The diverging epigenomic landscapes of honeybee queens and workers revealed by multiomic sequencing

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The diverging epigenomic landscapes of honeybee queens and workers revealed by multiomic sequencing

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Abstract

The role of the epigenome in phenotypic plasticity is unclear presently. Here we used a multiomics approach (RNA-seq, ChIP-seq, ATAC-seq and Hi-C) to explore the nature of the epigenome in developing honeybee (*Apis mellifera*) workers and queens. Our data showed that the distinct queen and worker epigenomic landscapes form during the developmental process. Differences in gene expression between workers and queens precede other epigenomic modifications, but the epigenomic differences between workers and queens become more extensive and more layered during development. Genes known to be important for caste differentiation were more likely to be multiply differentially regulated by more than one epigenomic system than other differentially expressed genes. This indicates a multidimensional regulation of expression of key genes, presumably to canalise differences in gene expression. Our data indicate that the epigenome interacts with diverging developmental trajectories rather than controlling them since different epigenomic landscapes form in concert with different developmental outcomes.

Keywords:

honeybees; caste differentiation; development; epigenetic modifications; gene expression

Introduction

Our growing appreciation of epigenomics has transformed our understanding of development and genome environment interactions. The epigenome describes the interacting mechanisms that collectively regulate the expression of the genome[1-3]. These mechanisms include biochemical modifications of the genome (such as DNA methylation), modulation of the affinity of DNA to proteins, (such as histones), that provide structural support to the DNA molecule, and changes to the folding of the DNA molecule and the chromosome to alter the accessibility of sections of DNA to gene transcription machinery[4-8]. Multiple different specific mechanisms exist in each of these broad classes. The complexity of the epigenome is such that metaphors of "epigenomic landscape" and "epigenomic architecture" have been used to capture the expression of the structural complexity and diversity of the epigenome[9-11].

The significance of the epigenome is apparent in naturally occurring phenotypic plasticity. Here one genome can yield different phenotypes in different environments with no change to the DNA sequence of the genome[12, 13]. The interactions between the genome and the environment, and the developmental pathways that lead to different phenotypic outcomes are all functions of the epigenome[14, 15].

Honeybee (*Apis mellifera*) caste differentiation is a striking example of naturally occurring phenotypic plasticity, and it has become an important model for studies of genomics and epigenomics. Young female larvae can develop into two very different castes: queens and workers, which differ in morphology, physiology, longevity, life-history, social function and other traits[16]. The different developmental outcomes come about because larvae are raised by workers in different developmental environments. These are different sized cells and different diets that differ in sugars, fatty acids, para-coumaric acid (*p*-coumaric acid) and specific royal jelly proteins[17, 18].

We now understand that these nutritional difference cause many changes in the epigenomic regulation of several key genomic signaling pathways. These include mitogen-activated protein kinase signaling pathway (MAPK), target of rapamycin signaling pathway (TOR) and insulin receptor substrate signaling pathway (IRS)[19, 20]. Developing queen and worker larvae are recognized to differ in extent and distribution of DNA methylation, histone modification and microRNA (miRNA) expression[21-24]. Collectively these studies illustrate very different epigenomic landscapes in developing workers and queens.

The queen and worker developmental pathways are flexible, to a degree. Transplanting young larvae from worker cells to queen cells can result in a normal queen phenotype, but transplanting larvae that are more than 3.5 days old from the worker rearing environment to the queen rearing environment result in inter-castes or workers rather than queens[25, 26]. There is a point in developmental beyond which the worker developmental trajectory cannot be successfully redirected to yield the queen phenotype.

As this introduction illustrates, it is common in studies of epigenomics to discuss epigenomic or genomic programs regulating development, environmental triggers for these and switching or reorientation between these programs[27-29]. Are these the best analogies for the epigenome in development? The analogy of "programs" gives the impression that phenotypic plasticity is achieved by different epigenomic configurations controlling different developmental outcomes, with "switches" transforming the epigenomic landscape from one configuration to another. An alternative model views the epigenome as part of, rather than controlling of, the developmental process[30-33]. In this model the epigenomic landscape develops along

with the developing phenotype, and shifts between landscapes/phenotypes are more progressive and fluid than a switch or trigger analogy might suggest.

In this study we examined how the epigenomic landscapes of the queen and worker honeybee form in development. We used a multiomics approach to obtain different views of the epigenomic landscapes of the same samples of workers and queens. We compared gene expression levels with RNA sequencing. We compared histome modifications using chromatin immunoprecipitation with high-throughout sequencing (ChIP-seq). We compared which regions of the chromosome were tightly wound with proteins and which regions were more available for transcription using the assay for transposase accessible chromatin with high-throughout sequencing (ATAC-seq). Finally we compared the three dimensional chromosome structure of samples with the high-throughput chromosome conformation capture (Hi-C) assay. Hi-C can detect topologically associated domains (TAD) in the genome. TADs are influenced by the three-dimensional configuration of the chromosome and genes within TADs are more likely to interact with each other and to be co-regulated than genes at random[34, 35]. ATAC-seq can distinguish the compartments of the genome in which genes are more or less likely to be transcribed[36, 37]. ChIP-seq assays histone modifications associated with different genes, which might influence the affinity of the DNA for the histone protein[38, 39]. These three assays each give different perspectives on biochemical processes that regulate gene expression, whereas RNA-seq measures the outcome of that regulation: the amount of expression of each gene. Using these four methods we compared the epigenomic landscapes of queen and worker larvae that were two and four days old to measure developmental timepoints before and after the age at which a worker could no longer be successfully

converted to a queen. This approach allowed study of both the nature and the development of the distinct epigenomic landscapes of queen and worker bees.

Results

Data quality control

This study performed and compared four different omics analyses (Hi-C, ATAC-seq, ChIP-seq and RNA-Seq) on worker larvae sampled when 2-day (2W) and 4-day old (4W), and queen larvae sampled when 2-day (2Q) and 4-day old (4Q). The Q30 value of each sample in all four omics was all over 90 % (Table S1-4). The Pearson's correlations between the three biological replicates of four omics were all over 0.85 (see Fig.S1). These results indicated that the sequencing was of acceptable and the biological replicates were reliable.

Comparison of Hi-C and RNA-seq analysis

Chromosome interactions were similar between 2Q and 2W (Fig. 1a, 1c), but 4Q had significantly stronger cis interaction while 4W had significantly stronger trans interaction (Fig. 1b, 1c). The 4Q/4W comparison also had more genes that switched between the A/B bins (325) than the 2Q/2W comparison (247) (Table S5). Compared with 2Q/2W, the 4Q/4W comparison had more A/B switched regions and more genes within the A/B switched region (Fig. S2A). The 4Q/4W comparison had longer TAD boundaries than the 2Q/2W comparison (Fig. 1f). These indicated that the differences of 3D genomic structure between honeybee queens and workers were greater at the 4 day larval stage compared to the earlier two-day larval stage.

The A/B compartment differences between queens and workers were positively related to differences in gene expression at both 2 day and 4 day stages. Active

compartments were strongly associated with up-regulated genes in queen larvae whereas inactive compartments were associated with down-regulated genes in queen larvae (Fig. 1d, 1e). More significantly differentially expressed genes (DEGs) were located in the TAD boundaries of 4Q and 4W than 2Q and 2W, and more DEGs were located in the TAD boundaries of 4W compared to 4Q (Fig. 1g). Many of these DEGs were enriched in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways involved in honeybee caste differentiation, including insect hormone biosynthesis, TOR signaling and MAPK signaling (Fig. S2e, S2f).

The number of 4Q unique loops increased compared to 2Q, whereas this was decreased in 4W (Fig.1h). There were more DEGs located in unique loops of the 4Q/4W comparison than the 2d comparison (Fig. 1i).

Comparison of ATAC-seq and RNA-seq analyses

ATAC-seq results showed that 1.02 Mb (0.46%), 1.52Mb (0.69%), 8.65Mb (3.92%) and 1.50Mb (0.68%) of the honeybee genome was detected as unique open accessible chromatin in 2Q, 2W, 4Q and 4W respectively (Fig. 2a). 4Q had more open accessible regions in its genome compared to the other samples.

In 2Q/2W comparisons we identified 253 unique ATAC peaks from 2Q and 382 from 2W (Table S6, Fig. S3a). In 4Q/4W comparisons, more unique peaks from 4Q (4,618) were identified but only 448 from 4W (Table S7, Fig. S3a). When mapping these unique peaks to gene expression differences, considerably more DEGs which contain significantly different ATAC peaks were identified in the 4 day comparison compared to 2 day comparison (Fig. 2b, S3b). There was a strong positive correlation between the differences of ATAC and biased gene expression in queen-worker comparisons (Fig. 2c, S3c-S3f). 4Q had the largest number of DEGs containing unique ATAC peaks, and also the most DEGs associated with caste differentiation

(Fig. 2d). There were more DEGs containing unique ATAC peaks in 4Q or 4W compared to the 2 day samples. More DEGs containing significantly different ATAC peaks in the 4 day comparison were involved in honeybee caste differentiation than in the 2 day comparison (Fig. 2e). DEGs which contain significantly different ATAC peaks from 4Q/4W were enriched in eight key pathways involved in honeybee caste differentiation, whereas DEGs which contain significantly different ATAC peaks from 2Q/2W were enriched in three key pathways only (Fig. S3g, S3h). These findings suggest that chromatin accessibility is strongly related to the biased gene expression that is known to be causal of honeybee caste differentiation (Fig. 3).

