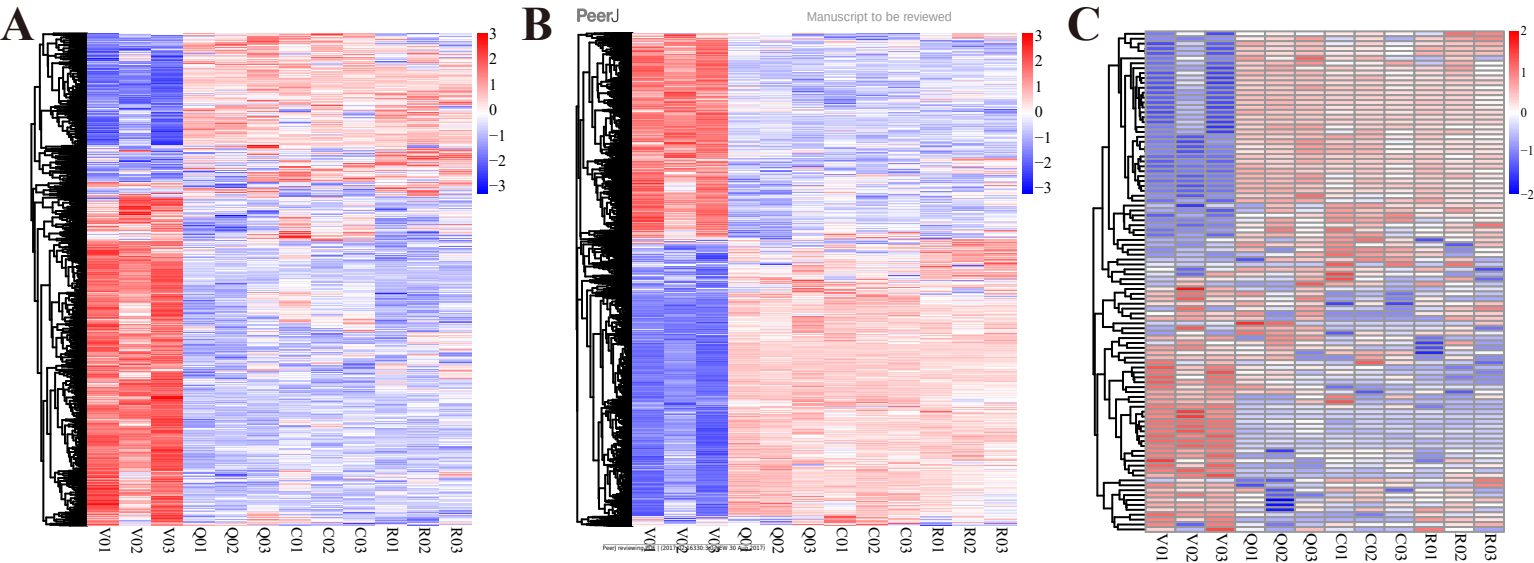
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