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Transcriptome comparison between inactivated and activated ovaries of the honey bee *Apis mellifera* L.

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Abstract

Ovarian activity not only influences fertility, but is also involved with the regulation of division of labour between reproductive and behavioural castes of female honey bees. In order to identify candidate genes associated with ovarian activity, we compared the gene expression patterns between inactivated and activated ovaries of queens and workers by means of high-throughput RNA-sequencing technology. A total of 1615 differentially expressed genes (DEGs) was detected between ovaries of virgin and mated queens, and more than 5300 DEGs were detected between inactivated and activated worker ovaries. Intersection analysis of DEGs amongst five libraries revealed that a similar set of genes (824) participated in the ovary activation of both queens and workers. A large number of these DEGs were predominantly related to cellular, cell and cell part, binding, biological regulation and metabolic processes. In addition, over 1000 DEGs were linked to more than 230 components of Kyoto Encyclopedia of Genes and Genomes pathways, including 25 signalling pathways. The reliability of the RNA-sequencing results was confirmed by means of quantitative real-time PCR. Our results provide new insights into the molecular mechanisms involved in ovary activation and reproductive division of labour.

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Introduction

A fundamental goal of sociobiology is to explain the regulation of complex social behaviour (Robinson *et al.*, 2005), especially in social insects. As eusocial insects, the honey bees (*Apis mellifera* L.) are characterized by the extreme reproductive division of labour (Michener, 1974). Although the queen and worker develop from the same genome, their reproductive functions exhibit significant differences. The queen monopolizes reproduction, whereas sterile workers have lost the ability to lay eggs in the presence of a queen, performing all the tasks related to colony maintenance (Winston, 1987). The onset of ovary activation is tightly regulated in queens. Five to 10 days after emergence, queens reach sexual maturity and initiate mating flights, during which the queen will mate with an average of 12 drones (Tarpy *et al.*, 2004). Once the queen completes the mating process, her ovaries become fully activated and she initiates egg laying (Tanaka & Hartfelder, 2004).

Insect ovaries are composed of functional units called ovarioles, which contain sequentially developing egg chambers (Büning, 1994). In the honey bee, the number of ovarioles varies between and within castes, with workers having two to 26 and queens having 100–180 ovarioles per ovary (Snodgrass, 1956; Sakagami & Akahira, 1958; Jackson *et al.*, 2011). This difference arises primarily during the final larval instars in which ovariole number in workers is reduced (Reginato & Cruz-Landim, 2001). Activated ovarioles are noticeably swollen and are characterized by having eggs in successive stages of development: the extreme upper part contains multinucleate protoplasmic mass in which cell boundaries are not clearly defined, whereas the lower part contains defined oocytes (eggs), trophocytes (nurse cells) and follicle cells (Snodgrass, 1956; King & Büning, 1985). Inactivated ovaries, by contrast, are composed of thin ovarioles that lack mature eggs (Snodgrass, 1956).

Workers are functionally sterile but retain vestigial ovaries that can be activated under certain conditions. When the colony loses its queen and fails to rear a new queen, 5–24% of workers can activate their ovaries and lay unfertilized eggs that will develop into males (Miller & Ratnieks, 2001). Worker reproductive traits greatly differ amongst different subspecies of *A. mellifera*. Workers of the South African honey bee *Apis mellifera capensis* are thelytokous and therefore produce diploid female offspring from unfertilized eggs (Anderson, 1963; Verma & Ruttner, 1983; Beekman *et al*., 2009). Variation in worker reproductive traits also occurs in populations of European honey bees although it is restricted to the production of haploid males. These variations have a genetically inherited component as strains with different ovarian activity have been selected from wild-type populations (Barron & Oldroyd, 2001; Châline *et al*., 2002; Holmes *et al*., 2013). An extreme example is the mutant strain of 'anarchistic' bees where workers are reproductively active even in the presence of the queen (Oldroyd *et al*., 1994; Thompson *et al*., 2006; Oldroyd & Beekman, 2008).

Ovarian activity influences the mechanisms of food collection and division of labour of honey bees. Selected strains of workers with larger ovaries and higher titres of vitellogenin (Vg) show a pollen foraging bias and are more likely to forage at younger ages compared with workers with smaller ovaries and lower titres of Vg (Amdam *et al*., 2004). Studies with mutant 'anarchist' bees have confirmed the association amongst ovarian activity, Vg expression and division of labour; however, it was found to have an inverse effect on behavioural development: anarchist bees with active ovaries forage later in life compared with wild-type honey bees (Thompson *et al*., 2006; Oldroyd & Beekman, 2008). As these experiments were performed with selected strains with different genetic backgrounds, the use of wild-type bees is expected to clarify the relationship between ovarian activity and behavioural development (Tsuruda *et al*., 2008).

Several studies have been conducted with the aim of identifying candidate genes associated with workers' reproductive status (Grozinger *et al*., 2007; Oxley *et al*., 2008; Thompson *et al*., 2008). In particular, studies comparing expression patterns between anarchist and functionally sterile wild-type workers have identified a large number of genes differentially expressed between these reproductive phenotypes (Thompson *et al*., 2006, 2008).

During the transition from a virgin queen to a fully mated queen, there are large-scale transcriptional changes in the brain and the ovary (Kocher *et al*., 2008; Niño *et al*., 2011, 2013; Vergoz *et al*., 2012a). Interestingly, postmating changes in gene regulation in the brain and the ovary were uncoupled: the initiation of mating triggers immediate changes in the ovary, whereas changes in the brain may require additional stimuli or take a longer time to complete (Kocher *et al*., 2010; Vergoz *et al*., 2012a). In an effort to uncover genes that are associated with ovary activation in both queens and workers, a study explored differences in gene expression in brains of same-aged virgin queens, sterile workers and reproductive workers (Grozinger *et al*., 2007). The results showed that there were about 2000 differently expressed genes between queen and workers and only 221 genes between sterile and reproductive workers. The expression pattern of the genes associated with reproduction in workers resembles those of queens and may represent a core group of genes associated with reproductive physiology.

With a complete genomic sequence for the honey bee *A. mellifera*, microarray and quantitative real-time PCR (qRT-PCR) technologies have been used for screening the genes that are involved in ovary activation and/or reproduction. Studies investigating expression of selected candidate genes in abdomens/ovaries of queens and workers by means of qRT-PCR found that biogenic amine receptors were affected by their reproductive status and showed differential expression in the same direction in both female castes (Thompson *et al*., 2007; Brito *et al*., 2010; Vergoz *et al*., 2012a, b), suggesting that ovary activation of queens and workers is regulated by similar genetic pathways. However, a deep sequencing approach would extensively extend the known repertoire of genes and molecular pathways.