Comparison of ChIP-seq and RNA-seq analyses

The number of unique ChIP peaks increased with age, and were more abundant in queen than worker samples (Fig. S4a, Table S10). Similar to the ATAC results, 4d comparison had more DEGs containing significantly different ChIP peaks than 2d comparison (Fig. 3a, 3b, S4b). There was a strong positive correlation between H3K27ac modification and gene expression (Fig. 3c, 3d, S4c-S4f). Compared with 2 day comparisons, 4d comparison had more genes containing unique peaks, and the genes associated with caste differentiation were also more abundant (Fig. 3e). Compared with 2Q/2W, there were more DEGs containing significantly different peaks associated with caste differentiation in 4Q/4W (Fig. 3f). Many DEGs containing significantly different ChIP peaks were enriched in caste-differentiation related pathways, with 8 pathways in 2Q/2W and 9 in 4Q/4W (Fig. S4g, S4h). These results suggest that H3K27ac also partly contributes to the determination of honeybee caste differentiation and differences are more pronounced at day 4 stage than day 2.

Multiomics analysis of caste differentiation

193 In the 2W/2Q comparisons, Hi-C, ATAC and H3K27ac histone modification all
194 differed between honeybee queens and workers, and the numbers of genes related to
195 the significant differences of Hi-C, ATAC and histone modification were 228, 344
196 and 4308 respectively. A very low proportion of these differences overlapped with the
197 DEGs (Fig. 4a). In the 4W/4Q comparison more genes were related to the significant
198 differences of Hi-C (813), ATAC (4096) and histone modification (7275), and a
199 higher proportion of these genes overlapped with DEGs (Fig. 4b).

200 We selected 58 key DEGs with functions that have been reported to be involved
201 in honeybee caste differentiation. More of these genes were differentially regulated by
202 at least one epigenomic system in 4 day comparisons compared to 2 day comparison
203 (Fig. 4c and 4d). These suggest that there is a small divergence in gene expression
204 between queen and workers at an early developmental stage that is not widely
205 reinforced by differential genomic regulation. At the 4 day larval stage the differences
206 in genes expression between workers and queens are more profound, and many more
207 of the differences are reinforced by at least one form of genomic regulation.

208 In the 2d comparison, only a few of the 58 key DEGs were regulated by three
209 different epigenetic modifications and most of them were influenced by one or two
210 types of modifications (see the color-marked symbols in Fig. 4e). By contrast, more
211 key DEGs were regulated by three epigenomic modifications in the 4 day comparison,
212 and more genes were differentially regulated by more than one genomic system (see
213 the color-marked symbols in Fig. 4e). These suggest that epigenomic control of
214 honeybee caste differentiation involves complex multi-omics interactions that develop
215 as the worker and queen phenotypes diverge.

216

217 **Gene expression differences associated with multiple epigenetic modifications**

In 2d comparison, genes containing significant differences of three epigenetic modifications between queen and worker larvae were rarely overlapped (Fig. 5a), but these notably increased in 4d comparison (Fig. 5b). The proportion of DEGs that were associated with at least one other epigenomic difference was lower in 2d comparison (51.42 %, Fig. 5c) than the 4d comparison (76.15 %, Fig. 5d). Moreover, the proportion of DEGs associated with more than one epigenomic difference was also higher in the 4d than 2d comparison (Fig. 5c, 5d). These results show that epigenomic differences between queens and workers become more extensive, more layered and more complex at a later developmental stage compared to an earlier stage. This pattern was also seen in the 58 key DEGs that are involved into the determination of honeybee caste differentiation (Fig. 5e, 5f), A higher proportion of these key DEGs were differentially regulated by at least 2 types of epigenetic modifications (Fig. 5f) compared to total DEGs (Fig. 5d), suggesting that the key genes involved into queen-worker dimorphism are likely regulated by more layers of epigenetic modification compared to other genes. The highest proportion was DEGs was associated with differences in chromatin accessibility and histone modification rather than 3D chromosome structure (Fig. 5f), perhaps suggesting an epigenetic pattern that gene expression is more closely regulated by chromatin accessibility and histone modification and remotely influenced by chromosome structure.

Effects of a key candidate gene on caste differentiation

We selected one key gene [*4-coumarate--CoA Ligase* (*4CL*, Loc726040)], which significantly differed in all four omics analyses for an RNA interference study (Fig. 6a). This gene has been reported involved in *p*-coumaric acid synthesis in honeybee larval diets and may be causal of honeybee caste differentiation[17, 40-42]. Knocking

down this gene decreased the weight (Fig. 6b, 6c), and body size (Fig. 6d), as well as the number of ovarioles (Fig. 6e) of newly-emerged queens. This gene plays an important role in the determination of honeybee caste differentiation.

Discussion

The worker and queen castes of the honeybee are highly divergent phenotypic outcomes from the same genome. They have become an important system for exploring how epigenetic systems enable phenotypic plasticity. Here we used a multiomics approach to obtain different perspectives on the divergent epigenomic landscapes of worker and queen bees.

The methods we used (Hi-C, ATAC-seq, ChIP-Seq and RNA-Seq) gave us different types of information on differences in genomic regulation between the worker and queen castes. We found differences in 3D chromosome structure and dramatically different proportions of accessible chromatin between queen and worker larvae (Fig.1 and 2). The Hi-C results detected hundreds of differences in A/B compartments, TAD boundaries and loops between queen and worker larvae at both 2-day and 4-day larval stages. Changes in A/B compartment, TAD boundaries and loops and play a critical role in regulation of gene expression[34, 35, 43-45], and previous studies in other animals have also shown that chromosome structure is involved in phenotypic plasticity [46, 47]. Here we found many DEGs related to these distinct A/B switch areas, TAD boundaries and loops (Fig. 1f-1i, Fig. S2a). These DEGs were enriched for key pathways such as TOR, IRS, Notch, Hippo etc[19, 20, 48] (see Fig. S2c-S2f). Therefore our findings suggest that differences in chromosome structure are involved in honeybee caste differentiation.

ATAC-seq assays genome-wide accessible chromatin. Our results found differences of unique peaks and significantly different peaks between queen and workers (Fig. 2a and 2b). Thousands of DEGs were associated with these unique or significantly different peaks, of which many were involved into the determination of caste differentiation (Fig. 2d and 2e). In our 4Q sample we observed a notable increase in unique accessible chromatin regions compared to 4W, 2Q and 2W (Fig. 2a). The 4Q samples were fed with royal jelly and were undergoing extremely rapid growth and development[16]. The large-scale open accessible chromatin in 4Q could indicate increased transcriptional processing to support their active metabolism. In support of this conclusion more DEGs were up-regulated in 4Q (1355) than 4W (951), (Table S10). These results indicate that the development of queen-worker dimorphism involves distinct patterns of accessible chromatin.

ChIP-seq allowed us to explore differences in histone modification between workers and queens. Our results were similar to Hi-C and ATAT-seq results and were consistent with a previous study that H3K27ac participates in the regulation of honeybee caste differentiation [24].

All three epigenetic modifications we explored (histone modification, chromatin accessibility and chromosome structure) had positive correlations with gene expression (Fig. 1d, 1e, 2c, 3c, 3d). Chromosome structure was positively correlated with chromatin accessibility and histone modification (Fig. S5), emphasizing how these epigenetic modifications can interact with each other to regulated gene expression[49-53]. Our data suggest that gene expression differences underlying caste differentiation are regulated by an interacting system of epigenetic modifications rather dominated by a single modification (Fig. 4 and 5) revealing very different epigenomic landscapes for developing worker and queen bees.

These different epigenomic landscapes clearly develop during the course of larval development. The differences in chromosome structure, chromatin accessibility and histone modification between workers and queens at the early developmental stage (see Fig. 1e-1i, 2d, 2e, 3a, 3e, 3f) , were not very pronounced and they had little association with DEGs (Fig. 4a) or key DEGs known to be involved in the determination of caste differentiation (Fig. 4c). By contrast, at the later day 4 developmental stage differences in all three types of epigenetic modifications increased and were more highly overlapped with DEGs (Fig. 4b).

