Report

Current Biology

# A Maternal Effect on Queen Production in Honeybees

## Highlights

- Honeybee queens lay larger eggs in queen cells than in worker cells
- Egg size influences both gene expression and adult queen weight
- The *in ovo* environment is a maternal effect influencing caste development in bees

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## In Brief

Wei et al. show that the *in ovo* environment in honeybees is a maternal effect on queen development. Honeybee queens selectively lay larger eggs in queen cells to be raised by workers as queens. Egg size influences both gene expression and the adult weight of the queen.

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# A Maternal Effect on Queen Production in Honeybees

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## SUMMARY

**Influences from the mother on offspring phenotype, known as maternal effects, are an important cause of adaptive phenotypic plasticity [1, 2]. Eusocial insects show dramatic phenotypic plasticity with morphologically distinct reproductive (queen) and worker castes [3, 4]. The dominant paradigm for honeybees (*Apis mellifera*) is that castes are environmentally rather than genetically determined, with the environment and diet of young larvae causing caste differentiation [5–9]. A role for maternal effects has not been considered, but here we show that egg size also influences queen development. Queens laid significantly bigger eggs in the larger queen cells than in the worker cells. Eggs laid in queen cells (QE), laid in worker cells (WE), and 2-day old larvae from worker cells (2L) were transferred to artificial queen cells to be reared as queens in a standardized environment. Newly emerged adult queens from QE were heavier than those from the other two groups and had more ovarioles, indicating a consequence of egg size for adult queen morphology. Gene expression analyses identified several significantly differentially expressed genes between newly emerged queens from QE and those from the other groups. These included a disproportionate number of genes involved in hormonal signaling, body development, and immune pathways, which are key traits differing between queens and workers. That egg size influences emerging queen morphology and physiology and that queens lay larger eggs in queen cells demonstrate both a maternal effect on the expression of the queen phenotype and a more active role for the queen in gyne production than has been realized previously.**

## RESULTS AND DISCUSSION

Honeybee queens and workers are radically different phenotypes. While both are female and develop from fertilized eggs, queens are typically the sole female reproductive in the colony [10, 11]. No genetic difference separates queens and workers; instead, the differentiation is controlled epigenetically [5–7]. Thus far, attention has focused on the role of the larval developmental environment in the differentiation of workers and queens. Workers and queens develop in wax cells of different sizes and are fed different diets. Both diet and the amount of space available to developing larvae cause changes in methylation of the larval genome [5, 6, 12]. The resulting differences in gene regulation (particularly involving signal transduction, gland development, carbohydrate metabolism, and immune function pathways [13–15]) establish the divergent queen and worker developmental paths [16]. Here, we examined whether the queen herself might influence caste development via maternal effects.

Maternal effects are a causal influence of the maternal genotype or phenotype on the offspring phenotype [1, 2] and are an important mechanism of adaptive phenotypic plasticity [1]. Vertebrate examples have shown that females can adaptively vary investment in eggs according to the perceived quality of their mate in order to invest more in young of higher quality males [17–19]. Insects can also adjust their investment in their eggs [20, 21], or even egg coloration [22], to better adapt offspring to their environment. Flanders in 1945 [23] proposed that maternal effects could influence caste development in social insects via differential investment in eggs, but surprisingly, there are very few reports of maternal effects from the hymenoptera.

Passera [24] reported that queens of the ant *Pheidole pallidula* tended to lay larger eggs at the time of year at which colonies raised a generation that included sexuals, and Schwander et al. [25] reported a maternal effect on female caste determination in Pogonomyrmex ants. A suggestion of a possible maternal effect on queen production in honeybees came from Borodacheva [26] in 1973 with the observation that some of the variation in the size of adult queens could be attributed to variation in egg size. Honeybee queens lay between 1,500–2,000 eggs a day [27] in small worker cells that develop as the next generation of workers. When a colony is ready to reproduce by swarming, a few (10–20) larger queen cells are constructed [28]. Eggs laid in these are fed more and richer food and develop as queens [10]. Here we tested whether queens lay larger eggs in queen cells.