In this study, we used the high-throughput RNA-sequencing technology to describe gene expression differences between inactivated and activated ovaries of queens and workers. This is the first report comparing gene expression patterns between inactivated and activated ovaries of queens and workers using high-throughput gene expression technology. Whole ovaries (composed of oocytes, trophocytes and follicle cells; Snodgrass, 1956), were used to prepare five mRNA libraries: inactivated ovaries of virgin queens (VQI), activated ovaries of egg-laying queens (EQA), inactivated ovaries of queenright workers (WIQR), inactivated ovaries of queenless workers (WIQL) and activated ovaries of queenless workers (WAQL). All of the differentially expressed genes (DEGs) between inactivated and activated ovaries of queens and workers were then subjected to gene ontology (GO) category and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. Our results reveal that ovarian activation in queens and potentially reproductive workers is associated with the regulation of common genetic pathways. In addition, our study demonstrates that the use of deep RNA-sequencing technology on dissected ovaries provides a powerful method to get insights into the molecular mechanisms involved in honey bee ovarian function and reproduction division of labour.

Table 1. Summary of read numbers based on the RNA-sequencing data from five ovary libraries

Category	VQI	EQA	WIQR	WIQL	WAQL
Raw reads	7248198	7086698	8436752	8342893	8778449
Clean reads	7079017	6755666	8320697	8219489	8666446
Mapped reads	6064466 (85.67%)	5795539 (85.79%)	6846587 (82.28%)	6776750 (82.45%)	7199653 (83.08%)
Perfect match	5188382 (73.29%)	5045414 (74.68%)	5543990 (66.63%)	5647115 (68.70%)	6006554 (69.31%)
≤2 bp mismatch	876084 (12.38%)	750125 (11.10%)	1302597 (15.65%)	1129635 (13.74%)	1193099 (13.77%)
Unique match	5904315 (83.41%)	5670011 (83.93%)	6726531 (80.84%)	6698340 (81.49%)	7136983 (82.35%)
Multiposition match	160151 (2.26%)	125528 (1.86%)	120056 (1.44%)	78410 (0.95%)	62670 (0.72%)
Unmapped reads	1014551 (14.33%)	960127 (14.21%)	1474110 (17.72%)	1442739 (17.55%)	1466793 (16.92%)

EQA, activated ovaries of egg-laying queens; VQI, inactivated ovaries of virgin queens; WAQL, activated ovaries of queenless workers; WIQL, inactivated ovaries of queenless workers; WIQR, inactivated ovaries of queenright workers.

Results

Ovaries with the most extreme differences in activation states (fully inactivated and activated) were dissected from 11/12-day-old queens and 14-day-old workers. The following five libraries were constructed and used for RNA-sequencing analysis: 23 pairs of ovaries from VQI, 17 pairs from EQA, 1000 pairs from WIQR, 600 pairs from WIQL and 260 pairs from WAQL.

Raw data processing

Illumina HiSeq 2000 platform was used to identify DEGs amongst the five ovary libraries. More than 7.0 million raw reads per library were obtained and over 95.33% of these reads were identified as clean reads (Fig. S1) before they were mapped to the reference database (Table 1).

Of these total clean reads, more than 82.28% successfully matched to either unique or multiple locations of the honey bee genome; the remaining were unmatched. In addition, 66.63–74.68% of the clean reads mapped perfectly, whereas 11.10–15.65% of the clean reads had ≤2 bp mismatches (Table 1). We detected 9765 (VQI), 9314 (EQA), 10 107 (WIQR), 10 137 (WIQL) and 9739 (WAQL) expressed genes in these five libraries (unique reads number ≥3). There were 8824 genes expressed in all of the samples, but some genes were either exclusively or specifically expressed amongst the five samples (Fig. 1; File S1).

Gene coverage, the percentage of a gene covered by reads, was determined by the ratio of the number of bases in a gene covered by unique mapping reads to the total number of bases of that gene. The distribution of distinct reads over different read abundance categories showed similar patterns for all five RNA-sequencing libraries, and more than 58% of the total genes had gene coverage of greater than 50% (Fig. S2).

We performed a cluster analysis for a crosswise comparison of five DEG groups, including WIQR vs. VQI, WIQR vs. EQA, WIQR vs. WIQL and WIQR vs. WAQL. 7314 DEGs were classified into several expression cluster groups according to the similarity of their expression patterns (Fig. 2). Further inspection of these expression groups revealed diverse and complex patterns of regulation. These clusters contained genes positively or negatively modulated throughout ovary development, whereas genes expressed in the five groups showed certain differences. There were clusters with relatively minor differences between WIQR vs. VQI and WIQR vs. WAQL, but marked differences with WIQR vs. WIQL. The cluster between WIQR vs. EQA showed greatest differences, indicating that gene expression patterns in the queenless worker ovaries were more like those of VQI than those of the other DEG groups.

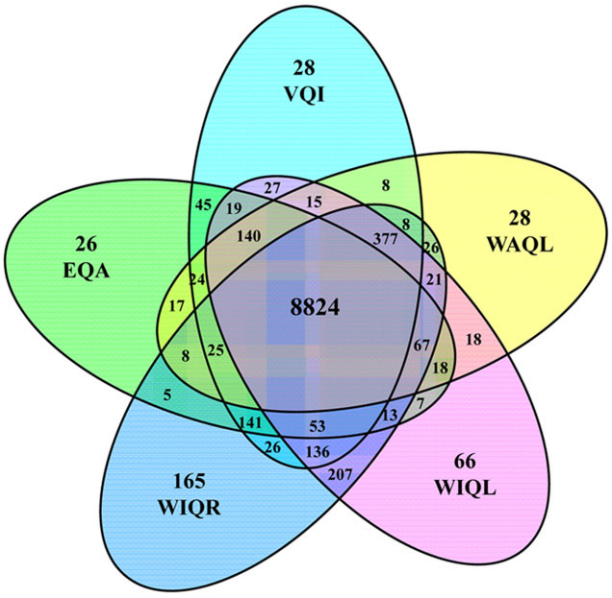

Figure 1. The number of genes expressed in inactivated ovaries of virgin queens (VQI), activated ovaries of egg-laying queens (EQA), inactivated ovaries of queenright workers (WIQR), inactivated ovaries of queenless workers (WIQL) and activated ovaries of queenless workers (WAQL). Numbers inside diagram stand for the gene number commonly and uniquely expressed amongst the five samples. The details are shown in File S1.