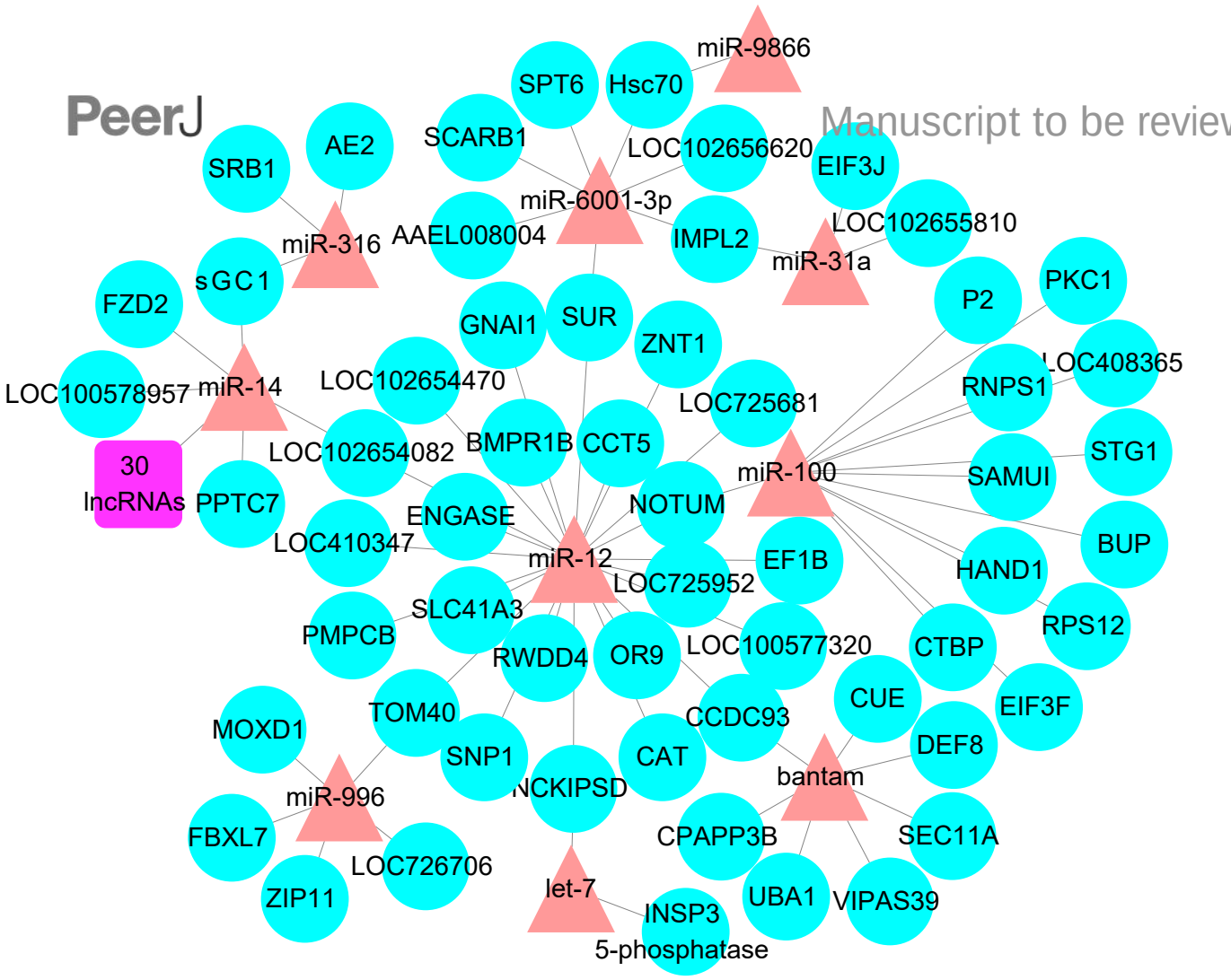
Figure 2(on next page)

