Insect Biochemistry and Molecular Biology 155 (2023) 103929

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

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

Yong Zhang [a,b,1], Xu Jiang He [a,b,1], Andrew B. Barron [c], Zhen Li [a,b], Meng Jie Jin [a,b], Zi Long Wang [a,b], Qiang Huang [a,b], Li Zhen Zhang [a,b], Xiao Bo Wu [a,b], Wei Yu Yan [a,b], Zhi Jiang Zeng [a,b,*]

[a] Honeybee Research Institute, Jiangxi Agricultural University, Nanchang, Jiangxi, 330045, PR China
[b] Jiangxi Province Honeybee Biology and Beekeeping Nanchang, Jiangxi, 330045, PR China
[c] Department of Biological Sciences, Macquarie University, North Ryde, NSW, 2109, Australia

ARTICLE INFO

Keywords:
Honeybees
Caste differentiation
Development
Epigenetic modifications
Gene expression

ABSTRACT

The role of the epigenome in phenotypic plasticity is unclear presently. Here we used a multiomics approach to explore the nature of the epigenome in developing honey bee (*Apis mellifera*) workers and queens. Our data clearly showed distinct queen and worker epigenomic landscapes during the developmental process. Differences in gene expression between workers and queens become more extensive and more layered during the process of development. Genes known to be important for caste differentiation were more likely to be regulated by multiple epigenomic systems than other differentially expressed genes. We confirmed the importance of two candidate genes for caste differentiation by using RNAi to manipulate the expression of two genes that differed in expression between workers and queens were regulated by multiple epigenomic systems. For both genes the RNAi manipulation resulted in a decrease in weight and fewer ovarioles of newly emerged queens compared to controls. Our data show that the distinct epigenomic landscapes of worker and queen bees differentiate during the course of larval development.

1. 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 (Skvortsova et al., 2018; Nacev et al., 2020; Tsai and Cullen, 2020). These mechanisms include biochemical modifications of the genome (such as DNA methylation), modulation of the affinity of DNA to proteins, that provide structural support to the DNA molecule, and changes to the folding of the DNA molecule and/or the chromosome to alter the accessibility of sections of DNA to gene transcription machinery (Robertson, 2005; Karlić et al., 2010; Krijger and De Laat, 2016; Verhoeven et al., 2016; Luo et al., 2022). Multiple different specific mechanisms exist in each of these classes. The complexity of the epigenome is such that metaphor of the "epigenomic landscape" has been used to capture the multidimensional structural complexity and diversity of the epigenome (Berdasco and Esteller, 2010; Rijnkels et al., 2010; Herman et al., 2014). The epigenomic landscape was first described by Conrad Waddington, who believed that during development, cells are affected by different epigenetic factors, resulting in different forms of cell development (Waddington and Kacser, 1957).

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 (West-Eberhard, 1989; Whitman and Agrawal, 2009). The interactions between the genome and the environment, and the developmental pathways that lead to different phenotypic outcomes are all functions of the epigenome (Duncan et al., 2014; Schlichting and Wund, 2014).

Waddington famously considered the process of development as a progressive series of developmental decisions, which he conceptualized as forked paths in an imagined developmental landscape. Two identical entities (two single cells in an organism or two embryos) that took

* Corresponding author. Honeybee Research Institute, Jiangxi Agricultural University, Nanchang, Jiangxi, 330045, PR China.
E-mail address: bees1965@sina.com (Z.J. Zeng).
[1] Y.Z. and X.J.H. contributed equally to this work.

https://doi.org/10.1016/j.ibmb.2023.103929
Received 7 December 2022; Received in revised form 27 February 2023; Accepted 2 March 2023
Available online 10 March 2023

different paths at a fork early in development could find themselves channeled (or canalized) along progressively more divergent paths to ultimately develop into irreversibly distinct forms. In this way an undifferentiated cell could give rise to different tissues, or organisms could show developmental plasticity.