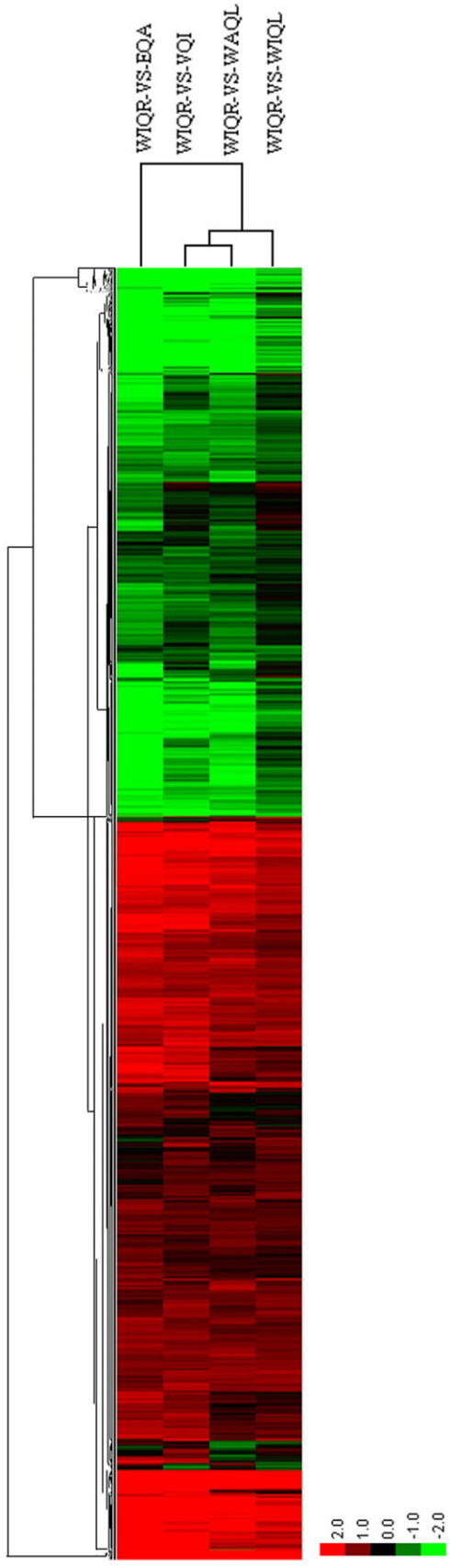

Figure 2. Crosswise comparison of the gene abundance in the five compared groups, including inactivated ovaries of queenright workers (WIQR) vs. inactivated ovaries of virgin queens (VQI), WIQR vs. activated ovaries of egg-laying queens (EQA), WIQR vs. inactivated ovaries of queenless workers (WIQL) and WIQR vs. activated ovaries of queenless workers (WAQL). The red colour represents up-regulated genes and the green colour represents down-regulated genes. The highest difference was found between WIQR vs. EQA and the lowest between WIQR vs. VQI and WIQR vs. WAQL.

Analysis of DEGs within castes

We used strict statistical criteria [false discovery rate (FDR) ≤ 0.001 and the absolute value of $\log_2$Ratio ≥ 1] to screen for genes that were differentially expressed at significant levels between two libraries. To identify changes in gene expression associated with ovary activation, we compared the VQI and EQA libraries. Our results showed 1615 DEGs, with 409 (25.33%) up-regulated and 1206 (74.68%) down-regulated in EQA (Fig. 3, File S2a).

To identify changes in gene expression associated with ovary activation in workers, we compared expression changes between WIQR vs. WIQL and WIQR vs. WAQL. A total of 4006 genes were considered significantly differentially expressed between the WIQR and WIQL libraries. From them, 2794 (69.7%) were up-regulated in WIQL, and the remaining 1212 (30.3%) were down-regulated (Fig. 3, File S2b). Between the WIQR and WAQL libraries, 5342 genes were significantly differentially expressed, with 2824 (53.0%) up-regulated and 2518 (47.3%) down-regulated genes in WAQL (Fig. 3, File S2c). The number of DEGs between WIQR and WAQL was higher than between WIQR and WIQL, and there were more DEGs up-regulated than down-regulated. To identify genes associated with ovary activation in workers of queenless colonies, we compared the WIQL and WAQL libraries. We found that 2104 genes were expressed differentially at significant levels, with 409 (19.4%) up-regulated and 1695 (80.6%) down-regulated in WAQL (Fig. 3, File S2d).

Genes associated with honey bee ovary activation

To further explore genes that are associated with ovary activation in honey bee queens and workers, overlap analysis of DEGs were performed amongst three groups: VQI vs. EQA, WIQR vs. WAQL and WIQL vs. WAQL. This analysis showed that 175 DEGs were up-regulated and 649 DEGs were down-regulated in both EQA and WAQL. The genes that were up-regulated or down-regulated by at least fivefold in activated ovaries relative to inactivated ovaries in both female castes are listed in Table S1.

Analysis of DEGs between castes

We identified the DEGs between queen and worker ovaries at the same developmental stage, namely inactivated and activated ovaries. In order to identify DEGs associated with inactivated ovaries of both castes, we analysed gene expression differences amongst the WIQR, WIQL and VQI libraries. 5441 genes were significantly differentially expressed between the WIQR and VQI libraries. 3346 (61.50%) DEGs were up-regulated, and the remaining 2095 (38.50%) were down-regulated in VQI (Fig. 4, File S3a). Between the WIQL and VQI libraries,