The reproductive associated lncRNA-miRNA-mRNA network.

The network was constructed with DE lncRNAs, DE miRNAs and DE mRNAs which have known or suspected roles in reproduction and/or located in the QTL region for ovary size. Purple square nodes represent lncRNAs. Red triangle nodes represent miRNAs. Blue circle nodes represent mRNAs.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SPT6
Hsc70
SCARB1
LOC102656620
EIF3J
AE2
SRB1
miR-6001-3p
LOC102655810
miR-316
AAEL008004
IMPL2
miR-31a
sGC1
PKC1
FZD2
P2
GNAI1
SUR
ZNT1
LOC408365
LOC100578957
miR-14
LOC102654470
RNPS1
LOC725681
STG1
LOC102654082
BMPR1B
CCT5
miR-100
SAMUI
30
lncRNAs
PPTC7
NOTUM
ENGASE
BUP
LOC410347
miR-12
EF1B
HAND1
LOC725952
SLC41A3
RPS12
PMPCB
RWDD4
OR9
LOC100577320
CTBP
CUE
EIF3F
TOM40
CCDC93
MOXD1
DEF8
SNP1
CAT
bantam
miR-996
NCKIPSD
FBXL7
CPAPP3B
SEC11A
LOC726706
UBA1
VIPAS39
ZIP11
let-7
INSP3
5-phosphatase
PRKDC
CAD
EXOC6B
miR-252a
L(2)EFL
ALAD
NADPME
SRSF1B
miR-125
NFYA
NUBP2
miR-6059
1
lncRNA
miR-2796
miR-10
U5-SNRNP
miR-1
UNC79
MPPE1
miR-9884
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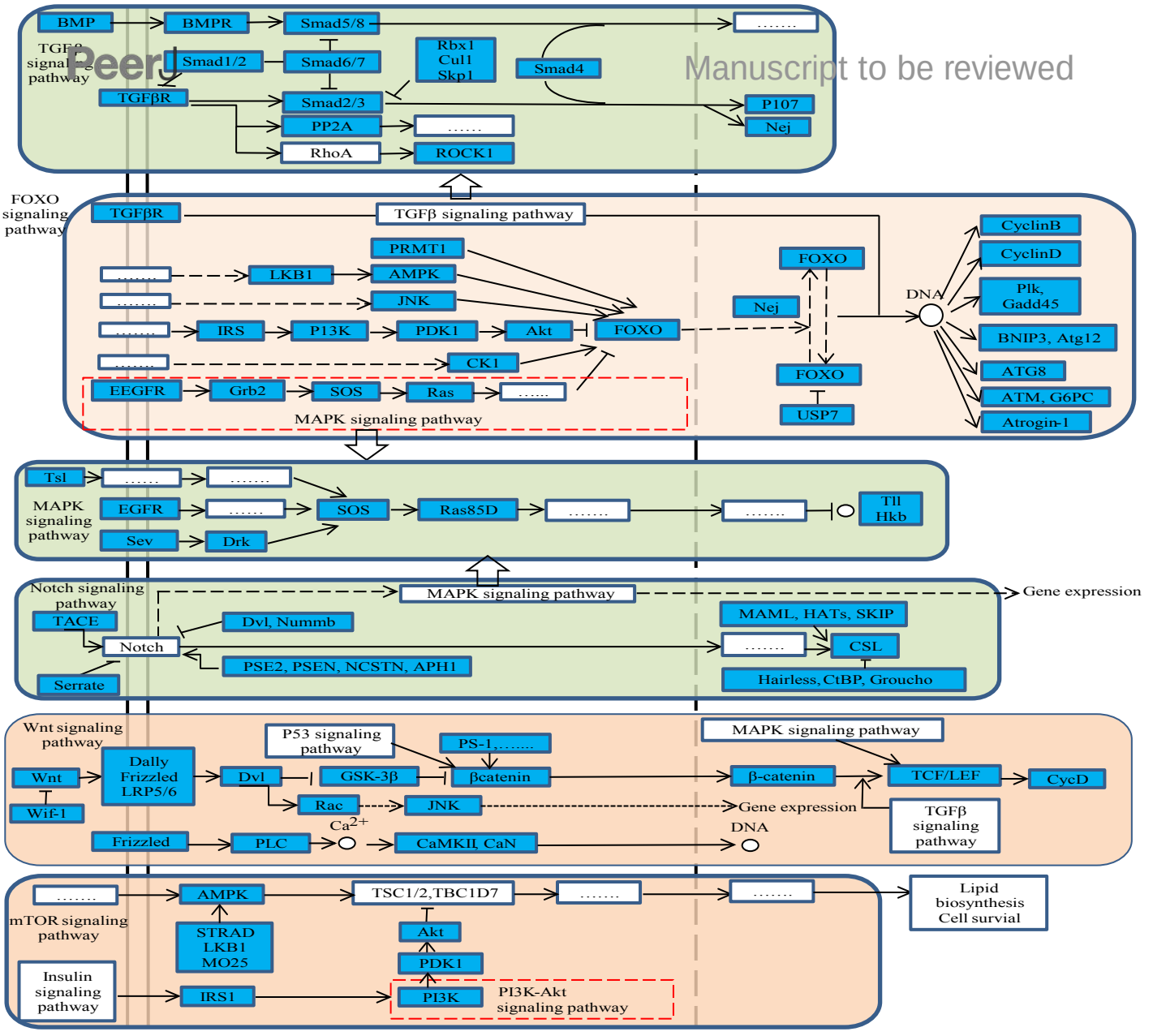
lncRNAs
miR-184
ADAMTS2
miR-92a
4
lncRNAs
miR-315
6
lncRNAs
miR-9a
LRRC24
miR-306
16
lncRNAs
miR-71
7
lncRNAs
miR-3718a
32
lncRNAs
miR-33

Figure 3(on next page)

The representative enriched pathways map.

DE genes were marked with blue color. Genes without color and "……" stand for genes that involved in the pathway but not differentially expressed in our results.