It is clear that there are not distinct pre-formed epigenomic programs for workers and queens that control different developmental pathways. Instead the distinct epigenomic landscapes of workers and queens developed in concert with the diverging worker and queen phenotypes in development. A similar phenomenon is seen in tissue development in early embryogenesis. In early blastula stages different regions of the embryo are pre-patterned by epigenomic markers that accumulate more and more epigenomic changes as the embryo grows and develops to ultimately establish different tissue types[30-32].

As the worker and queen larvae developed, we documented an increase in both extent and type of epigenomic differences, and a greater correlation between types of epigenomic modification and gene expression differences. By the 4d developmental point, many more DEGs were also differentially regulated by more than one epigenomic system than at the 2d developmental stage (Fig. 5a, 5b). This pattern was even more pronounced for our 58 key DEGs that have been associated with caste differentiation (Fig. 5e, 5f). The implication is the development of a layering of epigenomic control of gene expression differences.

316 Many studies have shown that different epigenetic modifications can interact
317 with each other to regulate gene expression[49-53]. This might serve to canalise critical
318 functional differences in gene expression between worker and queen phenotypes.
319 Phenotypic plasticity depends on the establishment of stable divergent developmental
320 outcomes. The stability of epigenetic regulation of gene expression depends on both
321 the degree of environmental change and the state of the epigenomic architecture[10]. We
322 see some evidence of the establishment of this stability during queen development. In
323 honeybees, young worker larvae (< 3.5 day old) can be successfully transformed into
324 adult queens by feeding them with more nutritional diets, but once worker larvae are
325 older than this no dietary change is capable of transforming them into queens[25, 26]. Our
326 4d samples were beyond this point at which a worker is committed to the worker
327 developmental path. Genomic-wide chromosome structures were significantly
328 different between workers and queens by this point (Fig. 1b and 1c), indicative of
329 extensive and layered changes in the epigenomic landscape by this later
330 developmental stage that cannot be easily reversed.

331 In support of this hypothesis is the observation that DEGs that have previously
332 been linked to honeybee caste differentiation had both more extensive and more
333 diverse differences in epigenomic regulation than all other DEGs (Fig. 5d). In the 4d
334 worker/queen larval comparison a higher proportion of the 58 key DEGs related to
335 caste differentiation differed in two or three different epigenetic modifications (Fig.
336 5f). From this we infer that gene expression differences that are critical to the
337 formation of the distinct worker and queen phenotypes become canalized in
338 development through regulation by multiple degenerate epigenomic systems.

339 To test our inference that genes of key importance for queen/worker differences
340 are subject to the most extensive epigenomic regulation we selected the candidate

DEG (*4CL*) for RNA interference (RNAi) verification. This important gene can catalyze *p*-coumaric acid synthesis in honeybee larval diets and potentially affect their ovary development, energy metabolism and lipid metabolism[17, 40-42]. This gene differed between 4W and 4Q in all four omics assays (Fig.6a). RNAi knock down of expression of this key gene resulted in newly emerged queens of lower weight, smaller body size and fewer ovariole numbers than controls (Fig. 6b-6e). This confirms the importance of expression of this gene for caste differentiation, and it is notable that this gene was also subject to the most extensive epigenomic differences between workers and queens.

Honeybee phenotypic plasticity and caste differentiation is an outcome of millions of years of evolution[54]. This is achieved through different epigenomic regulation of the bee genome during development. Our results combined with previous studies demonstrated that various epigenetic modifications including DNA methylation, RNA methylation, histone modification, non-coding RNA, chromosome structure, chromatin accessibility and poly(A) tails all involved in bee phenotypic plasticity[21-24, 44, 55-57]. The key insights from this present study are that different kinds of epigenomic modification work together to establish the different worker and queen phenotypes. The very different epigenomic landscapes of the worker and queen are not programs. They are not pre-established to control the running of different developmental paths. Instead the epigenomic landscapes diverge during development as the worker and queen phenotypes diverge. The epigenomic landscape appears to be simultaneously regulatory of development and part of the developmental process. Rather than considering the epigenome as a program controlling development it appears to be part of the developmental program. Rather than considering epigenomics as programs controlling phenotypic plasticity, it is perhaps more useful

to consider epigenomics as part of a developmental autoshaping process. In autoshaping, a change in shape cause subsequent changes that reinforce the change, or small differences case a cascade that increase the divergence. Workers and queens seem to develop in this way. Differences in developmental environment cause a cascade of reinforcing developmental and epigenomic differences that result in the divergent worker and queen phenotypes.

Methods

Insects

Honeybees (*Apis mellifera*) were from Jiangxi Agricultural University (28.46uN, 115.49uE), Nanchang, China, in 2020. A queen was restricted for 6h (8 am-2 pm) to a plastic frame designed by Pan et al[58] to lay eggs in worker cells. The queen lays her eggs on a removable plastic base, which could be transferred to a plastic queen cell without touching the egg itself. Half of the eggs were transferred to queen cells at 2 pm on the second day after laying and before hatching, while the other half remained in the worker cells. All eggs (in both queen and worker cells) were cared for by workers. Eggs hatch on the third day after laying. To collect two-day and four-day old larvae, we sampled larvae from both queen and worker cells at 4 pm on the 5th and 7th day after laying. Larvae were picked with sterilized tweezers and rinsed in ddH2O 3 times. Filter paper was used to drain the water from the larvae, and larvae were placed immediately in liquid nitrogen.

Hi-C

The reference genome used throughout this paper was Amel_HAv3.1 (GCF_003254395.2) downloaded from the NCBI. In situ Hi-C was performed using larval samples, with minor modification to previously described methods[59]. Hi-C

libraries were sequenced with paired-end, 150 bp reads on an Illumina Hiseq3000. Reads were filtered for contaminants, then were processed with Homer (version 4.11; http://homer.ucsd.edu/homer/) to produce genome-wide contact maps[60]. Principal component values were produced using the HiCExplorer, and A and B compartments were assigned to each 100-kb window according to the sign of the first component (PC1) values. We would reverse the sign of eigenvalues based on gene density content[61]. After correction, the positive value was the A compartment and the negative value was the B compartment.

ATAC-seq

ATAC-seq was performed using a modification of the method described by Corces et al[62]. Briefly, approximately 100 mg of larvae were ground and the nuclei were extracted, then the nuclei pellet was re-suspended in Tn5 transposase reaction mix. The transposition reaction was incubated at 37°C for 30 min. Equimolar Adapter1 and Adatper2 were added after transposition. PCR was then performed to amplify the library. ATAC libraries were sequenced on an Illumina Hiseq3000 and 150 bp paired-end reads were generated.

We employed Burrows Wheeler Aligner (BWA, version 0.7.12) to align the ATAC-seq reads to the honeybee genome as above. After mapping reads to the reference genome, we used the Model-based Analysis of ChIP-Seq (MACS) (version 2.1.0; https://github.com/taoliu/MACS/) to identify peaks with parameters as reported previously[62, 63]. A P-adj enrichment threshold of 0.05 was used for all data sets. The analysis of different peaks (Fold change ≥ 2) is based on the folding enrichment of different experimental peaks. ChIPseeker[64] was used to identify the nearest Transcription Start Sites (TSS) of every peak and the distance distribution between peaks and TSS was shown. An analysis of the distribution of peak summits on

different functional regions of the gene, such as 5'-untranslated region (5'UTR), 3'-untranslated region (3'UTR), distal intergenic, coding DNA sequence (CDS), was performed. Peak related genes were confirmed by PeakAnnotator[65], and then KOBAS software (version 3.0) was used to test the statistical enrichment of peak related genes in KEGG pathways [66].

ChIP-seq

Chromatin immunoprecipitation was performed as described by Wojciechowski et al[24] with slight modifications. Approximately 800 mg of larvae were cross-linked for 10 min in 1% ChIP-seq–grade formaldehyde. The antibody used for immunoprecipitation was H3K27ac (ab4729, Abcam). The H3K27ac library was sequenced (50 bp single-end reads or 150 bp paired-end reads) on an Illumina Hiseq3000 sequencer.

We employed Burrows Wheeler Aligner (BWA; version 0.7.12) to align the ChIP-Seq reads to the honeybee genome. After mapping reads to the reference genome, we used the MACS finding algorithm to identify regions of Immunoprecipitation (IP) enrichment over background (inputs). A p-adj enrichment threshold of 0.05 was used for all data sets. The analysis of different peaks (Fold change $\geq$ 2) was based on the folding enrichment of different experimental peaks. ChIPseeker[64] was used to identify the nearest TSS of every peak and the distance distribution between peaks and TSS was shown. Besides, the distribution of peak summits on different function regions of the gene structure, such as 5' UTR, 3'UTR, distal intergenic, CDS, was performed. Peak related genes were confirmed by PeakAnnotator[65], and then KOBAS software was used to test the statistical enrichment of peak related genes in KEGG pathways.