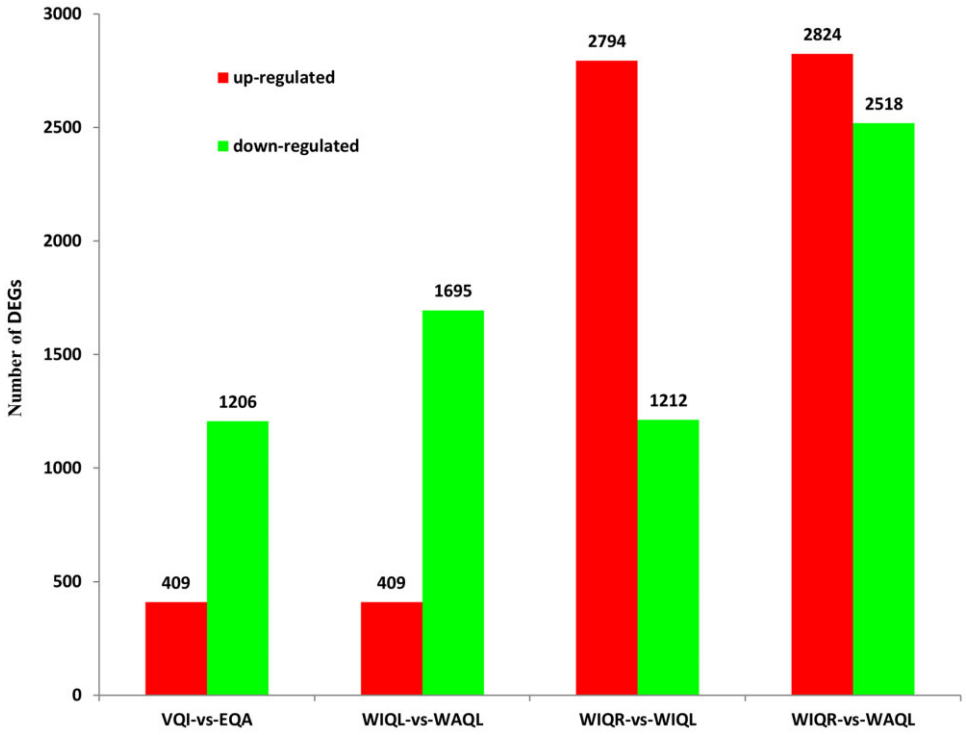

Figure 3. Numbers of differentially expressed genes (DEGs) (within castes) in the inactivated ovaries of virgin queens (VQI), activated ovaries of egg-laying queens (EQA), inactivated ovaries of queenright workers (WIQR), inactivated ovaries of queenless workers (WIQL) and activated ovaries of queenless workers (WAQL) libraries.

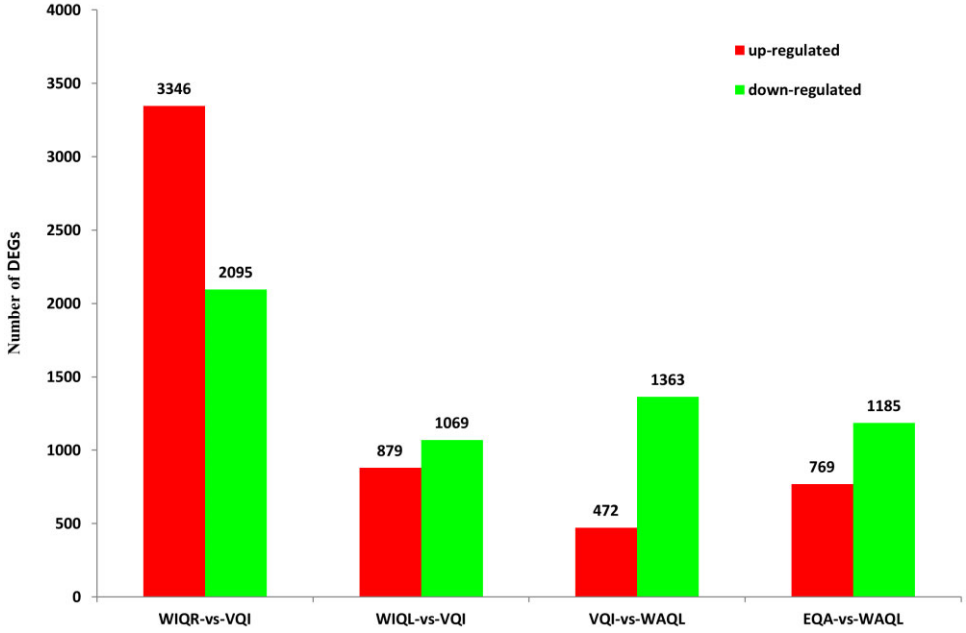

Figure 4. Numbers of differentially expressed genes (DEGs) (between castes) in the inactivated ovaries of virgin queens (VQI), activated ovaries of egg-laying queens (EQA), inactivated ovaries of queenright workers (WIQR), inactivated ovaries of queenless workers (WIQL) and activated ovaries of queenless workers (WAQL) libraries.

1948 genes were expressed differentially, with 879 (45.12%) up-regulated and 1069 (54.88%) down-regulated in VQI (Fig. 4, File S3b). There was a substantial amount of overlap between the genes that differed between VQI and both worker groups: 936 genes were significantly up-regulated in VQI vs. both worker groups, and 818 genes were significantly down-regulated (1754 genes total). These regulated genes may represent a core group of genes that are associated with ovarian inactivation.

In this study, the sets of genes that were significantly different between VQI and WAQL were compared with the sets of genes that were significantly different between EQA and WAQL. We found that 1835 genes were differentially expressed between the VQI and WAQL libraries, with 472 (25.7%) up-regulated and 1363 (74.3%) down-regulated genes in WAQL (Fig. 4, File S3c). When we compared the EQA and WAQL libraries, 1954 genes were expressed differentially, with 769 genes (39.36%) up-regulated and 1185 genes (60.64%) down-regulated in WAQL (Fig. 4, File S3d). Amongst these DEGs, 239 genes were significantly up-regulated in WAQL vs. both queen groups, and 603 genes were significantly down-regulated (842 genes total). This analysis revealed a core group of genes associated with ovarian activation in both female castes.

GO annotation of DEGs within castes

All of the DEGs within castes were subjected to GO functional annotation; GO terms were generated for 785, 745 and 2132 genes for VQI vs. EOA, WIQR vs. WAQL and WIQL vs. WAQL, respectively. These genes were divided into three categories: molecular function, cellular component and biological process. We can see that for all DEGs in each comparison group, the percentage of genes distributed to functional clusters were similar. There was significant overlap in the GO functional annotation of the identified DEGs between inactivated and activated ovaries. Amongst these groups, the terms cellular process, cell and cell part and binding were dominant in each of the three main categories (biological process, cellular component and molecular function), respectively. In terms of biological processes, GO analysis revealed an over-representation of the genes involved in cellular, metabolic, developmental, immune and reproduction processes as well as response to stimuli and cell proliferation. The details are shown in Table S2.

Pathway annotation of DEGs within castes

To understand the functions of the DEGs within castes, we mapped them using the KEGG database for signalling pathways analysis. More than 230 KEGG pathways were identified, including 25 signalling pathways, which included over 1000 DEGs. Specifically, 1062 DEGs were identified between VQI and EOA, 3792 between WIQR and WAQL, and 1401 between WIQL and WAQL. The top 10 most abundant differentially expressed signalling pathways from each comparison group (VQI vs. EOA, WIQR vs. WAQL, and WIQL vs. WAQL) are listed in Table S3. In both female ovaries, the cluster for metabolic pathways represented the largest group, which agreed with the previous notion that metabolic pathways have an important role in honey bee division of labour and behavioural maturation (Winston, 1987; Ament *et al.*, 2008). Differential expression of the genes involved in insect hormone biosynthesis may play important roles in ovary activation, as discussed below.