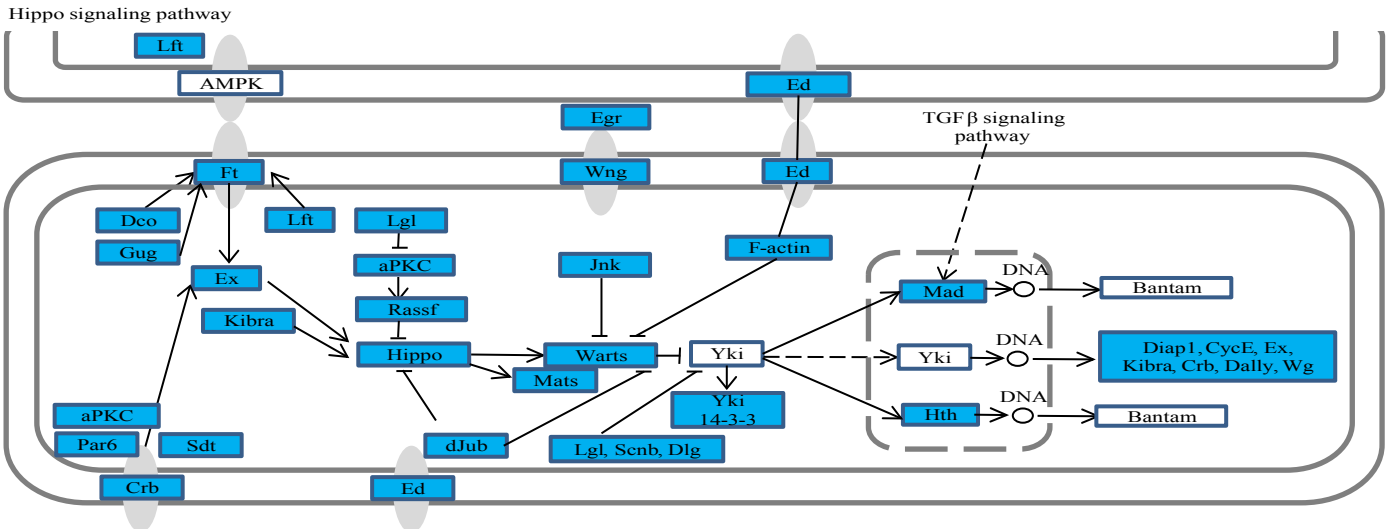


Table 1(on next page)

Number of differentially expressed coding and non-coding RNAs identified from each comparison.

Number of differentially expressed RNAs	Ovary activation (Egg-laying queens compared with virgin queens)		Oviposition inhibition (Egg-laying inhibited queens compared with egg-laying queens)		Oviposition recovery (Egg-laying recovery queens compared with egg-laying inhibited queens)	
	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated
mRNAs	3218	2263	266	72	256	241
lncRNAs	224	516	57	31	40	60
miRNAs	39	42	9	4	2	2

1

Table 2(on next page)

Intersection set of significantly enriched pathways with DE lncRNAs, DE mRNAs and DE miRNAs.

Note: V, group of virgin queens; Q, group of egg-laying queens; C, group of egg-laying inhibited queens; R, group of egg-laying recovery queens.

Significantly enriched pathways	Q_V	C_Q	R_C	Significantly enriched pathways	Q_V	C_Q	R_C
Arginine and proline metabolism		√	√	mTOR signaling pathway	√	√	√
Base excision repair	√		√	Mucin type O-Glycan biosynthesis	√		
Biosynthesis of amino acids	√	√		Neuroactive ligand-receptor interaction	√	√	√
Circadian rhythm - fly	√	√	√	N-Glycan biosynthesis	√	√	
Cysteine and methionine metabolism	√	√		Nitrogen metabolism	√	√	
Dorso-ventral axis formation	√	√	√	Notch signaling pathway	√	√	√
Drug metabolism - other enzymes	√	√		Other glycan degradation			
ECM-receptor interaction	√	√		Peroxisome	√		
Endocytosis	√	√		Phenylalanine metabolism	√	√	
Folate biosynthesis		√		Phosphatidylinositol signaling system	√	√	√
FoxO signaling pathway	√	√	√	Phototransduction - fly	√	√	√
Galactose metabolism	√			Proteasome	√	√	
Glycerolipid metabolism	√	√	√	Protein processing in endoplasmic reticulum		√	
Glycerophospholipid metabolism	√	√	√	Purine metabolism	√	√	√
Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate		√		Retinol metabolism		√	
Glycosaminoglycan biosynthesis - heparan sulfate / heparin		√	√	RNA degradation	√	√	√
Hedgehog signaling pathway	√	√		RNA transport	√	√	√
Hippo signaling pathway - fly	√	√	√	Spliceosome	√	√	√
Inositol phosphate metabolism	√	√	√	Starch and sucrose metabolism	√		
Jak-STAT signaling pathway			√	Sulfur metabolism	√		
Lysine degradation	√			TGF-beta signaling pathway	√	√	√
MAPK signaling pathway - fly	√	√	√	Tyrosine metabolism	√	√	
Metabolic pathways	√	√	√	Ubiquinone and other terpenoid-quinone biosynthesis	√	√	
mRNA surveillance pathway	√	√	√	Wnt signaling pathway	√	√	√