### Honeybee Queens Lay Larger Eggs in Queen Cells

To test for an effect of the queen-laid egg on caste development, we provided queens with artificial standardized plastic cells that were the size and shape of either worker cells or queen cells

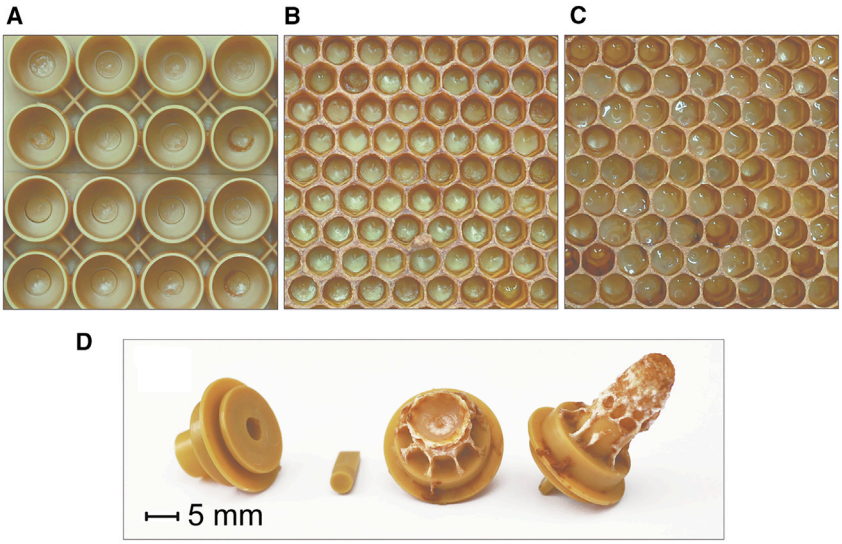


**Figure 1. To Sample QE and WE, Arrays of Standardized Plastic Cells the Same Size and Shape as Queen Cells or Worker Cells Were Placed in Colonies**

(A and B) Queens and attendant workers were restricted to these arrays for 6 h to lay in queen cells (A) and worker cells (B). After that time, the arrays were removed.

(C) To sample 2L, arrays remained in the colony for five days, by which time eggs hatched and 2-day-old larvae occupied each cell.

(D) The base of each plastic cell was removable, which allowed easy transfer of either eggs or larvae to new artificial queen cells. Queen cells containing QE, WE, or 2L were arranged randomly on a common rack and inserted into a queenless colony where the workers fed and raised each as a queen.

The scale bars (5 mm) were shown in (A), (B), (C), and (D).

(Figure 1). After six hours, eggs laid in the two cell types were collected and weighed. This study was repeated across 3 colonies; in total, 152 eggs were measured. Eggs laid in queen cells (QE) were 13.26% heavier (157.51 ± 12.37 versus 138.93 ± 10.90, mean ± SD, μg) and 2.43% longer (1.56 ± 0.04 versus 1.52 ± 0.05, mean ± SD, mm) and 4.18% thicker (0.374 ± 0.010 versus 0.359 ± 0.013, mean ± SD, mm) than eggs laid in worker cells (WE) (Figure 2).

## Adult Queens from QE Are Heavier Than Queens from WE and 2L

To determine whether this difference in egg size had any consequence for adult queen morphology, six hours after laying, QE and WE were transferred by moving the base of each plastic cell (so the egg was not touched, Figure 1) into artificial queen cells. Some WE remained in worker cells for 5 days until the larvae were 2 days old (2L). The larvae were then similarly transferred to artificial queen cells. All queen cells were placed in a common queenless colony to be reared as queens by workers. Sixteen days later, adult queens were collected on emergence from the sealed cells (Figure 1D) and weighed. This study was replicated using five colonies across two years (Figure 3). Adult queens from QE were heaviest in all five colonies, and queens from QE were significantly heavier than queens from WE (258.65 ± 22.82 versus 234.50 ± 36.00, mean ± SD, mg) in three colonies out of five (Figure 3).

## Adult Queens from QE Had the Greatest Number of Ovarioles

The number of ovarioles is an important index of queen fecundity [29]. Our hematoxylin-eosin (HE) staining results (Figure 3C) showed that five-day-old adult queens from QE had the greatest number of ovarioles in the right ovary, significantly more than queens from 2L (165.50 ± 10.65 versus 145.90 ± 14.89, mean ± SD) in all three colonies, but there was no significant difference between queens from QE and from WE (165.50 ± 10.65 versus 160.00 ± 9.48, mean ± SD).

## Differences in Gene Expression among Queens from QE, WE, and 2L

To further examine the consequences of egg source on adult queen phenotype, we compared the gene expression profiles of newly emerged adult queens from QE, WE, and 2L using RNA-seq. The heads and thoraces of two newly emerged queens from each group were collected and pooled for RNA-seq. This experiment was repeated twice using two colonies, and both repeats were considered together in our analyses of gene expression differences in 2016. This RNA-seq experiment was repeated again in 2018 with two extra colonies using same methods. Methods for sample preparation, mRNA isolation, and sequencing followed those of He et al. [14].

A small number of differentially expressed genes (DEGs) were detected in comparisons between groups from both 2016 and 2018 RNA-seq results (Figure 4 and Tables S1 and S2). Of the 121 DEGs identified across all comparisons in 2016 RNA-seq experiments, 6 genes with a high expression level were selected and gene expression differences assessed with qRT-PCR (following methods in [14]) to affirm the results of our RNA-seq analyses (Figure S2).