Confirmation of DEGs by qRT-PCR

Ten genes associated with reproduction, ecdysteroid synthesis and the target of rapamycin (TOR) signalling pathways were used for qRT-PCR validation in the five groups. These genes were differentially expressed between inactivated and activated ovaries (Fig. 5), consistent with the RNA-sequencing findings (Table S4). These results demonstrate the reliability of the RNA-sequencing performed here.

Discussion

In this study, we employed high-throughput RNA-sequencing transcriptional profiling to compare ovarian gene expression between virgin and mated queen bees as well as between inactivated and activated ovaries in worker bees. We identified common sets of genes associated with ovarian activation in queens and workers. Our findings show differential ovarian expression of genes encoding proteins involved in nutrition related-functions and hormone biosynthesis between different reproductive states. Our study demonstrates that the use of deep RNA-sequencing technology on dissected ovaries is a powerful method to investigate the molecular mechanisms involved in honey bee ovarian activation.

More than 6.7 million clean reads per library were generated, which corresponds to approximately 10 000 expressed genes. A comparative expression profiling strategy between inactivated and activated ovaries of *A. mellifera* queens and workers was used to identify a subset of genes that were differently expressed. The largest difference occurred between WIQR and WAQL, whereas the minimum difference occurred between VQI and EQA. This result suggests that worker transition from sterile to reproductive state is associated with higher changes in gene expression compared with queen transition from the nonlaying to egg-laying state. In addition, genes differentially expressed in VQI vs. EQA were similar

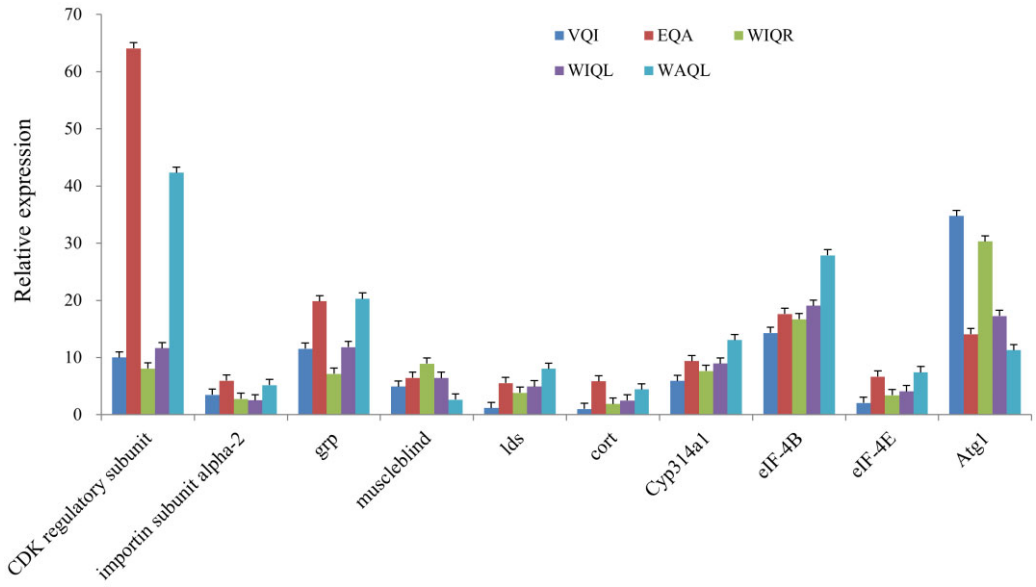

Figure 5. Quantitative real-time PCR confirmation of differentially expressed genes detected by RNA-sequencing. These genes are involved in the following processes and pathways: *CDK regulatory subunit*, *importin subunit alpha-2*, Serine/threonine-protein kinase grapes, transcript variant 2 (*grp*), *muscleblind*, transcription termination factor 2 (*lds*) and *cortex* (*cort*) in reproduction; *cytochrome P450 314A1* (*Cyp314a1*) in ecdysteroid biosynthesis; *eukaryotic translation initiation factor 4B* (*eIF-4B*), *eukaryotic translation initiation factor 4E* (*eIF-4E*) and *autophagy-specific gene 1* (*Atg1*) in the target of rapamycin pathway. EQA, activated ovaries of egg-laying queens; VQI, inactivated ovaries of virgin queens; WAQL, activated ovaries of queenless workers; WIQL, inactivated ovaries of queenless workers; WIQR, inactivated ovaries of queenright workers.

to WIQL vs. WAQL, indicating that a common set of core genes are involved in ovary activation in queens and workers. Altogether these comparisons suggest that although a common set of genes is involved in ovary activation in queens and workers, the presence of a queen is a major influence on worker gene expression.

Overlap analysis of DEGs was performed amongst three groups: VQI vs. EQA, WIQR vs. WAQL and WIQL vs. WAQL. 175 DEGs were up-regulated and 649 DEGs down-regulated in both EQA and WAQL. It is feasible that these genes play specific roles in honey bee ovary activation. Of these genes, five [*yellow-g*, *yellow-g2*, *chemosensory protein 5* (*CSP5*), *CSP6*, *odorant-binding protein 7* (*Obp7*)] were selected for further discussion, because of their likely significance in reproduction (Drapeau *et al.*, 2006; Forêt & Maleszka, 2006; Forêt *et al.*, 2007).

yellow-g and *yellow-g2* code for members of the yellow protein family. These genes are preferentially expressed in ovaries of honey bees, flies and ants and are involved in several functions related with ovarian function, including nutrient transport (Claycomb *et al.*, 2004; Tian *et al.*, 2004; Drapeau *et al.*, 2006). In this study we found that *yellow-g* and *yellow-g2* expression levels were higher in activated ovaries than in inactivated ovaries, suggesting that increased nutrient transport provided by yellow proteins is required for full ovarian activity in honey bees.

Small chemosensory proteins (CSPs) and odorant-binding proteins (OBPs) are two major classes of soluble proteins expressed not only in insect sensory organs, but also in other tissues that lack gustatory and olfactory neurones (Wanner *et al.*, 2004; Forêt *et al.*, 2007). CSPs and OBPs have similar roles in insect chemical communication (Calvello *et al.*, 2003, 2005; Forêt & Maleszka, 2006; Forêt *et al.*, 2007). The genome of the honey bee harbours six CSP genes involved in a wide range of cellular processes from embryonic development to chemosensory signal transduction (Danty *et al.*, 1998; Briand *et al.*, 2002; Kamikouchi *et al.*, 2004; Wanner *et al.*, 2004; Forêt *et al.*, 2007).