Waddington, and other researchers building on his frameworks, imagined that functional networks of genes could shape the bifurcating paths and valleys of the imagined developmental landscape (Waddington, 1942; Waddington and Kacser, 1957; Moris et al., 2016). Waddington's work preceded our contemporary understanding of epigenetic mechanisms. In the mid-1970s Riggs and Holliday both proposed that DNA methylation could affect gene expression, hence serve as an epigenetic regulatory mechanism. Further discoveries have added chromatin modifications, noncoding RNAs and miRNAs as other epigenetic modifications affecting gene expression and altering the operation of Waddington's functional gene networks. We might conceptualise the function of differential gene regulation in development in two different ways. Does differential gene regulation set the developmental landscape, establishing the valleys along which different developmental trajectories can flow? Or is differential gene regulation a response to moving through a developmental landscape, with different configurations of gene expression forming as diverging developmental paths canalize?

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 in phenotypic plasticity (Corona et al., 2016; Maleszka, 2018; Duncan et al., 2020). Young female larvae (less than 3.5 days old) can develop into two very different castes: queens and workers (Winston, 1991). The queen is large in size, with a long lifespan, large spermatheca and ovaries, and is usually the only female in the colony that can produce male and female offspring. The worker is small in size, with a short lifespan and usually infertile (Wilson, 1971; Michener and Michener, 1974). These 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 (Kamakura, 2011; Shi et al., 2011). We now understand that these nutritional differences cause many changes in the epigenetic 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) (Patel et al., 2007; Mutti et al., 2011). Developing queen and worker larvae are recognized to differ in extent and distribution of DNA methylation, histone modification, microRNA (miRNA) expression, m^6A modification and poly (A) tails (Kucharski et al., 2008; Shi et al., 2011, 2013; Wojciechowski et al., 2018; Yi et al., 2020; Wang et al., 2021a; He et al., 2022). Collectively these studies illustrate very different epigenomic profiles 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 (a caste that is morphologically intermediate between the queen and workers), or workers rather than queens (Weaver, 1957, 1966). There is a point (3.5 day) in developmental beyond which the worker developmental trajectory is irreversibly canalized and cannot be successfully redirected to yield the queen phenotype.

In this study we examined how the epigenomic profiles of the queen and worker change during honeybee during development. We used a multiomic approach to assess different aspects of the epigenome of workers and queens sampled before and after the 3.5 day developmental point at which the worker/queen trajectories are canalized. We compared gene expression levels with RNA sequencing. We compared histone modifications using chromatin immunoprecipitation with high-throughout sequencing (ChIP-seq). We compared which regions of the chromosome 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 (Sikorska and Sexton, 2020; Eres and Gilad, 2021). ATAC-seq can distinguish the compartments of the genome in which genes are more or less likely to be transcribed (Klemm et al., 2019; Agbleke et al., 2020). ChIP-seq assays histone modifications associated with different genes, which might influence the affinity of the DNA for the histone protein (Grant, 2001; Zentner and Henikoff, 2013). These three assays each give different perspectives on biochemical processes that regulate gene expression, whereas RNA-seq measures the outcome of that regulation: the 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 pathway is canalized. This approach allowed us to study how the distinct epigenomic profiles of queen and worker bees develop. We also selected two key genes which were significantly different in all four omics for RNA interference (RNAi) to verify the functions of these genes in honeybee caste differentiation.