RNA-seq

RNA-seq was performed as previously reported[67]. Total RNA was extracted from 100mg larvae. Paired-end 150-cycle sequencing was performed on Illumina Hiseq3000 sequencers according to the manufacturer's directions.

We employed Hierarchical Indexing for Spliced Alignment of Transcripts (HISAT; version 2.0.5) to align the RNA-Seq reads to the reference genome. Expression levels were reported as Fragments Per Kilobase Million (FPKM) to normalize for the length of annotated transcripts and for the total number of reads aligned to the transcriptome. Analysis of differential expression was performed using the DESeq2 R package (1.32.0). The p-value was corrected for multiple comparisons with a false discovery rate (FDR< 0.05). Genes with P-adj ≤ 0.05 were defined as differentially expressed genes.

Application of RNAi

One-day-old worker larvae were fed with semi-artificial diet in a petri dish according to previous method[17] and were incubated at 34℃and 75% humidity. Artificially manufactured siRNA for *4CL* (F: GGUGAAAGAUAUGCUAAUATT; R: UAUUAGCAUAUCUUUCACCTT) was added to the semi-artificial diet, with a final concentration of 100 μg/ml. Similarly the NCsiRNA (F: UUCUUCGAACGUGUCACGUTT; R: ACGUGACACGUUCGG AGAATT) was added into semi-artificial diet and fed other larvae as a control group. Each group had four biological replicates. Some of the larvae fed with the above siRNA diets for two days and four days were taken for qRT-PCR validation to verify the effect of RNAi on the expression of the *4CL* gene. Other larvae were reared until emergence and these newly emerged bees were weighed using an analysis balance (accuracy: 0.1 mg, ME204, METTLER) and photographed under a microscope (6.5X, GL99TI, VISHENT). Samples then were used for ovariole counts.

Paraffin sectioning of the queen ovary

The left ovaries of the newly emerged samples were dissected under a microscope. Ovaries were then fixed in 4% paraformaldehyde fix solution for 16 h and paraffin sections of the ovary were created using methods described by Yi et al[68]. The sections were photographed and overioles counted under the same microscope (40X) according to our previous methods[68].

Data availability

Raw sequencing reads for Hi-C ATAC-seq, ChIP-seq and RNA-seq are available at SRA accession PRJNA770835.

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Figure legends

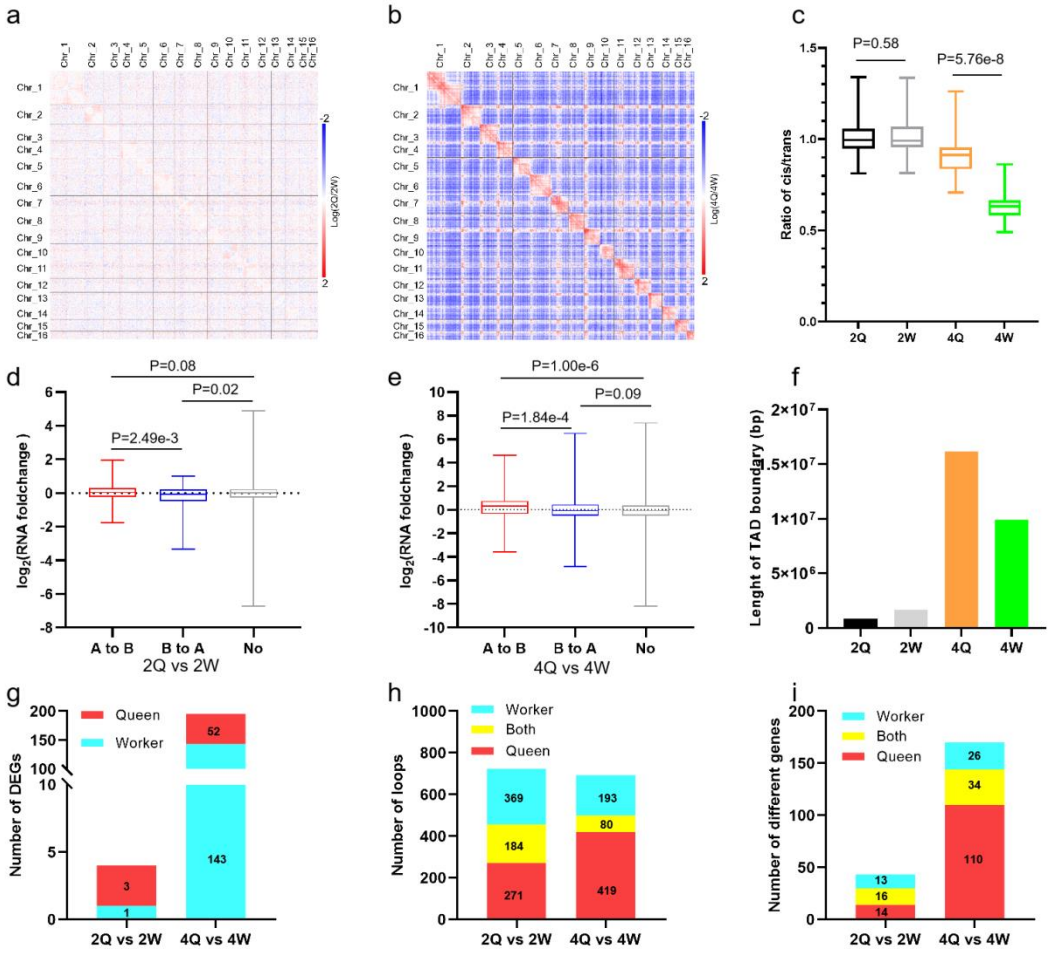


Figure 1. Hi-C analysis of queen and worker larvae. (a) Interaction map of 2Q vs 2W (observed/control). ChIP-seq for H3K27ac, as well as ATAC-seq and RNA-seq are shown below each stage of 2Q and 2W. **(b)** Interaction map of 4Q vs 4W (observed/control). In A and B The sixteen *Apis mellifera* chromosomes (chr) are shown from left to right and top to bottom. Chromosomes are separated by thin black bars. **(c)** The cis and trans ratios in queen and worker larvae. **(d and e)** Box plot comparing gene expression fold changes between genes in switch regions (A-B and B-A) and no switch regions (A-A and B-B) in 2Q/2W and 4Q/4W, respectively. **(f)** Length of TAD boundaries in larval samples. **(g)** Number of DEGs located at TAD boundary, **(h)** Number of loops and **(i)** Number of DEGs located at loop in comparisons of 2Q and 2W larvae and 4Q and 4W larvae.