Two years' RNA-seq results both showed that the greatest differences were detected in comparisons between queens reared from QE and 2L, followed by WE against 2L and QE against WE comparisons (Figure 4; Tables S1 and S2). This is of interest because raising queens from 2L rather than from WE has already been shown to have a significant impact on queen reproductive development and morphology [14]. Of the DEGs identified in the QE against WE comparison, 31 (2016, Figure 4A) and 19 (2018, Figure 4B) have been documented previously in comparisons of queen and worker honeybees or queen honeybees varying in caste development or reproductive condition (Figure 4; Tables S1 and S2). Besides, 59 of 191 DEGs from three comparisons of the 2016 RNA-seq results were also identified in the 2018 RNA-seq results (Table S1).

This suggests that the gene expression differences between adult queen from QE and WE are reflective of variation in the

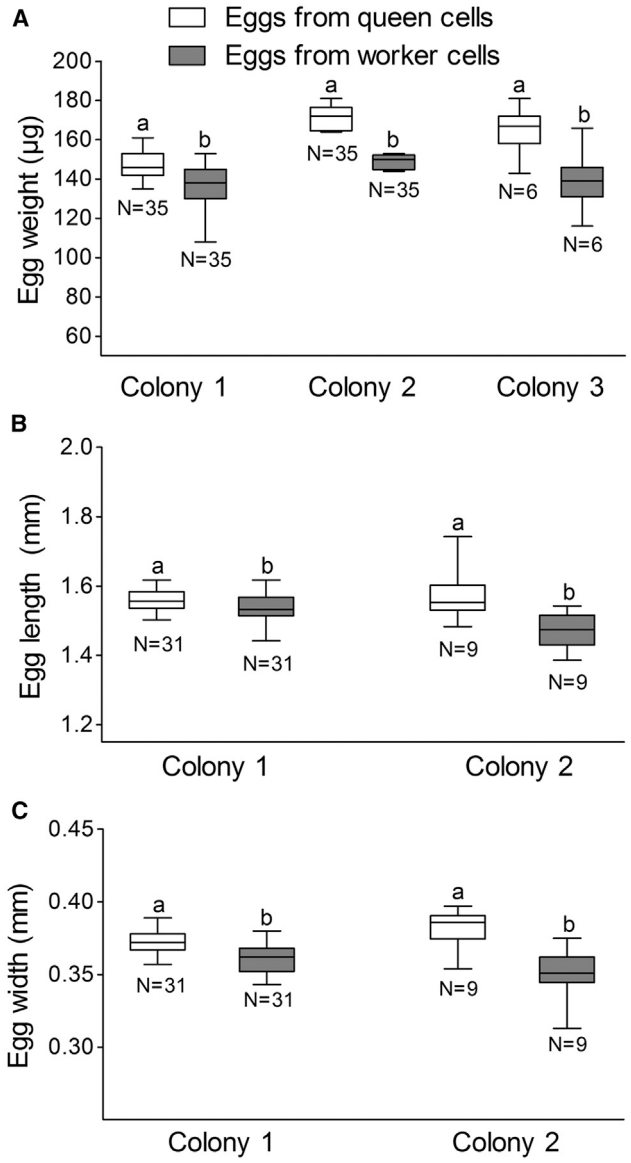


**Figure 2. QE and WE Differed in Size and Weight**

(A) For experiment 1, egg weights were recorded from 3 different colony replicates.

(B and C) For experiment 2 we recorded egg size from two of the colony replicates, but the third colony failed and could no longer be used. Eggs from these colonies were also used for queen rearing (Figure 3). For all panels, boxplots show median, quartiles, and range. The sample size for each group is marked below boxes. Data from three colonies were combined for analysis. The egg weight, length, and width of QE and WE were compared using ANOVA tests followed with Fisher's PLSD test. The critical p value was adjusted to 0.0167 according to the Bonferroni correction. Sample sizes are shown. Groups that did not differ ($p > 0.0167$) are marked with the same superscript.