2. Material and methods

2.1. Insects

Honeybees (*Apis mellifera*) were from Jiangxi Agricultural University (28.46μN, 115.49μE), Nanchang, China, in 2020. Queens were restricted for 6h (8 a.m.-2 pm) to a plastic frame designed by Pan et al. (2013) to lay eggs in worker cells. The queen laid 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 p.m. on the second day after laying, while the other half remained in the worker cells. All eggs (in both queen and worker cells) were cared for by workers. Eggs hatched on the third day after laying. To collect 2-day and 4-day old queen and worker larvae, we sampled larvae from both queen and worker cells in each of three colonies at 4 p.m. on the 5th and 7th day after laying. Larvae were picked with sterilized tweezers and rinsed in ddH_2O three times. Filter paper was used to drain the water from the larvae, and larvae were placed immediately in liquid nitrogen. Whole larvae were used for sequencing, as in previous studies (Wang et al., 2021a; He et al., 2022), since they were very small (2-day worker and queen larvae: 1.63 mg/larva; 4 day worker larvae: 11.60 mg/larva; 4 day queen larvae: 25.92 mg/larva) (Stabe, 1930; Liu et al., 2021; Wang et al., 2021a; He et al., 2022). Because larvae of different ages differed in mass, we sampled different numbers of 2-day and 4-day larvae so that each of our samples delivered similar amounts of DNA or RNA before sequencing. The reference genome used throughout this paper was Amel_HAv3.1 (GCF_003254395.2) downloaded from the NCBI. The detailed methods of high-throughput sequencings (Hi-C, ATAC-seq, ChIP-seq and RNA-seq) described below. All omic analyses had three biological replicates.

2.2. Hi-C

In situ Hi-C was performed using larval samples, with minor modification to previously described methods (Rao et al., 2014). In 2-day queen (2Q) and worker (2W) larval groups each sample had 80 larvae, whereas in 4-day queen and worker larval ones each sample mixed 8 larvae. Hi-C was performed using a modification of the method

described by Szabo et al. (2018). 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 (Heinz et al., 2010). 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 (Wolff et al., 2018). After correction, the positive value was the A compartment and the negative value was the B compartment. Lieberman-Aiden et al. (2009) first found that A compartment is more open and has more transcriptional activation regions whereas B dcompartment has more chromatin inhibited regions (Lieberman-Aiden et al., 2009). TAD is a topological association domain and the frequency of gene interactions within a TAD is significantly higher than that between the adjacent two TADs, which establishes patterns of gene co-regulation (Burgess, 2022). According to the following hidden Markov model formula, our data were divided into thousands of windows on the chromosome with a length of 100 KB, and the directionality index value of each window was calculated. The size of TAD can be determined according to the directionality index value. A loop is a ring structure of chromosomal interaction, and the entrance of the ring is two regions of the chromosome that are close in space. The approach frequency here is multiple times that of other random approaches (Rao et al., 2014), so a peak can be formed. Based on this, we distinguished loops from the background (random connection) using the algorithm (HiCCUPS) (Durand et al., 2016), which is called call-peak and is often the action site of CCCTC-binding factor (CTCF), and also the action site of other regulatory elements (enhancers, etc.).

2.3. ATAC-seq

ATAC-seq was performed using a modification of the method described by Corces et al. (2017). Briefly, approximately 100 mg of larvae (40 larvae per sample in 2Q and 2W; 4 larvae per sample in 4Q and 4W) 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 (Zhang et al., 2008; Corces et al., 2017). A P-*adj* enrichment threshold of 0.05 was used for all data sets. The analysis of different peaks (Fold change $\geq$2) is based on the folding enrichment of different experimental peaks. ChIPseeker (Yu et al., 2015) 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 (Zhu et al., 2010), and then KOBAS software (version 3.0) was used to test the statistical enrichment of peak related genes in KEGG pathways (Xie et al., 2011). HMMRATAC was used for range identification of single, double and triple nucleosomes in honeybees (Tarbell and Liu, 2019). We used MEME Suite to conduct sequence analysis based on Motif (Bailey et al., 2009). Motif is implemented by MEME using the search mode “anr”. It is assumed that each peak sequence can contain any number of non-overlapping occurrences of each motif. The most significant motif is firstly detected, and its significance is evaluated by the e value, which is the estimated value of the number of expected memes under a given log-likelihood ratio.

2.4. ChIP-seq

H3K27ac has been shown to be associated with caste differentiation of honeybees (Wojciechowski et al., 2018), therefore it was chosen in this study. Chromatin immunoprecipitation was performed as described by Wojciechowski et al. (2018) with slight modifications. Approximately 800 mg of larvae (320 larvae per sample in 2Q and 2W; 32 larvae per sample in 4Q and 4W) 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 (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 were used as a control for this experiment). 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 (Yu et al., 2015) 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 (Zhu et al., 2010), and then KOBAS software was used to test the statistical enrichment of peak related genes in KEGG pathways.