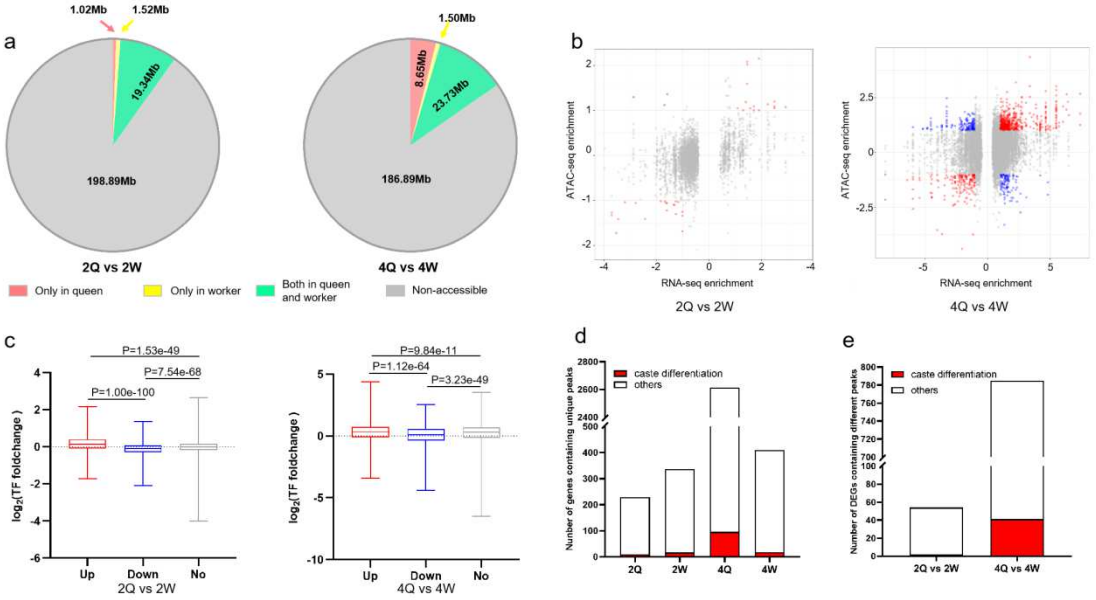
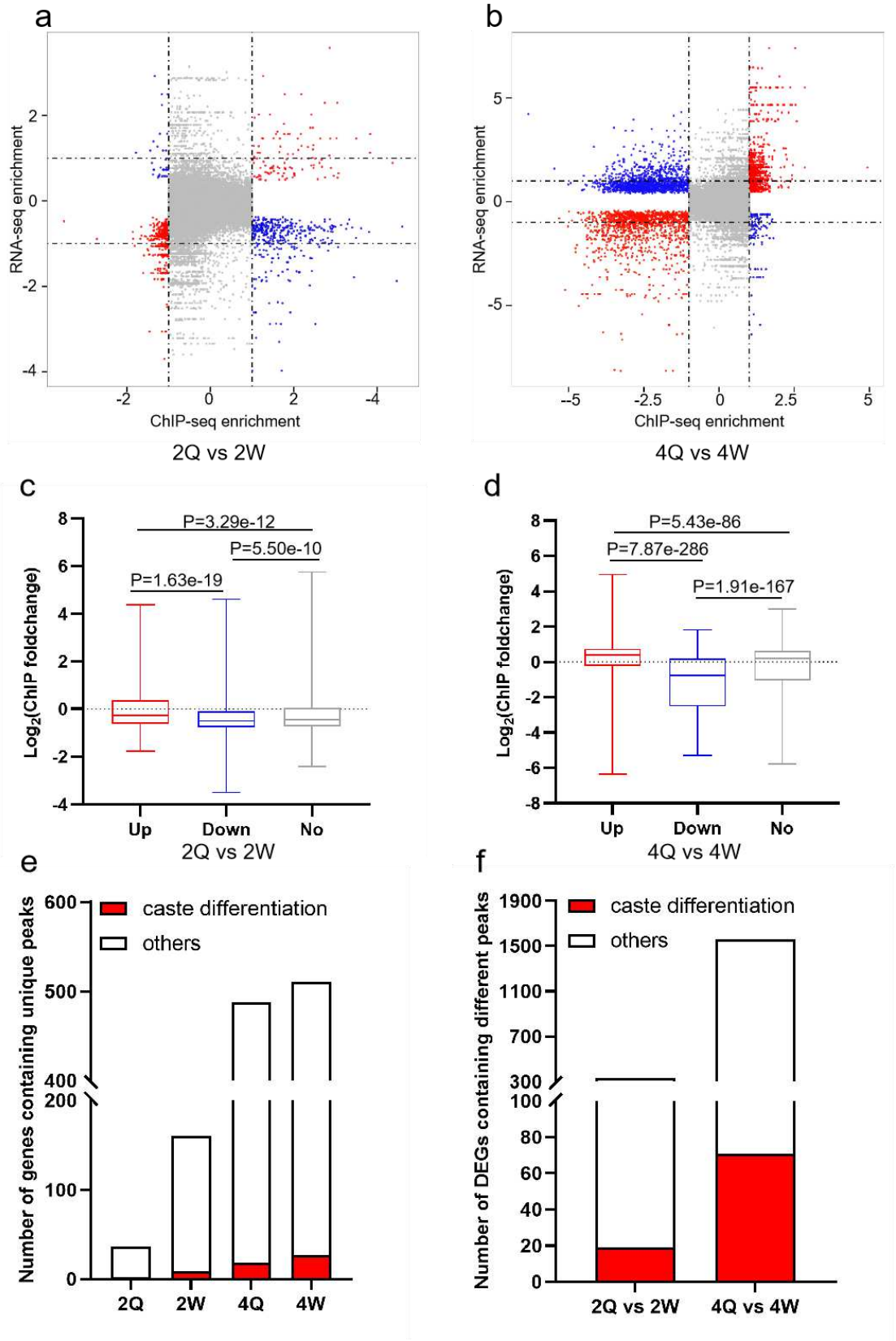


Figure 2. ATAC-seq analysis of queen and worker larvae. **(a)** Pie chart of comparison of accessible regions in 2Q and 2W, and 4Q and 4W. **(b)** Scatter plot of the difference in significant ATAC-seq enrichment between queen and worker (y-axis) against the Log2FC of transcript expression between queen and worker (x-axis). **(c)** Box plots comparing transcription factor (TF) expression fold changes between protein coding genes in comparisons of 2Q and 2W, and 4Q and 4W, respectively. **(d)** Number of genes containing unique ATAC-seq peaks in queen or worker larvae, and **(e)** Number of DEGs containing significantly different ATAC-seq peaks between queen and worker larvae. The red part in each bar represents the number of genes that are involved in caste differentiation.



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645 **Figure 3. ChIP-seq analysis of queen and worker larvae. (a and b)** Scatter

646 plots of the significant differences in ChIP-seq enrichment between queen worker

larvae samples (x-axis) against the Log2FC of transcript expression between queen worker larvae samples (y-axis). **(c and d)** Box plot comparing ChIP signal expression fold changes between protein coding gene in 2Q/2W and 4Q/4W, respectively. **(e)** Number of genes containing unique ChIP-seq peaks in queen or worker larvae. **(f)** Number of DEGs containing significantly different ChIP-seq peaks between queen and worker larvae. The red part in each bar represents the number of genes that are involved in caste differentiation.

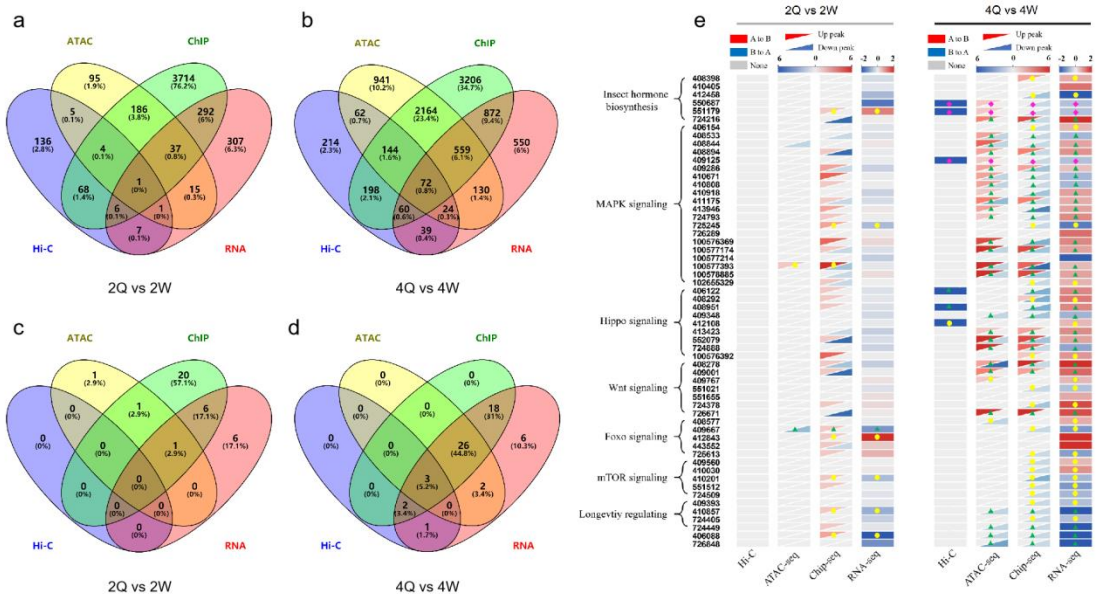


Figure 4. Multiomics analysis between queen and worker larvae. Venn diagram showing the overlap in DEGs identified by different omics methods in 2Q/2W comparisons **(a)** and 4Q/4W comparisons **(b)**. **(c and d)** As **(a)** and **(b)**, but focused only on DEGs associated with caste differentiation. **(e)** Summary of differences in epigenetic regulation of genes associated with caste differentiation in queen and worker larvae comparisons. Yellow circle means there were significant differences observed in two different omic analyses. Green triangle means there were significant differences observed in three different omic comparisons. Pink square means there were significant differences observed in four different omic comparisons.