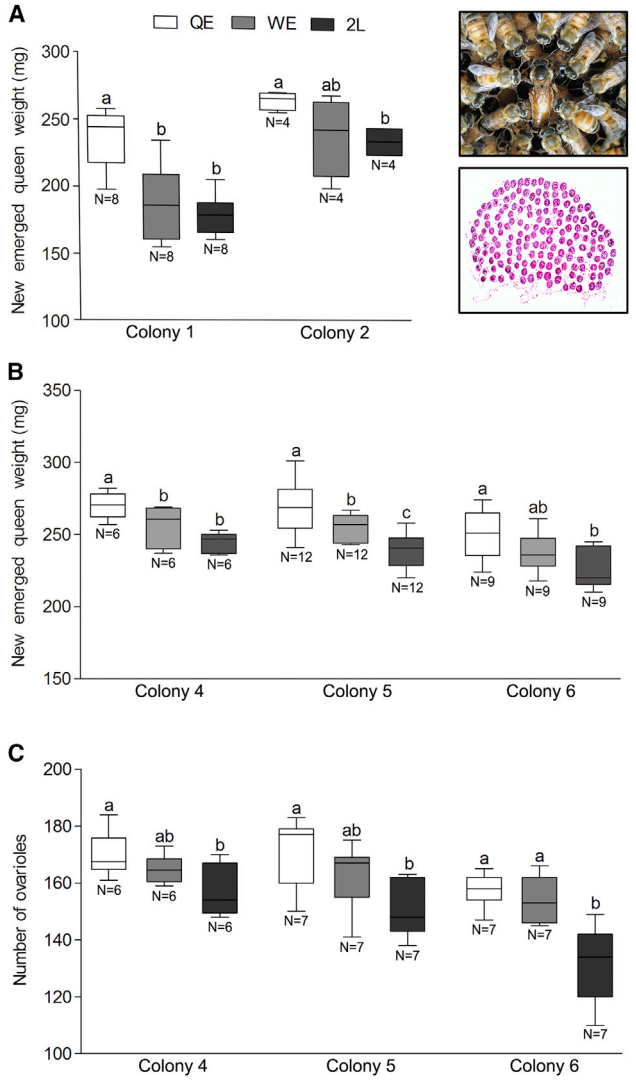


**Figure 3. Queens Collected on Emergence from Queen Cells from QE, WE, and 2L Differed in Weight**

(A) Data from two colonies measured in 2016.

(B) Data from three colonies repeated in 2018. The newly emerged queen is shown in inset.

(C) Measurements of queen ovaries from QE, WE, and 2L. The right ovary of each queen was used (cross section is shown in inset). For all panels, boxplots show median, quartiles, and range. The sample size for each group is marked below boxes. Data from each group were compared with ANOVA test followed by Fishers PLSD test. Groups that did not differ ($p > 0.05$) are marked with the same superscript.

caste development process. Our DEGs contained a disproportionately large number of genes such as *juvenile hormone methyltransferase*, *abaecin*, and *hexamerin* genes involved in hormone synthesis, ovary development, cuticle development, and immune functions (Figure 4; Tables S1 and S2) [14, 30–33].

## Conclusions

In summary, our data demonstrate a maternal effect on honeybee queen size and physiology, which is caused by queens laying larger eggs in queen cells than in worker cells. This could have significant consequences for colony function, since various authors [14, 26, 29, 34–36] have reported a relationship between queen weight and queen ovariole number and fecundity. Bilash [34] has even reported an influence of queen weight on colony honey production.