CSPs are expressed in diverse tissues throughout development. None is exclusively expressed in antennae. Interestingly, *csp5* and *csp6* are the highest expressed CSPs in queen ovaries. However, whereas *csp5* expression is restricted to the ovary (especially in trophocytes) and embryo, *cps6* is expressed in the ovary, embryo, larvae and pupae (Forêt *et al.*, 2007; Maleszka *et al.*, 2007). *csp5* gene expression is associated with the maternal to zygotic transition, as it is initially expressed by the ovarian maternal genome and subsequently by the embryonic genome (Maleszka *et al.*, 2007). In this study, we showed that *csp5* and *csp6* are highly expressed in both queen and worker ovaries, especially in activated ovaries. This pattern is consistent with specific functions in embryonic development previously demonstrated in other studies (Maleszka *et al.*, 2007). Increased expression levels of *csp5* and *csp6* in queen ovaries, compared with worker ovaries, are probably the result of the higher reproductive capability of queens. Compared with CSPs,

OBPs are expressed at lower levels in ovaries, suggesting that CSPs have a more predominant role in the process of honey bee ovary activation. OBP function in tissues other than sensory organs is unknown, but it is unlikely that their function in ovaries is related to their typical role in recognition of chemical stimuli. OBPs have been found to be a component of royal jelly (Fujita *et al*., 2012), opening the interesting possibility of a nutritional-related function. Thus, members of both the CSP and OBP families could have evolved novel nutritional functions in the ovary.

Previous studies used microarray and qRT-PCR technologies to search for DEGs associated with honey bee ovary activation and reproduction. Next generation sequencing provides an opportunity to compare DEGs previously identified with those identified in our study. Over 50 previously identified DEGs are listed to allow a comparison with our results (Table S5). Most of our results showed similar trends compared with previous studies in which brains and whole abdomens were analysed using high-throughput technologies. However, our study reveals a distinctive profile for a small subset of DEGs during ovary differentiation. Several factors may account for this difference. First, in contrast with those studies, in our study we used isolated ovaries. Thus, the observed differences may be the result of tissue-specific expression. Second, the results obtained using RNA-sequencing may differ with respect to other technologies for a variety of reasons, including differences in expression detection thresholds.

The honey bee genome harbours 10 genes encoding yellow proteins and nine encoding major royal jelly proteins (MRJPs). Interestingly, MRJP expression was strongly negatively associated with ovarian activation in both castes: all MRJPs were down-regulated in activated worker ovaries and most of them were not detected in egg-laying queens. Similarly, the expression of yellow protein genes has a trend towards a negative association with ovarian activation, especially in workers: only two genes were up-regulated in activated ovaries in both castes whereas the remaining eight were down-regulated in activated worker ovaries.

Transferrin (*trf*) encodes an iron-binding protein, which has been proposed to be imported into developing oocytes (Nichol *et al*., 2002). In our study, *trf* expression in ovaries was negatively associated with ovarian activity in queens and workers. These results are in good agreement with previous studies in which the expression of *trf* has been shown to be lower in mature queen abdomens relative to virgin queen abdomens (Kucharski & Maleszka, 2003). Contrary to *trf*, *Vg* expression in fat bodies positively correlates with ovary activity (Corona *et al*., 2007). This expression pattern contrasts with the observed expression of MRJPs (and yellow proteins to a lesser degree) in ovaries, which is in general negatively correlated with ovarian activation.

MRJPs are similar to Vg in being important proteins for the evolution of eusociality (Amdam *et al*., 2006; Drapeau *et al*., 2006; Thompson *et al*., 2006). Both proteins play important nutritive related-functions, but seem to be alternatively used under different developmental and physiological states: whereas maternally inherited Vg is the main nutritional source during embryonic development (Raikhel & Dhadialla, 1992), MRJPs are the main protein component of the glandular secretions used to feed developing larvae (Fujita *et al*., 2012). Our results support the notion that proteins involved in nutritionally related-functions display different tissue specific expression and are alternatively used as nutritional sources under different physiological and reproductive conditions.

The DEGs between inactivated and activated ovaries of queens and workers were subjected to GO category and KEGG pathway enrichment analysis. The results of the GO analysis suggest that the same gene set is active (but differentially expressed) in both queen and worker ovaries, which in turn results in dimorphic female ovaries. However, there is evidence that some factors involved in ovary activity have opposing effects in queen and workers: virgin queens react to CO_2 narcosis treatment by accelerating ovary activation and egg laying, whereas the same treatment inhibits ovary activation in queenless workers (Mackensen, 1947; Koywiwattrakul *et al*., 2005; Brito *et al*., 2010). Perhaps the fluctuation in the expression levels of several genes combined with the molecular interaction of their products is responsible for triggering honey bee ovary activation and reproduction. More than 230 KEGG pathways were identified, including 25 signalling pathways. Genes involved in hormone biosynthesis showed differences between inactivated and active ovaries, which were selected for further discussion (Table 2).

Juvenile hormone (JH) and ecdysteroids are two important endocrine hormones found in adult insects. They influence a wide range of developmental, reproductive, physiological and behavioural processes (Hiruma *et al*., 1999; Truman & Riddiford, 2002; Maki *et al*., 2004). JH and ecdysteroids have been studied in many insect species as they are associated with ovary development (Larrere & Couillaud, 1993; Bloch *et al*., 1996), oogenesis and vitellogenesis (Kozlova & Thummel, 2000; Guidugli *et al*., 2005; Paul *et al*., 2005), and reproductive behaviours (Wyatt & Davey, 1996; Bloch *et al*., 2000). However, in the highly eusocial honey bee, *A. mellifera*, JH has apparently changed its typical function from a reproductive hormone to an integrator of age polyethism in the worker caste (Robinson *et al*., 1992; Robinson & Vargo, 1997). Low levels of JH are associated with ovary development and egg-laying behaviour in honey bee queens and queenless workers (Robinson *et al*., 1991, 1992; Fahrbach *et al*., 1995).

Table 2. Genes involved in the juvenile hormone, ecdysteroid and insulin pathways

Pathway	Gene	Gene ID	Reads per kb per million reads)					Regulation	Definition
			VQI	EQA	WIQR	WIQL	WAQL		
Juvenile hormone	*jhe*	NM_001011563.1	3.95	0.23	53.72	15.96	3.93	DEQA*, DWAQL*	Juvenile hormone esterase
	jheh	XM_394922.4	14.96	17.84	68.33	29.90	16.73	UEQA, DWAQL*	Juvenile hormone epoxide hydrolase
	jhamt	XM_001119986.2	0.19	0.38	–	0.61	0.01	UEQA	Juvenile hormone acid methyltransferase
Ecdysteroids	*CYP306A1*	XM_391946.4	5.29	12.20	10.51	9.32	14.86	UEQA*, UWAQL*	Cytochrome P450 306A1
	Cyp314a1	NM_001040257.1	190.08	342.10	162.17	194.50	518.12	UEQA*, UWAQL*	Cytochrome P450 314A1
Insulin	*AmILP2*	NM_001177903.1	2.99	3.56	3.07	1.44	1.30	UEQA, DWAQL	Insulin-like peptide 2

EQA, activated ovaries of egg-laying queens; VQI, inactivated ovaries of virgin queens; WAQL, activated ovaries of queenless workers; WIQL, inactivated ovaries of queenless workers; WIQR, inactivated ovaries of queenright workers; D, down-regulated; U, up-regulated.
*Significant regulation.