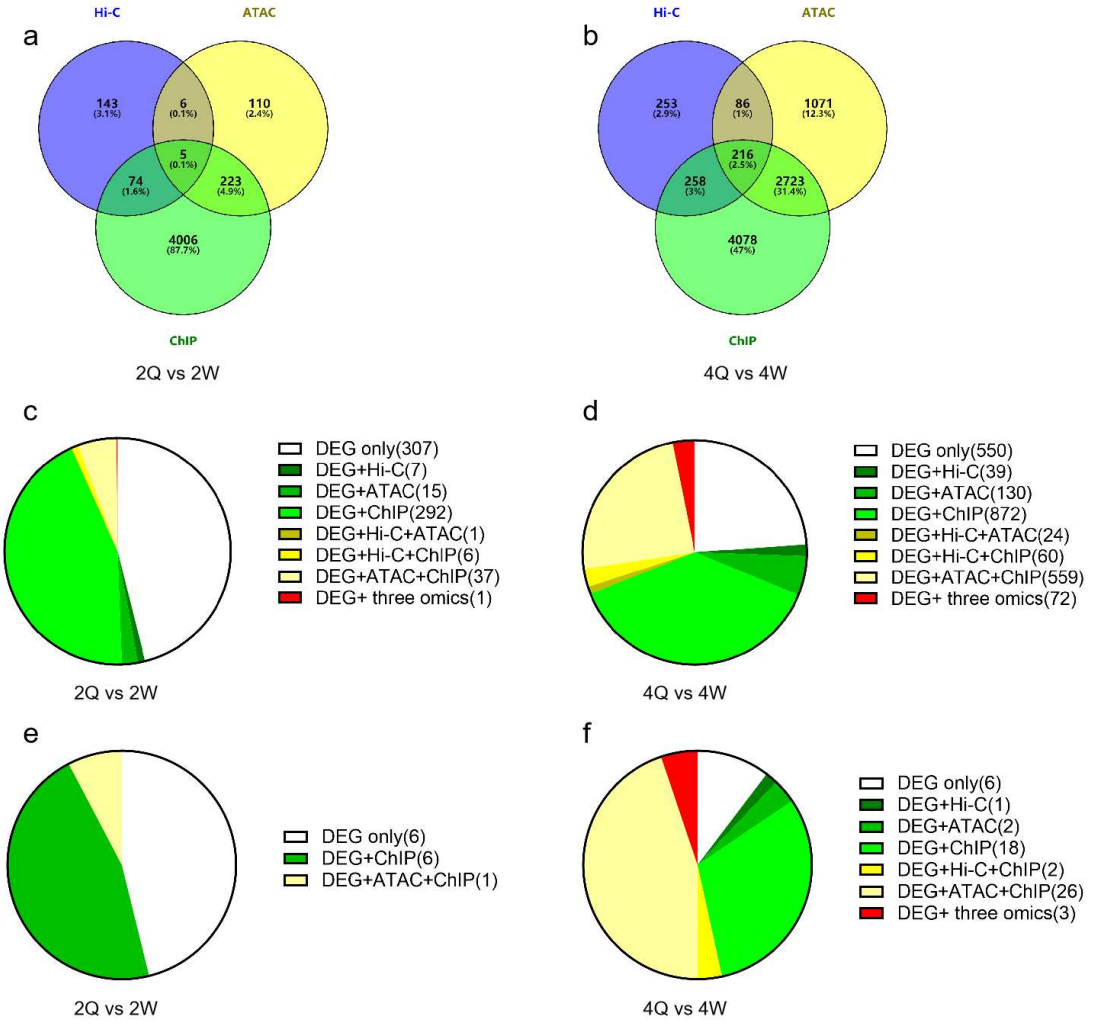
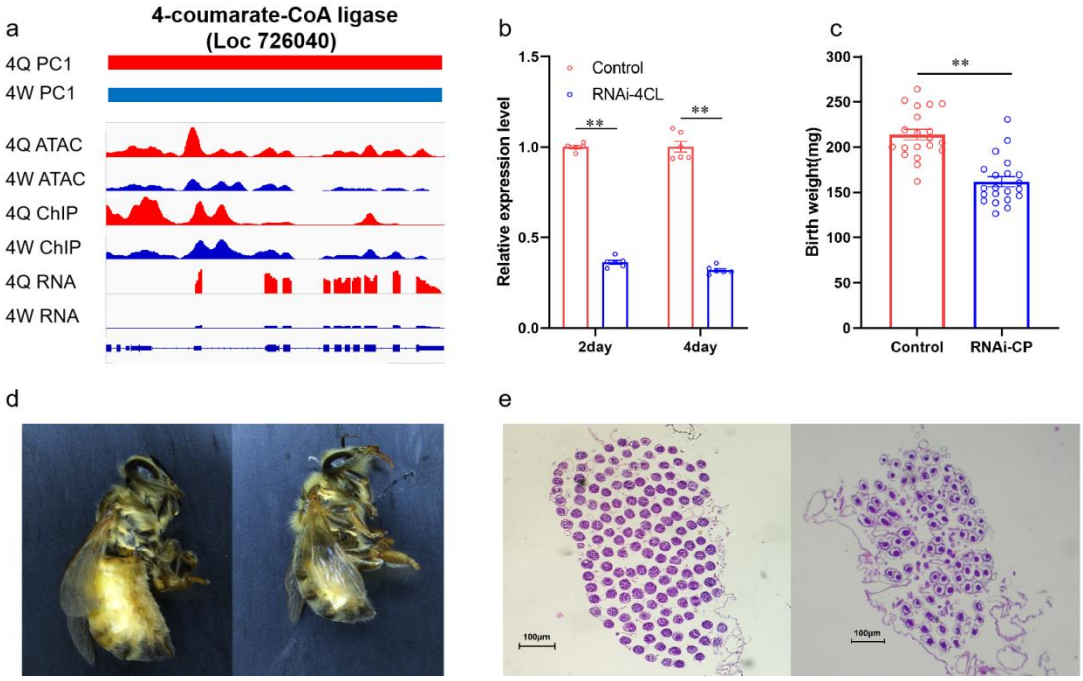


Figure 5. Three epigenetic modifications overlapped with gene expression. Venn diagrams showing the number of genes identified as deferentially regulated in Hi-C, ATAC-seq and ChIP-seq analyses of 2Q/2W **(a)** and 4Q/4W **(b)**. Pie chart of number of genes that differ in epigenetic regulation in comparisons of 2Q/2W (c) and 4Q/4W **(d)**. Pie chart of number of 58 key genes that differ in epigenetic regulation in comparisons of 2Q/2W **(e)** and 4Q/4W **(f)**.



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672 **Figure 6.** RNAi **verification of the *4CL* gene.** **(a)** Example showing the
673 correlation of Hi-C PC1, ATAC signal, ChIP signal and RNA-seq reads in 4Q and
674 4W for the *4CL* gene. The *4CL* gene located in the A compartment in 4Q and
675 switched to the B compartment in 4W. **(b)** Gene expression in siRNA fed and control
676 larvae. *p<0.01 by t test. **(c)** Birth weight of queens between siRNA group and
677 control group. *p<0.01 by Mann-Whitney U test. **(d)** Photo of queen after feeding
678 siRNA reagent, left is the control group and right is the siRNA group. **(e)** Section of
679 the newly-emerged queen's ovariole, left is the control group and right is the siRNA
680 group.

Supplementary figures

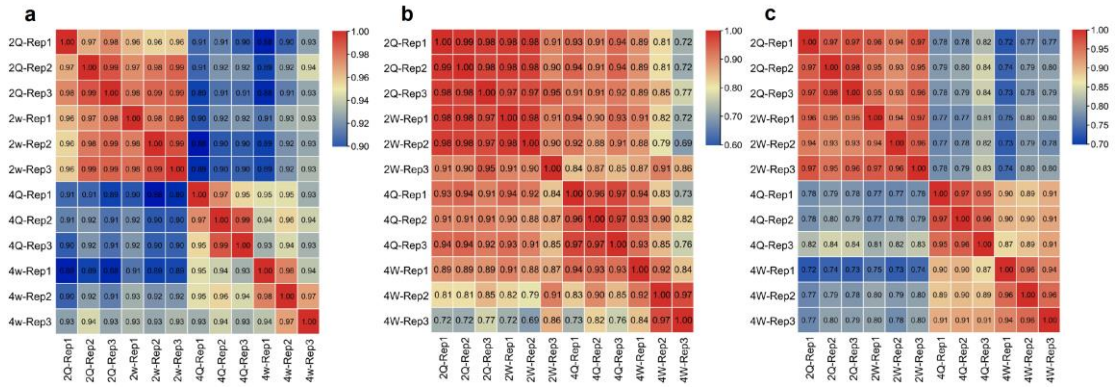


Figure S1. Multi-omics comparative analyses of queen and worker larvae. (a-c) Correlation analysis of ATAC-seq, ChIP-seq and RNA-seq sequencing libraries of queen and worker larvae. 2Q: second-instar queen larvae. 4Q: fourth-instar queen larvae. 2W: second-instar worker larvae. 4W: fourth-instar worker larvae.