2.5. RNA-seq

RNA-seq was performed as previously reported (He et al., 2019). Total RNA was extracted from 100 mg larvae (40 larvae per sample in 2Q and 2W; 4 larvae per sample in 4Q and 4W). 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) (Kim et al., 2015) 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* $\leq$ 0.05 were defined as differentially expressed genes. By integrating the Hi-C, ATAC-seq, ChIP-seq and RNA-seq data, we selected 58 key DEGs that also differed in one or more epigenetic modifications. These 58 DEGs were chosen because they have been associated with caste differentiation in other studies (Table S8).

2.6. Application of RNAi

One-day-old worker larvae were fed with semi-artificial diet in a petri dish according to the previous method (Mao et al., 2015) and were incubated at 34 °C and 75% humidity. Artificially manufactured siRNA for 4-coumarate–CoA Ligase (4CL) (F: GGUGAAAGAUAUGCUAAUATT; R: UAUUAGCAUAUCUUUCACCTT) and L-lactate dehydrogenase (*LLD*) (F: CGGUCGACAUUCUCACCUACG; R: UAGGUGAGAAUGUCGACCGGA) were added to the semi-artificial diet, with a final concentration of 100 μg/mL. Similarly the NCsiRNA (F: UUCUUCGAACGUGUCACGUTT; R: ACGUGACACGUUCGGAGAATT) was added into semi-artificial diet and fed other larvae as a control group. Totally 118 honey bee larvae with 36 in each group (4CL-RNAi, *LLD*-RNAi and the control) were used, and the mortality rate was 29.67%. At least 15 biological replicates were successfully reared and sampled in each group. 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 and *LLD* 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.

2.7. Gene expression differences were verified by qRT-PCR

2Q, 2W, 4Q and 4W larvae were sampled. In each case four larvae of one type were combined as one sample. We collected four samples for each type, with each sample coming from a different colony. Therefore, for this study we sampled a total of 16 of each larval type (2Q, 2W, 4Q and 4W) across four different colonies.

qRT-PCR was performed according to the method of Pan et al. (2022). Briefly, total RNAs were extracted by TransZol Up Plus RNA Kit (TransGen Biotech, China). Total RNAs were then transcribed into cDNA using a PrimeScript™ RT reagent Kit (Takara, Japan). GAPDH was used as a reference gene ((F: GCTGGTTTCATCGATGGTTT; R: ACGATTTC-GACCACCGTAAC). The primers were as follows: 4CL (F: CAAGTG-GACCTTTCGTGGTT; R: CGTCAACATGACACCTTTCG) and *LLD* (F: ATTCGAGCGTGTGCATAGTG; R: GACCGGATTCGAGACGATAA). The primer sequences were designed by Prime Primer 5.0. A 10 μL (5 μL of SYBR®Premix Ex Taq™ II, 3 μL of H_2O, 1 μL of cDNA, 0.4 μL each of forward and reverse primers and 0.2 μL of ROX) qRT-PCR reaction system was established. The PCR conditions were as follows: 95 °C, 5 min; 94 °C, 2 min; 40 cycles (95 °C, 10 s, Tm, 15 s, 72 °C, 15 s); 72 °C, 10 min. To establish the melting curve of the qRT-PCR product, the primers were heated slowly with a gradual increase of 1 °C every 5 s from 72 to 99 °C. The data were analyzed by $2^{-\triangle\triangle CT}$ (Livak and Schmittgen, 2001).

2.8. Paraffin sectioning of the honeybee ovaries

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. (2020). The sections were photographed and ovarioles counted under the same microscope (40X) according to our previous methods (Yi et al., 2020).