1

Table 3(on next page)

Analysis of DE genes with known or suspected roles in honey bee reproduction or related process.

V, group of virgin queens (n=3); Q, group of egg-laying queens (n=3); C, group of egg-laying inhibited queens (n=3); R, group of egg-laying recovery queens (n=3).

Gene id	Gene name	Correlate	*Ref.*	Expression level in this study		
				Q *vs.* V	C *vs.* Q	R *vs.* C
408961	Apolipophorins (known as *RFABP*)	*JH* response genes	(Pandey & Bloch 2015)	Down-regulated	Up-regulated	Down-regulated
552515	ATP-dependent RNA helicase WM6 (known as Helicase at 25E)	Higher expressed in ovaries of queen larvae compared with worker larvae in fourth and early fifth larvae	(Lago et al. 2016)	Up-regulated	Down-regulated but not significantly	Up-regulated but not significantly
408827	Carbonic anhydrase 1 (*CAH1*)	*JH* response genes	(Pandey & Bloch 2015)	Down-regulated	Up-regulated but not significantly	Down-regulated but not significantly
413762	Complement component 1 Q subcomponent-binding protein, mitochondrial	Higher expressed in ovaries of queen larvae compared with worker larvae in fourth and early fifth larvae	(Lago et al. 2016)	Down-regulated	Down-regulated but not significantly	Up-regulated
726690	Cytochrome P450 6AS3 (*CYP6AS3*)	Up-regulated in the *EcR* knock down bees	(Mello et al. 2014)	Down-regulated	Up-regulated	Down-regulated but not significantly
412209	Cytochrome P450 6AS4 (*CYP6AS4*)	Up-regulated in the *EcR* knock down bees	(Mello et al. 2014)	Down-regulated	Up-regulated	Down-regulated
409677	Cytochrome P450 6AS5 (*CYP6AS5*)	Up-regulated in the *EcR* knock down bees	(Mello et al. 2014)	Down-regulated	Up-regulated	Down-regulated
551560	Cytochrome P450 6BD1 (*CYP6BD1*)	Up-regulated in the *EcR* knock down bees	(Mello et al. 2014)	Down-regulated	Up-regulated	Down-regulated
411057	Cytochrome P450 314 A1 (*CYP314A1*)	coded for *Ec*dysone 20-hydroxylase	(Rewitz et al. 2006)	Up-regulated	No change	Down-regulated but not significantly
406143	Defensin 1 (*Def1*)	Up-regulated in mated queens compared with virgin queens	(Manfredini et al. 2015)	Down-regulated	Up-regulated	Down-regulated
406070	Dopamine r*ec*eptor 2 (*Dopr2)*	*Ec* response genes	(Rewitz et al. 2006)	Down-regulated	No change	Up-regulated
410309	*Ec*dysone-induced protein 75 (*E75*)	*Ec* response genes	(Rewitz et al. 2006)	Up-regulated	Up-regulated but not significantly	No change
406084	*Ec*dysone r*ec*eptor (*EcR*)	*Ec* response genes	(Rewitz et	Up-regulated	Not det*ec*ted	Not det*ec*ted