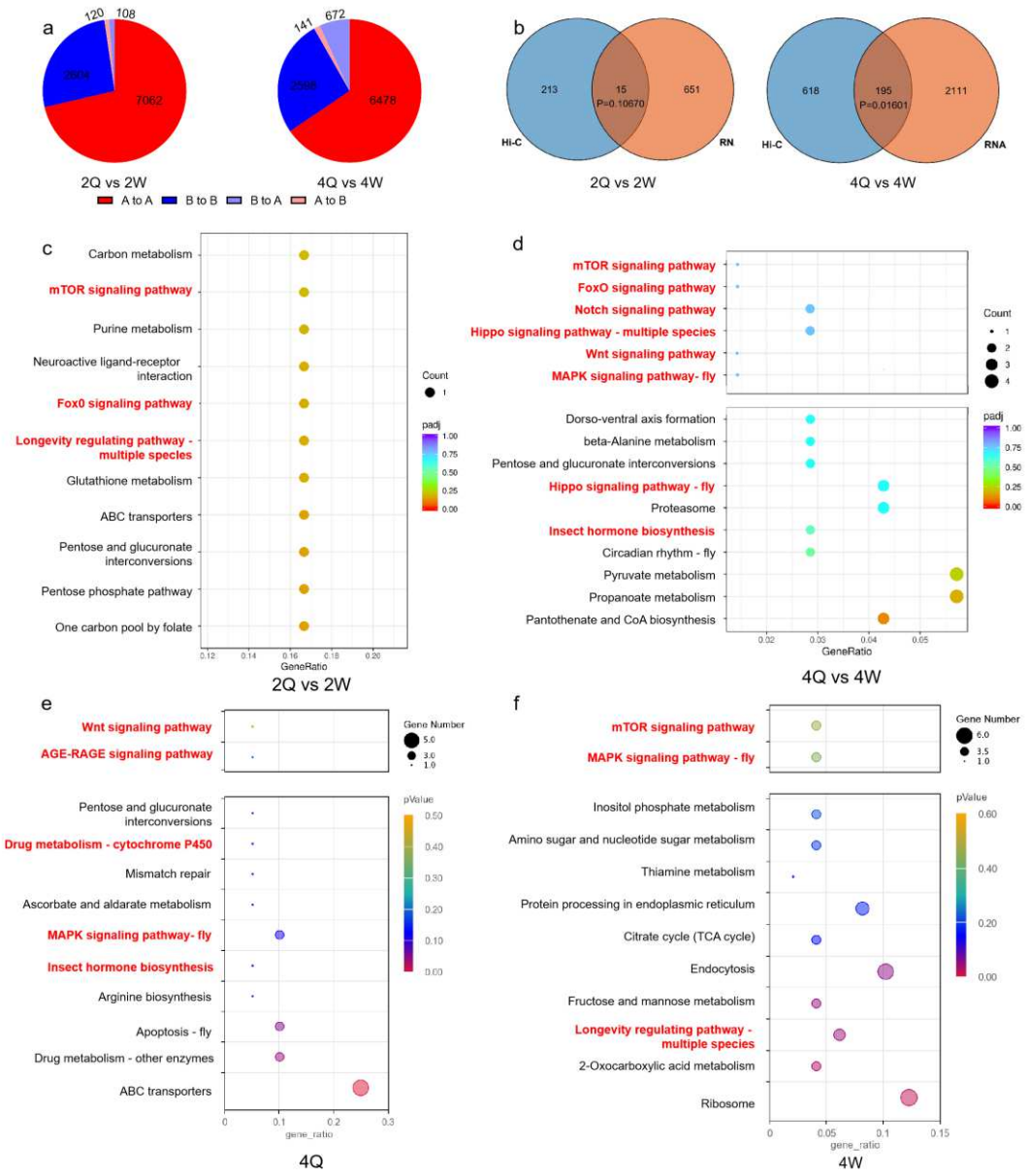


Figure S2. (a) Pie plot showing local compartment numbers divided by stable (A-A and B-B) and switching (A-B and B-A) compartments in 2Q vs 2W and 4Q vs 4W, respectively. **(b)** Venn diagrams of genes of A/B switched and differentially expressed genes. p values of the overlap between the two datasets were calculated by performing fisher's exact test with the total number of *Apis mellifera* genes we've detected (10366) as the reference. **(c-d)** KEGG pathway of differentially expressed genes identified by both Hi-C and RNA-seq in queen and worker. Bold red indicates that this pathway is associated with caste differentiation, and the bottom KEGG

696 pathway is the top 10 significant pathway. **(e)** KEGG pathway of DEGs in 4Q. **(f)**
697 KEGG pathway of DEGs in 4W. Bold red indicates that this pathway is associated
698 with caste differentiation, and the bottom KEGG pathway is the top 10 significant
699 pathway.

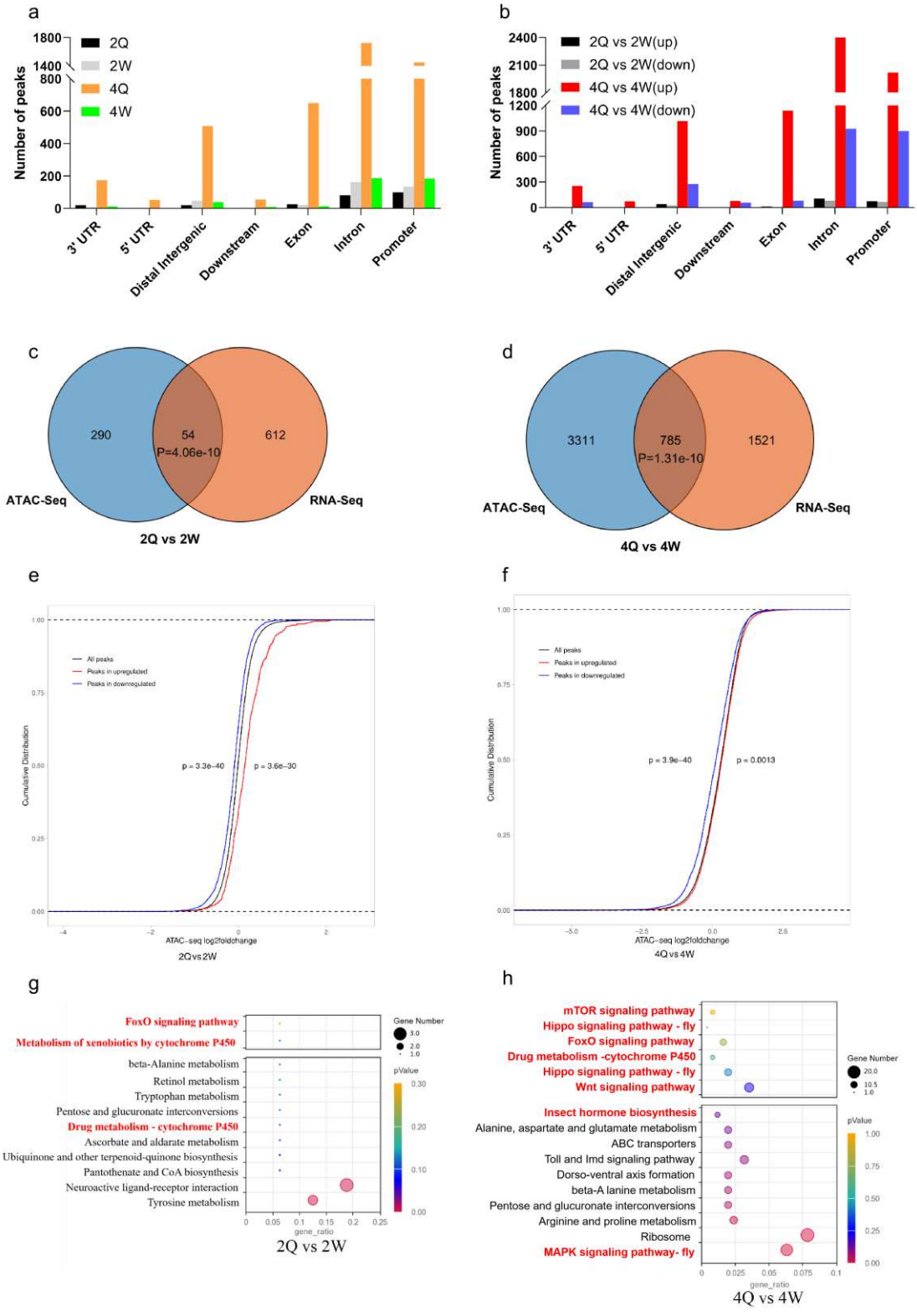
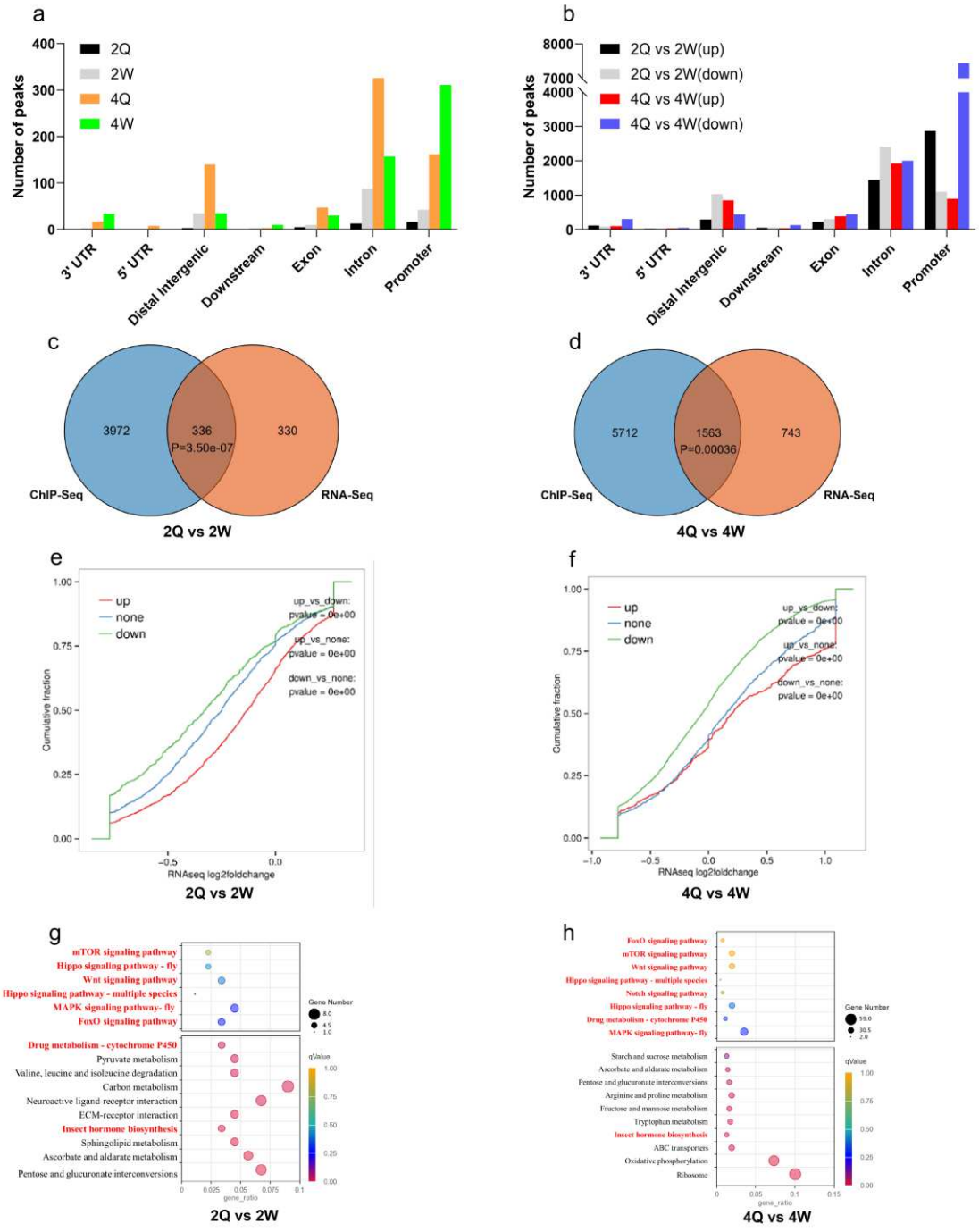