2.9. Quantification and statistical analysis

For the data analysis of qRT-PCR were calculated using $2^{-\Delta\Delta Ct}$ comparative Ct method (Livak and Schmittgen, 2001) and were transformed by taking their square root to be normally distributed. Data were analyzed by one-way *t*-test in SPSS (version 25.0). The p-value <0.05 was considered as significantly different. The relative expression was evaluated as mean ± SEM. Other data analyses were also processed using SPSS (version 25.0) and the methods used are also written in the figure legend.

2.10. Data and code availability

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

3. Results

3.1. Data quality control

This study performed four different sequencing techniques (Hi-C, ATAC-seq, ChIP-seq and RNA-Seq) to analyze 2Q, 2W, 4Q and 4W. The Q30 value of each sample in all four omics was all over 90% (Table S1). 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 acceptable and the biological replicates were reliable.

3.2. Comparison of Hi-C and RNA-seq analysis

Chromosome interactions were similar between 2Q and 2W (Fig. 1A and C), but 4Q had significantly stronger cis interaction while 4W had significantly stronger trans interaction (Fig. 1B and C). The 4Q/4W comparison also had more genes that switched between the A/B compartments (325) than the 2Q/2W comparison (247) (Table S2). 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 different at the 2-day larval stage and the 4-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 and E). 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 (Figs. S2E and 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 2 day comparison (Fig. 1I).

3.3. Comparison of ATAC-seq and RNA-seq analyses

ATAC-seq results showed that 1.02 Mb (0.46%), 1.52 Mb (0.69%), 8.65 Mb (3.92%) and 1.50 Mb (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 S3, Fig. S3A). In 4Q/4W comparisons, more unique peaks from 4Q (4,618) were identified but only 448 from 4W (Table S4, Fig. S3A). The proportion of mononucleosome, di-nucleosomes and tri-nucleosomes in queens dramatically increased with age ($p = 8.11e\text{-}22$), whereas workers showed an opposite trend ($p = 1.25e\text{-}39$, see Fig. 2B). 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. 2C, S3B). There was a strong positive correlation between the differences of ATAC and biased gene expression in queen-worker comparisons (Fig. 2D, S3C–S3F). 4Q had the largest number of DEGs containing unique ATAC peaks, and also the most DEGs associated with caste differentiation (Fig. 2E). 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. 2F). 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 (Figs. S3G and S3H). The most significantly enriched transcription factor in all larval groups was activating transcription factor 1 (Atf1). This transcription factor was predicted to regulate 3729 target genes including the 4-coumarate–CoA Ligase (4CL, Loc726040) which was selected for the later RNAi experiment (Fig. S3I, Table S9).