JH is synthesized in the *corpora allata* (CA) from precursors of the mevalonate pathway (Bellés *et al*., 2005). In honey bees, the last two enzymatic steps in JH synthesis are catabolized by juvenile hormone acid methyltransferase (JHAMT) and methyl farnesoate epoxidase (MFE); whereas JH is degraded predominantly by juvenile hormone esterase (JHE) and JH epoxidase hydrolase (JHEH) (Mackert *et al*., 2008, 2010; Bomtorin *et al*., 2014). Recently, Bomtorin *et al*. (2014) showed that the expression of the genes encoding for JHAMT and MFE in the CA of adult worker honey bees correspond with JH titres. These results are in line with early studies showing correlation between JH synthesis by the CA *in vitro* and haemolymph JH titres (Rachinsky & Hartfelder, 1990; Huang *et al*., 1991), and together indicate that the amount of circulating JH in adult bees is determined primarily by the rate of their synthesis in the CA in adult bees.

We found that only one gene involved in the last steps of JH synthesis (*jhamt*) was expressed in ovaries of VQI, EQA and WIQL, but no significant differences in its expression were found amongst these groups. By contrast, genes encoding for enzymes involved in JH degradation were detected in all of the groups, with *jhe* being down-regulated in activated ovaries of both queens and workers and *jheh* being down-regulated only in workers. These results suggest that low JH synthesis and high degradation rates, probably resulting in reduced JH levels, are associated with ovary activation.

The CA has been considered the only source of JH in insects, including honey bees (Sullivan *et al*., 2000). Although JH biosynthesis has also been shown in ovaries of beetles (Tian *et al*., 2010) and mosquitoes (Borovsky *et al*., 1994), Bomtorin *et al*. (2014) recently showed that expression of *mfe* in worker bee ovaries is much lower compared with in the CA, suggesting that the ovarian contribution to overall circulating JH titres is insignificant. Our results confirm the expression of genes involved in the JH pathway in honey bee ovaries and that lower JH synthesis and higher degradation is associated with ovary activation. The physiological significance of higher expression of genes involved in JH catabolism in activated ovaries remains to be determined in future studies.

20-hydroxyecdysone (20E) is the active ecdysteroid in most insects (Buszczak & Segraves, 1998), including honey bees (Yamazaki *et al*., 2011). *Cytochrome P450 314A1* (*CYP314A1*) codes for ecdysone 20-hydroxylase, the enzyme that catalyses the conversion of ecdysone into 20E (Rewitz *et al*., 2006). Several other enzymes participate in 20E biosynthesis, including the products of the *CYP307A1*, *CYP306A1*, *CYP302A1* and *CYP315A1* genes (Rewitz *et al*., 2007; Yamazaki *et al*., 2011). In this study, *CYP306A1* and *CYP314A1* were preferentially expressed in activated ovaries. The expression level of *CYP306A1* in EQA was approximately threefold higher

than in VQI, whereas its expression level in WAQL was similar to WIQL and WIQR. The expression level of *CYP314A1* in WAQL was approximately threefold higher than in WIQL and WIQR, whereas its expression level in EQA was slightly higher than in VQI. These results are consistent with previous reports that *CYP306A1* and *CYP314A1* are expressed in honey bee ovaries (Yamazaki *et al*., 2011) and that ecdysteroid titres are higher in egg-laying queens and workers than in nonreproductive honey bees (Robinson *et al*., 1991). Our results further support that ecdysteroids and ecdysteroid signalling are involved in ovary activation as well as reproduction behaviour in the honey bee. Our results also parallel previous studies reporting higher expression of genes involved in ecdysteroid synthesis in reproductive workers using whole bodies (Cardoen *et al*., 2011).

Insulin–IGF-1/TOR (IIS/TOR) signalling is a nutrient sensing pathway involved in the regulation of ageing, fertility and other important biological processes (Tatar *et al*., 2003). The insulin-like peptides (ILPs) are important members of the IIS/TOR pathway that have been shown to be involved in the regulation of caste determination (Wheeler *et al*., 2006; de Azevedo & Hartfelder, 2008), division of labour (Ament *et al*., 2008) and ageing (Corona *et al*., 2007). There are two genes encoding for ILPs in the honey bee genome: *AmILP1* and *AmILP2* (Honeybee Genome Sequencing Consortium, 2006). In our study, *AmILP2* was detected in all five ovary groups, although it was not differentially expressed at significant levels amongst them. To the best of our knowledge, this is the first report of any ILP expression in honey bee ovaries.

Our results reveal important information about the molecular mechanisms of honey bee ovarian activation. First, we found that genes involved in the major insect hormone signalling pathways, JH, ecdysteroid and insulin, are expressed in ovaries. Second, we found that genes encoding for important proteins with nutritional functions, such as MRJPs and yellow proteins, are up-regulated in activated ovaries. Overall, our results suggest interactions amongst nutritional and hormonal signals during the ovary activation process. The identification of candidate genes associated with ovarian activation provides new insights into processes underlying the evolution of sociality and reproductive division of labour.

Experimental procedures

Bee rearing and ovary sample collection

Honey bee (*A. mellifera*) colonies were reared at the Honey Bee Research Lab in the College of Animal Sciences, Zhejiang University, Hangzhou, China. Sister virgin queens were produced according to standard rearing practices (Laidlaw & Page, 1997). Newly emerged queens were introduced into prepared individual nucleus colonies and prevented from taking mating flights by a strip of 'queen excluder' material fastened across the entrance, which allows the passage of workers but not of the queens. When the queens were 6 days old [the typical age of the first mating flight (Winston, 1987)], the queen excluder was removed from half of the colonies to allow natural mating flights (mated-queen group), whereas the queen excluder remained on the entrance of the other half of the colonies (virgin queen group). Once the mated queens initiated egg laying (11–12 days after emergence), all surviving same-aged virgins and egg-laying queens were collected and returned to the laboratory for ovary dissection. Analysis of the queen ovarian activation state was performed as in Kocher *et al*. (2008). We confirmed queen-mating status by checking whether sperm was present or absent in the spermatheca.