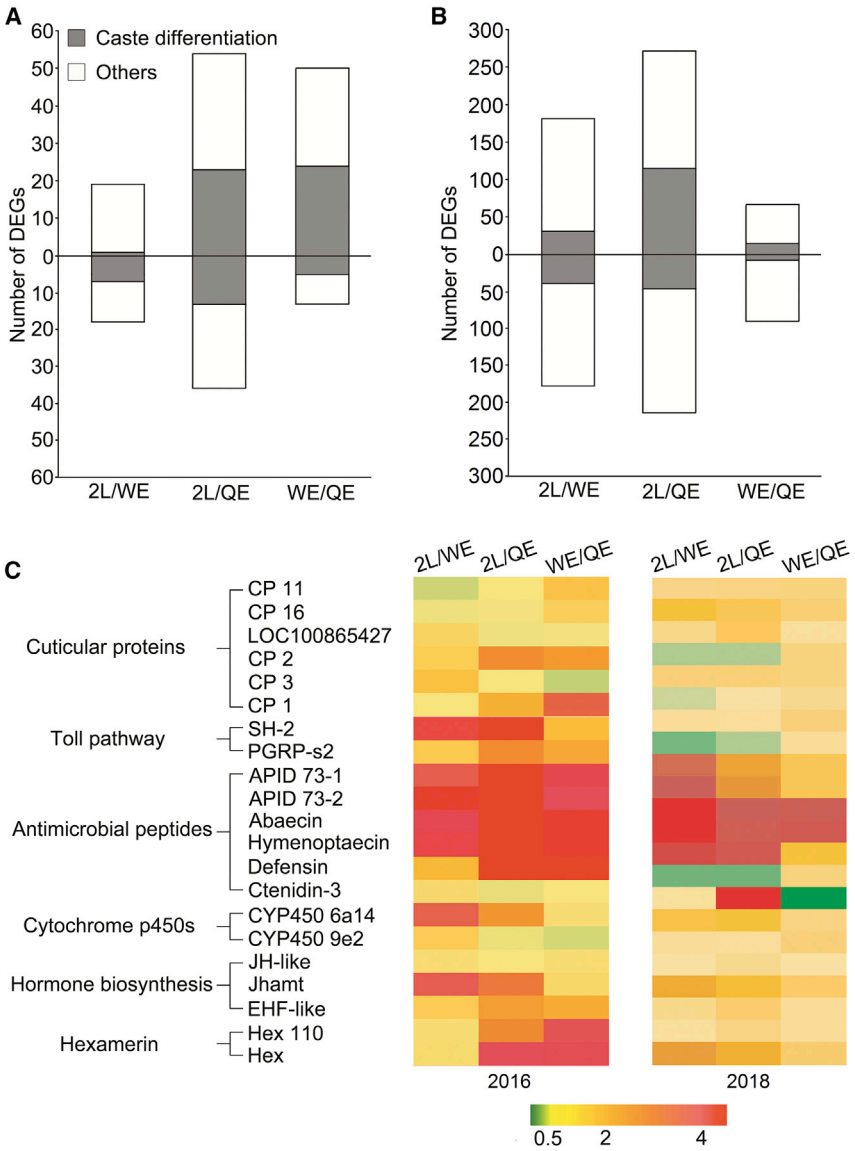


**Figure 4. Summary of Gene Expression Differences in Pairwise Comparisons between QE, WE, and 2L in 2016 and 2018**

(A) Total numbers of DEGs detected as significantly upregulated (above line) and downregulated (below line) in each comparison from 2016 RNA-seq. Grey areas mark the numbers of DEGs that have previously been identified as differing between either queens and workers or between queens of different quality (details and references are shown in Table S1).

(B) Total numbers of DEGs detected as significantly upregulated (above line) and downregulated (below line) in each comparison from 2018 RNA-seq (details and references are shown in Table S2).

(C) Gene expression ratios (color coded by scale bar) of selected DEGs with proposed functional roles in hormone synthesis, caste differentiation, immune function, and detoxification.

See also Tables S1 and S2.

We do not here propose that there is a special class of queen-destined eggs. The distribution of egg masses sampled from queen and worker cells was continuous, normal, and unimodal. Rather, we propose that fecund queens at any one time have more than one egg ready for laying [10] and that queens may lay the largest available egg in queen cells. Alternatively, queens may pause oviposition prior to laying in queen cells, since delaying oviposition causes bigger eggs with more yolk protein [37], but this possibility needs to be investigated.

An important inference of our data, however, is that queens can actively select larger fertilized eggs for oviposition in queen cells. It has been demonstrated previously that queens can control and withhold fertilization of eggs prior to laying in male (haploid) drone cells and that queens measure the larger drone cell with their foreleg prior to laying [38]. This is the first evidence that queens can select among fertilized eggs and that they differentiate between queen and worker cells.

We feel that the differences observed between QE and WE are attributable to the queen and not to interactions with workers for the following reasons. In the honeybee colony, worker-laid unfertilized eggs in worker cells will be removed by worker policing [39]; however, the queen-laid eggs we studied here have queen egg-marking pheromones and usually avoid worker policing [40, 41]. Workers are not expected to police these eggs. Even so, workers will sometimes consume queen-laid eggs if a colony is stressed [42]. However, there has been no report of selective queen egg removal based on size. There was no evidence of selective egg destruction by workers in our study. It is believed that the QE with more nutrition possibly results in better queens compared to WE, which is determined by the maternal effect.

Our data indicate that the *in ovo* environment influences queen development (Figure 3), but the *in ovo* environment is not necessary for queen formation. The queen developmental pathway proves to be quite robust [14], and queens can be reared from eggs or even larvae transplanted from worker cells [14]. Indeed, this capacity underlies commercial queen-rearing practices. Not all queens are the same quality, however, and queens reared from transplanted worker larvae are smaller and have less well-developed reproductive systems [14, 29, 43]. Rangel et al. [43] also reported that rearing queens from older worker larvae results in significantly lower production of worker comb, drone comb, and stored food compared to those by eggs [43]. Here, we provide the first evidence that the *in ovo* environment also influences adult queen morphology and physiology. It feels remarkable for a social insect as intensively studied as the honeybee that the possibility of maternal effects on caste has been overlooked until now. It has perhaps been assumed that the enormous difference in food provision to developing worker