			al. 2006)			
408758	*Ec*dysteroid-regulated gene E74 (*E74*)	*Ec* response genes	(Rewitz et al. 2006)	Up-regulated	No change	No change
409384	Heat shock protein 60 (*Hsp60*)	Higher expressed in ovaries of queen larvae compared with worker larvae in fourth and early fifth larvae	(Lago et al. 2016)	Up-regulated but not significantly	Down-regulated	Up-regulated
408928	Heat shock protein 90 (*Hsp90*)	Higher expressed in ovaries of queen larvae compared with worker larvae in fourth and early fifth larvae; candidate marker genes for caste-sp*ec*ific ovary development;	(Lago et al. 2016)	Up-regulated	Down-regulated	Up-regulated
408818	Hexokinase (*HK*)	*QMP* response genes	(Hoover et al. 2003)	Up-regulated	No change	Down-regulated but not significantly
406117	Hexamerin 70b (*Hex70b*)	*JH* response gene, highly expressed in fourth and early fifth-instar queen ovaries	(Lago et al. 2016)	Up-regulated	Up-regulated but not significantly	Down-regulated but not significantly
726542	Histone H3	*QMP* response genes; overrepresented in ovaries of queens in the fifth larval instar	(Humann & Hartfelder 2011)	Down-regulated	No change	Down-regulated but not significantly
102655073	Histone H4	*QMP* response genes	(Hoover et al. 2003)	Up-regulated	Up-regulated but not significantly	Down-regulated but not significantly
726965	*JH*-inducible protein	*JH* and *Ec* response gene, up-regulated in *Ec* knock down bess	(Mello et al. 2014)	Up-regulated	Down-regulated but not significantly	No change
100576395	Kruppel homolog 1 (*Kr-h1*)	an immediate response gene in the *JH* response cascade	(Lago et al. 2016)	Up-regulated	Down-regulated but not significantly	Up-regulated but not significantly
406121	Major royal jelly protein 3 (Mrjp3)	*Ec* response gene; down-regulated in *Ec* knock down bees	(Mello et al. 2014)	Down-regulated	Up-regulated but not significantly	Down-regulated but not significantly
409870	Minor histocompatibility antigen H13	Higher expressed in ovaries of queen larvae compared with worker larvae in	(Lago et al. 2016)	Up-regulated	Down-regulated but not significantly	No change

		fourth and early fifth larvae				
411820	Mitogen-activated protein kinase phosphatase-3 (*Mapk-3*)	Higher expressed in ovaries of queen larvae compared with worker larvae in fourth and early fifth larvae	(Lago et al. 2016)	Down-regulated	Up-regulated but not significantly	Up-regulated but not significantly
408572	Myophilin (*CHD64*)	*JH* response genes	(Rewitz et al. 2006)	Down-regulated	Down-regulated but not significantly	Up-regulated but not significantly
409881	Myosin regulatory light chain 2 (*Mlc2*)	Up-regulated in mated queens compared with virgin queens	(Manfredini et al. 2015)	Down-regulated	Down-regulated	Up-regulated but not significantly
552193	Proton-coupled amino acid transporter	*QMP* response genes	(Hoover et al. 2003)	Up-regulated	No change	Down-regulated but not significantly
409681	RWD domain-containing protein 1 (*RWDD1*)	*QMP* response genes	(Hoover et al. 2003)	Up-regulated	Down-regulated but not significantly	Down-regulated but not significantly
409227	Ultraspiracle (*USP*)	*Ec* and *JH* response genes	(Rewitz et al. 2006)	Up-regulated but not significantly	Down-regulated but not significantly	Up-regulated
406088	Vitellogenin (*Vg*)	The protein product serves as a yolk precursor in egg development	(Nunes et al. 2013)	Down-regulated	Up-regulated	Down-regulated

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Table 4(on next page)

Analysis of DE miRNAs with known or suspected roles in honey bee reproduction or related process.

V, group of virgin queens (n=3); Q, group of egg-laying queens (n=3); C, group of egg-laying inhibited queens (n=3); R, group of egg-laying recovery queens (n=3).