Figure S3. ATAC-Seq and RNA-Seq comparative analysis of queen and worker larvae. **(a)** The proportion of accessible chromatin in different regions of the genome. **(b)** The proportion of different peaks between queen and worker bees in each genome

region. **(c-d)** Venn diagrams of differentially expressed genes associated with differences in accessible chromatin peaks. p values of the overlap between the two datasets were calculated by performing fisher's exact test with the total number of *Apis mellifera* genes we've detected (10366) as the reference. **(e-f)** Differential gene-related ATAC-seq signal accumulative distribution diagram. **(g-h)** KEGG pathway of ATAC-seq and RNA-seq common differential genes in queen and worker. Bold red indicates that this pathway is associated with caste differentiation, and the bottom KEGG pathway is the top 10 significant pathway.



712

713 **Figure S4.** ChIP-Seq and RNA-Seq comparisons of queen and worker larvae. **(a)**
714 The proportion of accessible chromatin in different regions of the genome. **(b)** The
715 proportion of different peaks between queen and worker bees in each genome region.
716 **(c-d)** Venn diagrams of differentially expressed genes associated with differences in
717 peaks. p values of the overlap between the two datasets were calculated by performing
718 fisher's exact test with the total number of *Apis mellifera* genes we've detected (10366)

as the reference. **(e-f)** Differential gene-related ChIP-seq signal accumulative distribution diagram. **(g-h)** KEGG pathways of ATAC-seq and RNA-seq common differentially regulated genes in queen and worker. Bold red indicates that this pathway is associated with caste differentiation, and the bottom KEGG pathway is the top 10 significant pathway.

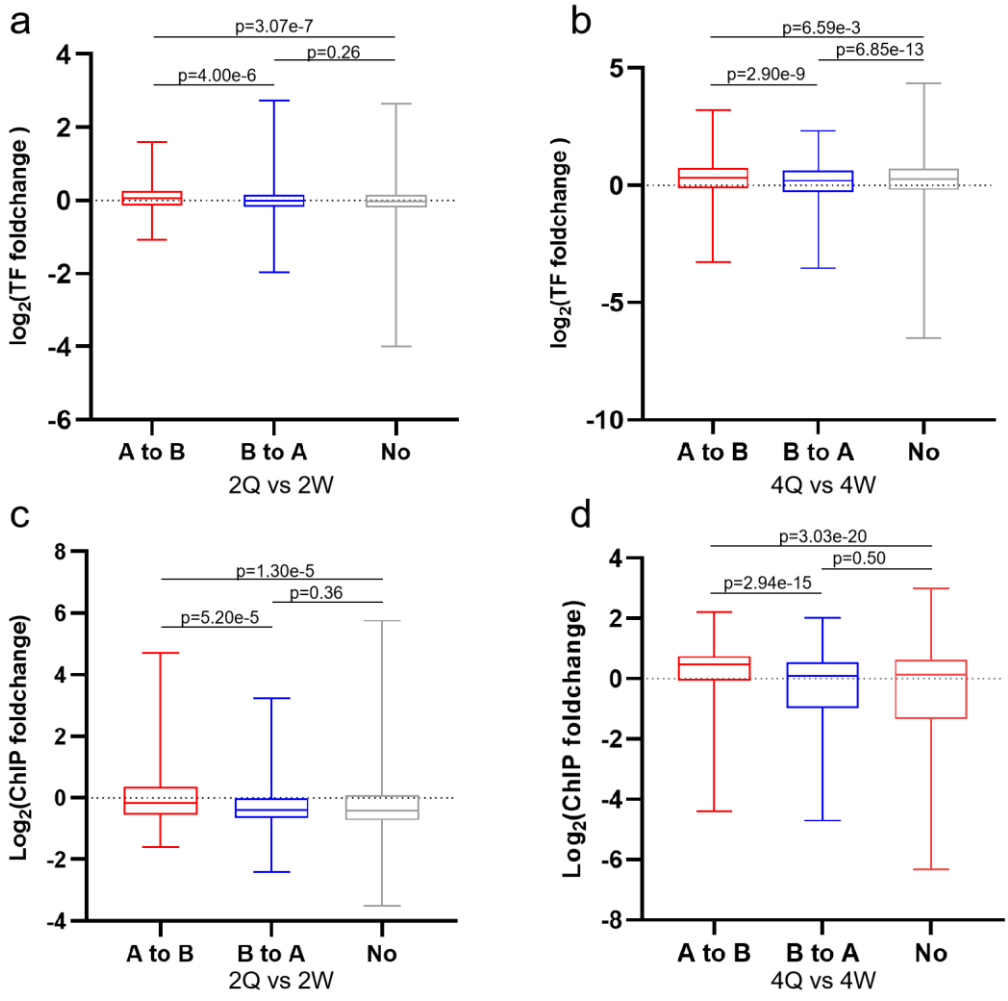


Figure S5. (a-b) Box plot comparing TE expression fold changes between TE in switch regions (A-B and B-A) in 2Q vs 2W and 4Q vs 4W. **(c-d)** Box plot comparing ChIP signal fold changes between switch regions (A-B and B-A) in 2Q vs 2W and 4Q vs 4W.

Table S1 Summary of Hi-C sequencing data quality

Table S2 Summary of ATAC sequencing data quality

731 Table S3 Summary of Chip sequencing data quality

732 Table S4 Summary of RNA sequencing data quality

733 Table S5 The position where A/B switched occurs between queen and worker

734 Table S6 Number of unique ATAC peaks from 2Q and 2W

735 Table S7 Number of unique ATAC peaks from 4Q and 4W

736 Table S8 Number of unique ChIP peaks from 2Q and 2W

737 Table S9 Number of unique ChIP peaks from 4Q and 4W

738 Table S10 The list of DEGs in 2d and 4d queen-worker comparisons

739

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS14.docx
- TableS6.xlsx
- TableS5.xlsx
- TableS8.xlsx
- Fig.S5.tif
- TableS10.xlsx
- Fig.S3.tif
- Fig.S4.tif
- Fig.S1.tif
- Fig.S2.tif
- TableS7.xlsx
- TableS9.xlsx