and queen larvae must swamp any differential provisioning during the egg. We now recognize, however, that the epigenetically differentiated worker and queen developmental pathways are sensitive to the early larval environment [14], and our data also indicate a sensitivity to the *in ovo* environment. This adds a new perspective on colony function and indicates that the queen has a more active role in the production of the next generation of queens than has been previously recognized. It will be important to assess whether similar maternal effects are at play also in other social eusocial insects.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- CONTACT FOR REAGENT AND RESOURCE SHARING
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
- METHOD DETAILS
  - Egg collecting and queen rearing methods
  - Histolological analyses of queen ovarioles
  - RNA-seq analysis
  - Real-time PCR validation
- QUANTIFICATION AND STATISTICAL ANALYSIS
- DATA AND SOFTWARE AVAILABILITY

### SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2019.05.059.

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### AUTHOR CONTRIBUTIONS

H.W., X.J.H., C.H.L., and W.J.J. conducted all experiments. Z.J.Z. designed experiments. X.J.H., A.B.B., and Z.J.Z. wrote the paper. X.B.W., B.Z., L.B.Z., and L.Z.Z. participated in experiments.

### DECLARATION OF INTERESTS

All authors declare no competing interests.

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Biological Samples | | |
| Honeybee (*Apis mellifera*) eggs and adult queens | This paper | N/A |
| Chemicals, Peptides, and Recombinant Proteins | | |
| 4% Paraformaldehyde Fix | BBI Life Sciences (China) | Lot#: E205fa0004 |
| Hematoxylin-Eosin | Boster biological Technology (USA) | Lot#: 12K01A80 |
| Neutral Balsam Mounting Medium | BBI Life Sciences (China) | Lot#: E122FD0271 |
| Xylene | Tianjin Damao Reagent Factory (China) | Analytical Reagent |
| Ethanol | Xilong Scientific (China) | Analytical Reagent |
| Paraffin with Ceresin | Sinopharm Chemical Reagent Co., Ltd (China) | Lot#: 69019461 |
| Critical Commercial Assays | | |
| TRIzolTM Reagent kit | Life technologies (USA) | Cat#:15596-026 |
| NEBNext® Poly(A) mRNA Magnetic Isolation Module | NEB (USA) | Cat#:E7490 |
| Oligo (dT) | NEB (USA) | Cat#:E7500 |
| Fragmentation Buffer | NEB (USA) | Cat#:E7530B/E7530L |
| Agencourt AMPure XP | Beckman Coulter (USA) | Cat#:A63880 |
| PrimeScriptTM Reagent Kit | Takara (Japan) | Cat#: RR047A |
| Deposited Data | | |
| Egg weight,length and width; queen weight and ovarioles | Mendeley datasets | https://data.mendeley.com/datasets/3xmkwh79gj/3 |
| RNA-seq data collected in 2016 | NCBI database | BioProject: PRJNA310321 |
| RNA-seq data collected in 2018 | NCBI database | BioProject: PRJNA530116 |
| Experimental Models: Organisms/Strains | | |
| High royal jelly producing honeybee strain (*Apis mellifera*) | Hangzhou Dexing bee industry Co., Ltd (China) | http://hzdexingtang.cn.gongxuku.com/ |
| Software and Algorithms | | |
| Statview | SAS Institute, Cary, NC, USA. | Version: 5.01 |
| EBSeq | R package. | https://doi.org/10.18129/B9.bioc.EBSeq |
| KOBAS software | Center for Bioinformatics, Peking University. | Version: 2.0 |
| Cufflinks software | Cole-trapnell-lab (GitHub) | Version: 2.2.1 |
| Other | | |
| Queen cell frame and excluder | This paper | N/A |
| Worker cell frame and excluder | This paper | N/A |

### CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Zhi Jiang Zeng (bees1965@sina.com).

### EXPERIMENTAL MODEL AND SUBJECT DETAILS

Six western honeybee (*Apis mellifera*) colonies, which are a high royal jelly producing honeybee strain, were used in this experiment. Honeybee colonies were maintained at the Honeybee Research Institute, Jiangxi Agricultural University, Nanchang, China (28.46 oN, 115.49 oE), according to standard beekeeping techniques. Each colony had 8 frames with approximately 12,000 bees and a mated queen. Three colonies were used for egg weight, length and width measurement, queen weight measurement and RNA-seq in 2016

(RNA-seq was performed by Beijing Biomarker Technologies Co., Ltd.). All experiments were performed in accordance with the guidelines from the Animal Care and Use Committee of Jiangxi Agricultural University, China. Other three were use for queen weight and ovariole measurement and repeated RNA-seq in 2018 (RNA-seq was performed by Guangzhou Gene Denovo Co., Ltd.).