miRNA id	Correlate	*Ref.*	Expression level in this study		
			Q *vs.* V	C *vs.* Q	R *vs.* C
Bantam	Caste determination; target of hippo and EGFR/MAPK signaling pathways; critical in embryonic development and the control of cell proliferation and survival; up-regulated in 4-day-old queen larvae compared with 4-day-old worker larvae; related to insulin and Wnt pathway.	(Ashby et al. 2016; Shi et al. 2015)	Down-regulated	Up-regulated but not significantly	Down-regulated but not significantly
Let-7	Caste determination; major target of steroid pathways; miRNA markers associated with the behavioural shift of worker bees from nurses to forages; immune-related; *Vg* positive correlation; participated in regulation of behavioral maturation in honey bees; associated with reproductive statuses; up-regulated in the inactive ovaries; up-regulated in 4-day-old queen larvae; related to Wnt pathway; down-regulated in *Ec* knock down bees	(Ashby et al. 2016; Macedo et al. 2016; Mello et al. 2014; Nunes et al. 2013; Shi et al. 2015)	Down-regulated	No change	Up-regulated but not significantly
Ame-mir-1	*Vg* positive correlation; associated with reproductive statuses; up-regulated in the inactive ovaries; down-regulated in *Ec* knock down bees	(Macedo et al. 2016; Mello et al. 2014; Nunes et al. 2013)	Down-regulated	No change	Down-regulated but not significantly
Ame-mir-10	Up-regulated in 4-day-old queen larvae	(Shi et al. 2015)	Up-regulated	Down-regulated	Up-regulated but not significantly
Ame-mir-100	20E and JH response miRNA; caste determination; associated with reproductive statuses; up-regulated in the inactive ovaries	(Ashby et al. 2016; Macedo et al. 2016)	Down-regulated	Down-regulated but not significantly	Up-regulated but not significantly
Ame-mir-12	Associated with reproductive statuses; up-regulated in 4-day-old queen larvae compared with 4-day-old worker larvae; related to insulin and MAPK pathway; down-regulated in *Ec* knock down bees	(Macedo et al. 2016; Mello et al. 2014; Shi et al. 2015)	Down-regulated but not significantly	Up-regulated	Down-regulated but not significantly
Ame-mir-125	20E and JH response miRNA; caste determination; up-regulated in the inactive ovaries; up-regulated in 4-day-old queen larvae compared with	(Ashby et al. 2016; Macedo et	Down-regulated	Down-regulated but not	Up-regulated but not significantly

	4-day-old worker larvae; related to insulin, MAPK and mTOR pathway	al. 2016; Shi et al. 2015)		significantly	
Ame-mir-133	Associated with the lipid loss in bees; participated in regulation of behavioral maturation in honey bees; up-regulated in 4-day-old queen larvae; related with MAPK pathway; down-regulated in *Ec* knock down bees	(Mello et al. 2014; Nunes et al. 2013; Shi et al. 2015)	Down-regulated	No change	Down-regulated but not significantly
Ame-mir-14	Caste determination; negatively related with *Ec*R expression and activity; up-regulated in 4-day-old queen larvae compared with 4-day-old worker larvae; related to insulin, MAPK, mTOR and Wnt pathway; down-regulated in *Ec* knock down bees; up-regulated in the activated ovaries	(Ashby et al. 2016; Macedo et al. 2016; Mello et al. 2014; Shi et al. 2015)	Down-regulated	No change	Up-regulated but not significantly
Ame-mir-184	Stable expression in active and inactive ovary; plays key roles in embryogenesis; the determination of the anteroposterior axis; embryo cellularization and stem cell determination; up-regulated in 4-day-old queen larvae compared with 4-day-old worker larvae; related to insulin pathway; down-regulated in *Ec* knock down bees; a miRNA in royal jelly and aff*ec*t caste determination	(Guo et al. 2013; Macedo et al. 2016; Mello et al. 2014; Shi et al. 2015)	Down-regulated	No change	Down-regulated but not significantly
Ame-mir-190	Caste determination; immune-related	(Ashby et al. 2016)	Down-regulated	No change	Down-regulated but not significantly
Ame-mir-252a	Up-regulated in 4-day-old queen larvae; up-regulated in the activated ovaries	(Macedo et al. 2016; Shi et al. 2015)	Down-regulated	Down-regulated but not significantly	Up-regulated but not significantly
miR-263a	Associated with reproductive statuses; up-regulated in the inactive ovaries; down-regulated in *Ec* knock down bees	(Macedo et al. 2016; Mello et al. 2014)	Up-regulated	Down-regulated but not significantly	Up-regulated but not significantly
Ame-mir-275	*Vg* positive correlation; up-regulated in 4-day-old queen larvae; related to insulin and MAPK pathway	(Nunes et al. 2013; Shi et al. 2015)	Down-regulated	Up-regulated	Up-regulated but not significantly