To obtain age-matched workers, we removed sealed brood combs containing emerging adult workers from four unrelated source colonies and placed them in an incubator overnight at 34.5 °C with high relative humidity. The following day, we marked approximately 1600 newly emerged (<24 h) workers from each source colony over two days (Day 0 and 1), with different source colonies and different days marked with different paints, and cofostered them into the prepared queenright and queenless host colonies equally (about 800 workers in each host colony). Marked workers from the four unrelated host colonies were mixed together to obtain a worker pool with a diverse genetic background. After 14 days, we collected a subsample of workers ($n = 4$) of the same colour from each host colony. These same-aged, paint-marked workers were dissected and their ovarian activation states were determined as in Velthuis (1970) and Martin *et al*. (2004).

Ovaries with the most extreme differences in activation states (fully inactivated and fully activated) were carefully removed with forceps under a stereomicroscope and then immediately flash-frozen in liquid nitrogen to stabilize their RNA in tissues and stored at −80 °C until further processing. The whole dissection process was performed on ice. In total, 23 pairs of ovaries from VQI, 17 pairs from EQA, 1000 pairs from WIQR (250 pairs × four source colonies), 600 pairs from WIQL (150 pairs × four source colonies) and 260 pairs from WAQL (65 pairs × four source colonies) were used for RNA-sequencing analysis.

RNA extraction, library construction and sequencing

Total RNA was extracted from the frozen ovaries using TRIzol reagents (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The concentration and quality of the RNA was determined using an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The total RNA groups were first treated with DNase I to degrade any possible DNA contamination. The mRNA was then enriched from the total RNA by using oligo (dT) magnetic beads. The mRNA was mixed with fragmentation buffer to obtain short fragments (about 200 bp). Using these short fragments as templates, the first-strand cDNA was synthesized by using random hexamer-primers. Buffer, deoxynucleotide triphosphates, RNase H and DNA polymerase I were added to synthesize the second strand. The double-stranded cDNA was purified with a QiaQuick PCR extraction kit (Qiagen, Valencia, CA, USA) and washed with elution buffer for end repair and single nucleotide A (adenine) addition. Finally, sequencing adaptors were ligated to the fragments. The resulting fragments were purified by agarose gel electrophoresis and enriched by PCR amplification. The library products were prepared for sequencing

analysis via Illumina HiSeq 2000 (Beijing Genome Institute, Beijing, China). Five libraries from inactivated and activated ovaries of queens and workers were constructed and sequenced.

Raw data processing and statistical analysis

The original image data produced by the sequencer were transformed into sequence data by base calling, which was defined as raw reads. We used the following procedure to obtain clean reads for data analysis: (1) removal of the reads with adaptor sequences; (2) filtering of the reads in which unknown bases were greater than 10%; and (3) removal of low quality reads in which the percentage of low quality bases (quality value ≤5) were greater than 50%.

The clean reads of five libraries were mapped to the most recently updated honey bee genome assembly (Amel_4.5; Elsik *et al.*, 2014) using SOAPaligner/SOAP2 (Li *et al.*, 2009). Mismatches of no more than two bases were allowed in the alignment.

Identification of DEGs

Gene expression levels were calculated by using the reads per kb per million reads (RPKM; Mortazavi *et al.*, 2008) method with the following formula:

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

where C is the number of reads that uniquely aligned to a gene, N is the total number of reads that uniquely aligned to all genes and L is the number of bases on a gene. All libraries of clean reads were normalized to the RPKM value to obtain the normalized gene expression level. A strict algorithm was performed to identify DEGs between two groups based on 'the significance of DGE profiles' (Audic & Claverie, 1997). A threshold of FDR ≤ 0.001 and absolute value of $\log_2$Ratio ≥ 1 was used to judge the significance of the gene expression difference.

Expression pattern, GO and KEGG pathway enrichment analysis of DEGs

We performed cluster analysis of gene expression patterns with cluster software (de Hoon *et al.*, 2004) and Java TREEVIEW software (Saldanha, 2004). For GO functional enrichment analysis and KEGG pathway enrichment analysis, all DEGs were mapped to GO terms in the database (http://www.geneontology.org/) and pathway terms in the KEGG database (http://www.genome.jp/kegg/), respectively.

Validation of RNA-sequencing data by qRT-PCR

To verify the RNA-sequencing results, qRT-PCR was used for the investigation of DEG expression levels. The RT reaction was performed using 1 μg of total RNA for each group, and the qPCR reactions were performed on an Masercycler ep realplex (Eppendorf, Hamburg, Germany) with SYBR *Premix Ex Taq* (TaKaRa, Dalian, China) according to the manufacturers' protocols. Each group was analysed in triplicate in a 20 μL total reaction volume containing 4 pmol of each primer, 10.0 μL SYBR Green and 1 μL diluted cDNA. The *actin* gene (*A. mellifera actin*, transcript variant 2, NM_001185145; Lourenço *et al.*, 2008) was used as a control housekeeping gene and the relative expression levels of genes were calculated by using the 2(−Delta Delta C(T)) method (Livak & Schmittgen, 2001). The primers of *actin* and DEGs for qRT-PCR are shown in Table S6.

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Supporting Information

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

Figure S1. Classification of total raw reads for the inactivated ovaries of virgin queens (VQI), activated ovaries of egg-laying queens (EQA), inactivated ovaries of queenright workers (WIQR), inactivated ovaries of queenless workers (WIQL) and activated ovaries of queenless workers (WAQL) groups.

Figure S2. Per cent of coverage representing the percentage of genes expressed at each of the groups. The distribution of distinct reads over different read abundance categories showed similar patterns for all five RNA-sequencing libraries. More than 58% of the total genes had gene coverage of greater than 50% in each library.

Table S1. Genes up-regulated and down-regulated by over fivefold in activated vs. inactivated ovaries in both queens and workers.

Table S2. Gene ontology (GO) annotation of the differentially expressed genes (DEGs) between inactivated ovaries of virgin queens (VQI) and activated ovaries of egg-laying queens (EQA), between inactivated ovaries of queenright workers (WIQR) and activated ovaries of queenless workers (WAQL), and between inactivated ovaries of queenless workers (WIQL) and WAQL.

Table S3. The top 10 most abundant differentially expressed signalling pathways.

Table S4. Information for the 10 differentially expressed genes (DEGs) in the five groups using RNA-sequencing methods.

Table S5. Comparison of the trend of the differentially expressed genes (DEGs) between those found in previous studies and in this study.

Table S6. Information for the quantitative real-time PCR primers.

File S1. Genes specifically expressed in the five groups.

File S2. Differentially expressed genes (DEGs) within castes.

File S3. Differentially expressed genes (DEGs) between castes.