## METHOD DETAILS

### Egg collecting and queen rearing methods

Mated queens were caged for six hours to lay in either a plastic frame of worker cells or a plastic frame of queen cells (Figure 1A). One side of the box was a queen excluder that allowed workers to pass through and attend the queen as normal. The plastic frame of worker cells was developed by Pan et al. [44] and designed such that the base of each cell could be removed allowing the egg or larvae within to be transferred to other plastic queen cells or worker cells without touching them (Figure 1B). Generally, queens were caged in the morning to lay queen cells eggs for 6 hr and were removed immediately to worker cell frames to lay worker cell eggs for 6 hr in the afternoon. Egg size changes during the incubation period and varies across inbred lines [45, 46], therefore queens were restricted to plastic frames of either queen-sized (internal diameter 9.7 mm) cells or worker-sized (internal diameter 4.9 mm) cells for only 6 h to lay (Figure 1) and measured immediately on collection. In total 152 eggs from three colonies were sampled and weighed.

For weighing the eggs, a plastic pen with a very thin and soft needle was developed to individually transplant eggs from cells to an analytical balance (Ax26 Comparator (Max = 22 g, d = 1 μg), Switzerland Mettler Toledo Co., Ltd.), and data was shown in Figure 2. Their width and length were measured with a zoom-stereo microscope system (Panasonic Co., Ltd.) according to the manufacturer's instructions and were shown in Figure 2. Since queens laid only dozens of eggs into queen cells, all eggs were measured. In worker cells, about 30 eggs from among 250-300 eggs laid were measured from each colony. We excluded the possibility that workers differentially cannibalize or remove eggs after they are laid by queens, since fertilized eggs laid by queens had queen egg-marking pheromones to avoid worker policing [40, 41].

Eggs sampled in this way were also used to rear queens. Eggs sampled from queen cells and worker cells were transplanted into standard plastic queen cells (Figure 1) Queen cell bars were placed into a strong queenless hive with 8 frames for queen rearing. For the 2L group, eggs laid in worker cells were allowed to develop for 30-36 hours after hatching. The larvae were subsequently transferred to queen cells and added to the same queenless hive to be reared. Cells from the three groups (QE, WE, 2L) were mixed randomly. After 11 days, queens were harvested immediately on emergence. Queen weights were measured using the methods above and were shown in Figure 3. Six of newly emerged queens were collected immediately on emergence for RNA-seq in 2016, and other six queens were collected from other three colonies for RNA-seq in 2018.

### Histolological analyses of queen ovarioles

For measuring the queen ovarioles, 60 newly emerged queens from those three groups were caged and kept into a colony for 5 days until their ovaries were fully developed. The methods of histopathologic observation were according to Zou et al. [47]. Ovaries from the right side of queen were collected and fixed in 4% paraformaldehyde for 18h at room temperature. These tissues were then embedded in paraffin after dehydration and permeabilization. Paraffin-embedded ovaries were sectioned serially at 4 μm on a microtome and dried. Subsequently, the slices were stained with HE after deparaffinization and rehydration. Histomorphology was assessed using a microscope (Qlympus-DP80, Olympus Corporation, Tokyo, Japan), data were shown in Figure 3.

### RNA-seq analysis

For RNA-seq, we sampled in total 12 newly emerged queens. Two queens of each of the QE, WE and 2L groups were taken from two different colonies. Each RNA-seq sample combined 2 queens from the same group from the same colony. Each experimental group had two biological replicates. Only the heads and thoraces of two queens were used and mixed for RNA extraction and sequencing, since microorganisms and food in queen midgut could interfere RNA-seq analysis. All samples were immediately flash-frozen in liquid nitrogen. Methods for sample preparation, mRNA isolation, RNA sequencing and data analysis followed those of He et al. [14]. First, total RNA was extracted using a TRIzol Reagent kit (Life technologies, California, USA) from each sample individually. Total RNA of each sample (around 6 μg) were used for RNA sequencing. The RNA quality was further checked using Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). mRNA was isolated from total RNA using a NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB, E7490) with Oligo(dT)(NEB, E7500). Then the enriched mRNA was randomly fragmented leading to approximately 200 nt RNA inserts by a fragmentation buffer (NEB, E7530B/E7530L). Fragmented RNA inserts were used to synthesize cDNA, which were purified with AMPure XP beads (Beckman Coulter, Inc.) for End-repair/dA-tail and adaptor ligation. Finally the constructed cDNA libraries were sequenced by an Illumina HiSeq 2500 sequencing platform.