Ame-mir-276	Associated with reproductive statuses; up-regulated in the inactive ovaries; down-regulated in *Ec* knock down bee; up-regulated in 4-day-old queen larvae compared with 4-day-old worker larvae	(Macedo et al. 2016; Mello et al. 2014; Shi et al. 2015)	Down-regulated	Up-regulated	No change
Ame-mir-279	Caste determination; immune-related; down-regulated in *Ec* knock down bees	(Ashby et al. 2016)	Up-regulated	No change	Down-regulated but not significantly
Ame-mir-2796	Participated in regulation of behavioral maturation in honey bees	(Nunes et al. 2013)	Down-regulated	Down-regulated but not significantly	Down-regulated but not significantly
Ame-mir-279a-3p	Up-regulated in the activated ovaries	(Macedo et al. 2016)	Down-regulated	Up-regulated but not significantly	Down-regulated but not significantly
Ame-mir-279b-3p	Up-regulated in the activated ovaries	(Macedo et al. 2016)	Up-regulated	No change	Down-regulated but not significantly
Ame-mir-2944-3p	Up-regulated in the activated ovaries	(Macedo et al. 2016)	Up-regulated	Down-regulated but not significantly	Up-regulated but not significantly
Ame-mir-305	Down-regulated in *Ec* knock down bees	(Mello et al. 2014)	Down-regulated but not significantly	Up-regulated	Up-regulated but not significantly
Ame-mir-306	Associated with reproductive statuses; up-regulated in the activated ovaries; targets ATPsyn-beta-PA; down-regulated in *Ec* knock down bees	(Macedo et al. 2016; Mello et al. 2014)	Up-regulated	Down-regulated but not significantly	Down-regulated but not significantly
Ame-mir-315	Caste determination; modulates tissue patterning and cell differentiation	(Ashby et al. 2016)	Up-regulated	Down-regulated but not significantly	Down-regulated but not significantly
Ame-mir-316	*Vg* negative correlation; related to Wnt pathway; down-regulated in *Ec*	(Mello et al.	Down-regulated	Up-regulated but	No change

	knock down bees	2014; Nunes et al. 2013)		not significantly	
Ame-mir-317	Associated with reproductive statuses; up-regulated in the activated ovaries; related to insulin pathway; down-regulated in *Ec* knock down bees	(Macedo et al. 2016; Mello et al. 2014)	Down-regulated	Up-regulated but not significantly	No change
Ame-mir-31a	*Vg* negative correlation; associated with reproductive statuses; up-regulated in the inactive ovaries	(Macedo et al. 2016; Nunes et al. 2013)	Down-regulated	Up-regulated but not significantly	Down-regulated but not significantly
Ame-mir-33	Caste determination; immune-related	(Ashby et al. 2016)	Down-regulated	Up-regulated but not significantly	No change
Ame-mir-3718a	*Vg* negative correlation	(Nunes et al. 2013)	Down-regulated	No change	Up-regulated but not significantly
Ame-mir-375	Up-regulated in 4-day-old queen larvae; related to MAPK pathway	(Shi et al. 2015)	Down-regulated	No change	Up-regulated but not significantly
Ame-mir-6001-3p	Up-regulated in 4-day-old queen larvae	(Shi et al. 2015)	Down-regulated	Not detected	Up-regulated but not significantly
Ame-mir-71	Participates in sp*ec*ific steps of the insulin/insulin-like signaling pathway	(Macedo et al. 2016)	Down-regulated but not significantly	Up-regulated	Up-regulated but not significantly
Ame-mir-8	Caste determination; immune-related; 20E and JH response miRNA; related to Wnt pathway; up-regulated in the activated ovaries	(Ashby et al. 2016; Macedo et al. 2016; Shi et al. 2015)	Down-regulated	Up-regulated	No change
Ame-mir-92a	*Vg* negative correlation; participated in regulation of behavioral maturation in honey bees; associated with reproductive statuses; down-regulated in *Ec* knock down bees	(Macedo et al. 2016; Mello et al. 2014; Nunes et al. 2013)	Up-regulated	Down-regulated but not significantly	No change
Ame-mir-92b	Up-regulated in the activated ovaries; related to insulin, MAPK and mTOR pathway; down-regulated in *Ec* knock down bees	(Macedo et al. 2016; Mello et al.	Up-regulated	Down-regulated	Up-regulated but not significantly

		2014; Shi et al. 2015)			
Ame-mir-993	Related to insulin pathway	(Shi et al. 2015)	Down-regulated	Up-regulated but not significantly	Down-regulated but not significantly
Ame-mir-996	Related to insulin pathway	(Shi et al. 2015)	Down-regulated but not significantly	Up-regulated	Down-regulated but not significantly

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