The reads with over 50% of its base pairs had a Q-score of less than 10 ($Q = -10 * \log_{10} Pe$) were filtered [48]. All clean reads were mapped to honeybee (*Apis mellifera*) reference genome (Amel 4.5) using Tophat2 package [49]. Gene expression levels were calculated and analyzed using read counts by the Cufflinks software [50] and normalized using FPKM values (fragments per kilobase of exon per million fragments mapped). Gene expression among three experimental treatments were evaluated and compared by using EBSeq [51]. Only those genes with an absolute value of log2 ratio $\geq 1$ and $P$ value $< 0.05$ were defined as significantly differentially expressed genes (DEGs), which were shown in Figure 4 and Tables S1 and S2.

The identified DEGs peptide sequences were aligned to NCBI non-redundant database (NCBI Nr), gene ontology database (GO), cluster of orthologous groups of proteins database (COG), kyoto encyclopedia of genes and genomes database (KEGG), Swiss-Prot database, using BLASTX and BLASTn with a cut-off E-value of 10-5. The Enrichment analysis of DEGs in KEGG pathways was performed using KOBAS 2.0 software [52]. The similarity of DEG results between each comparison (2L/QE, 2L/WE and WE/QE) were shown in Figure S1 and the number of DEGs in each section were marked with star key.

Twelve cDNA libraries were generated from our experimental groups. The Q30 of each sample was higher than 87% indicating the high quality in the saturation of RNA sequencing (2016: Table S3; 2018: Table S4). The Pearson correlation coefficient among two biological replicates of each experimental group were all $\geq$ 0.80 (2016: Table S5; 2018: Table S6), which is a conventionally accepted threshold for valid replicates indicating that there was acceptable sequencing quality and repeatability among the biological replicates of each group.

### Real-time PCR validation

RNA for qRT-PCR was taken from the RNA samples used for the RNA-seq, and was used as templates to synthesis cDNA by MLV reverse transcriptase (Takara Japan) according to the manufacturer's instructions. Six genes identified as highly and significantly differentially expressed among 2L, WE and QE were chosen for confirmation of expression differences with real-time PCR (Bio-Rad IQ2, USA). The gene Apr-1 was selected as an appropriate internal control [53]. Real-time PCR Primers of these six target genes were designed using Primer 5.0 software (Table S7). The internal standard and each target gene were run in the same plate to eliminate interplate variations. The qRT-PCR cycling conditions were as follows: preliminary 94°C for 2 min, 40 cycles including 94°C for 15 s, xx°C (varied according to the best annealing temperatures of each target gene, Table S7) for 30 s, and 72°C for 30 s. For each gene, two biological replicates with five technical replicates were performed. The Ct value for each biological replicate was obtained by calculating the mean of five technical replicates. The relative gene expression was calculated by $2^{-\Delta\Delta Ct}$ formula reported by Liu and Saint [54]. The results are presented in Figure S2.

## QUANTIFICATION AND STATISTICAL ANALYSIS

As the weigh, thorax width and length of eggs in this study were highly corrected, we therefore used a Bonferroni correction for the data analysis according to the format: $\alpha' \leq \alpha/K$ where $\alpha$ is the critical value ($p_{critical} = 0.05$) and k is the number of hypotheses. The adjusted significance value ($p_{adjusted} = 0.05/3 = 0.0167$) was employed as the critical p value. For the egg weight analysis, the weight, length and width of each egg was the response variable, two treatments were the explanatory factors and three colonies were the covariants. Data from three honeybee colonies was integrated together and analyzed by using ANOVA test (StatView 5.01) followed by fisher's PLSD, since there was no significant difference among three colonies in weight, length and width ($p = 0.1206$, $p = 0.2563$ and $p = 0.1918$ respectively). For the analysis of queen weight and number of ovarioles, the data were analyzed by ANOVA test using StatView 5.01 followed by a Fisher's PLSD test, and p value $< 0.05$ was considered as significance. The data from qRT-PCR of each group were analyzed by ANOVA test using StatView 5.01 followed by a Fisher's PLSD test.

## DATA AND SOFTWARE AVAILABILITY

The raw data of egg weight, egg length and width, queen weight and ovarioles are accessible through Mendeley database: https://data.mendeley.com/datasets/3xmkwh79gj/3.

2016 RNA-seq raw data are accessible through NCBI' database: BioProject: PRJNA310321; BioSamples: QE (SAMN04450256), WE (SAMN04450254), 2L (SAMN04450253)

2018 RNA-seq raw data are accessible through NCBI' database: BioProject: PRJNA530116 (SRP190001); BioSamples: QE-replicate 1 (SRR8823608), QE-replicate 2 (SRR8823607), WE-replicate 1 (SRR8823606), WE-replicate 2 (SRR8823605), 2L-replicate 1 (SRR8823604), 2L-replicate 2 (SRR8823603).