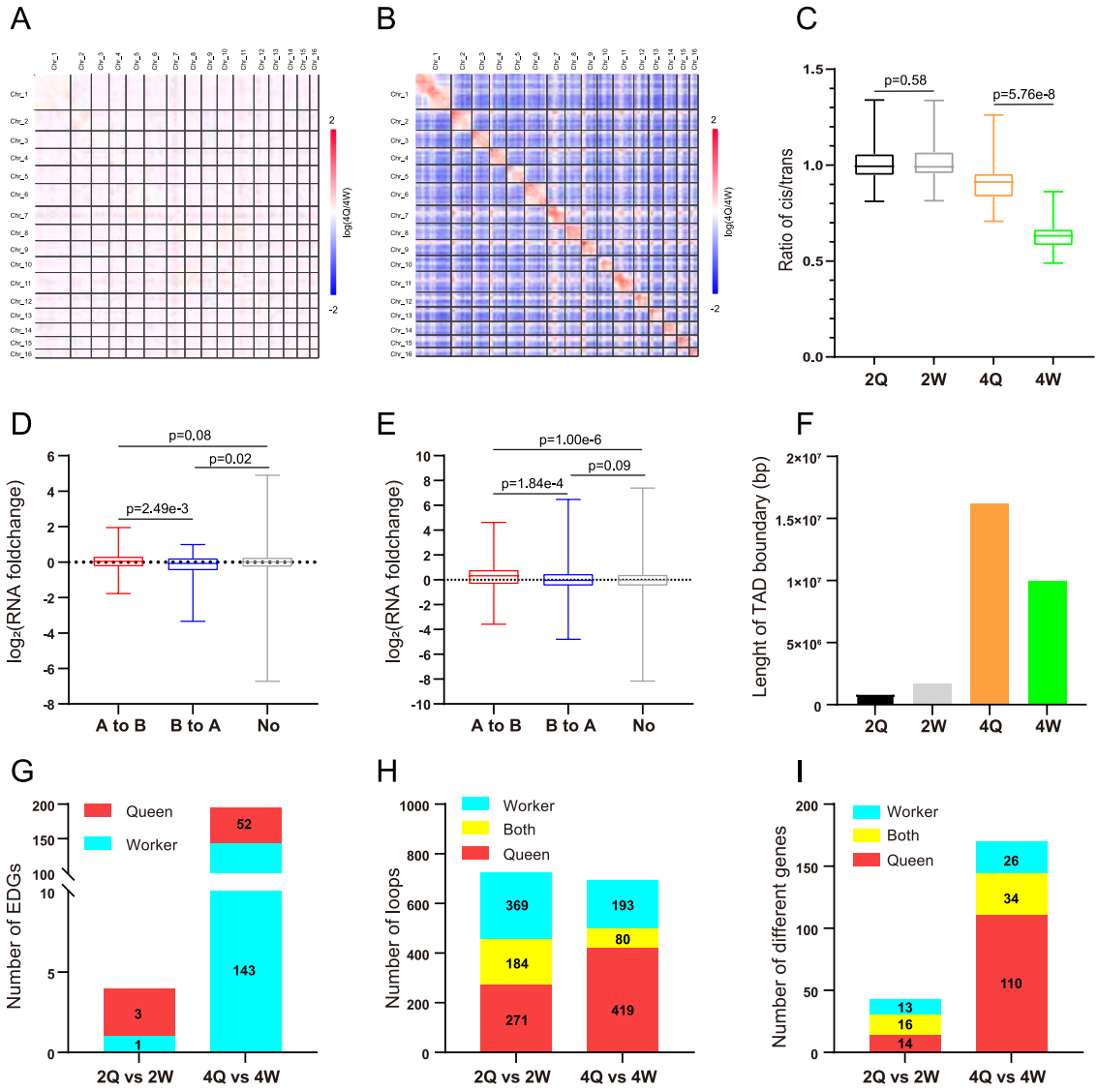

Fig. 1. Hi-C analysis of queen and worker larvae. (**A**) Interaction map of 2Q vs 2W (observed/control). (**B**) Interaction map of 4Q vs 4W (observed/control). In A and B, the sixteen *Apis mellifera* chromosomes (chr) are shown from left to right and top to bottom. Chromosomes are separated by thin black bars. (**C**) The cis and trans ratios in queen and worker larvae. Test by Mann-Whitney *U* test. (**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. Test by Kruskal-Wallis H test. (**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.

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. 2C and D, Figs. S3E and F).

3.4. 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 S7). Similar to the ATAC results, the 4-day comparison had more DEGs containing significantly different ChIP peaks than the 2-day comparison (Fig. 3A and B, S4B). There was a strong positive correlation between H3K27ac modification and gene expression (Fig. 3C and D, S4C–S4F). Compared with the 2 day comparison, the 4-day 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 (Figs. S4G and S4H). These results suggest that H3K27ac also partly contributes to the honeybee caste differentiation and differences are more pronounced at the day 4 stage than day 2.

3.5. Multiomics analysis of caste differentiation

In the 2W/2Q comparisons, Hi-C, ATAC and H3K27ac histone modification all differed between honeybee queens and workers, and the numbers of genes related to the significant differences of Hi-C, ATAC and histone modification were 228, 344 and 4308 respectively. A very low proportion of these differences overlapped with the DEGs (Fig. 4A). In the 4W/4Q comparison more genes were related to the significant differences of Hi-C (813), ATAC (4096) and histone modification (7275), and a higher proportion of these genes overlapped with DEGs (Fig. 4B). Among them, the 72 genes that were different in the four

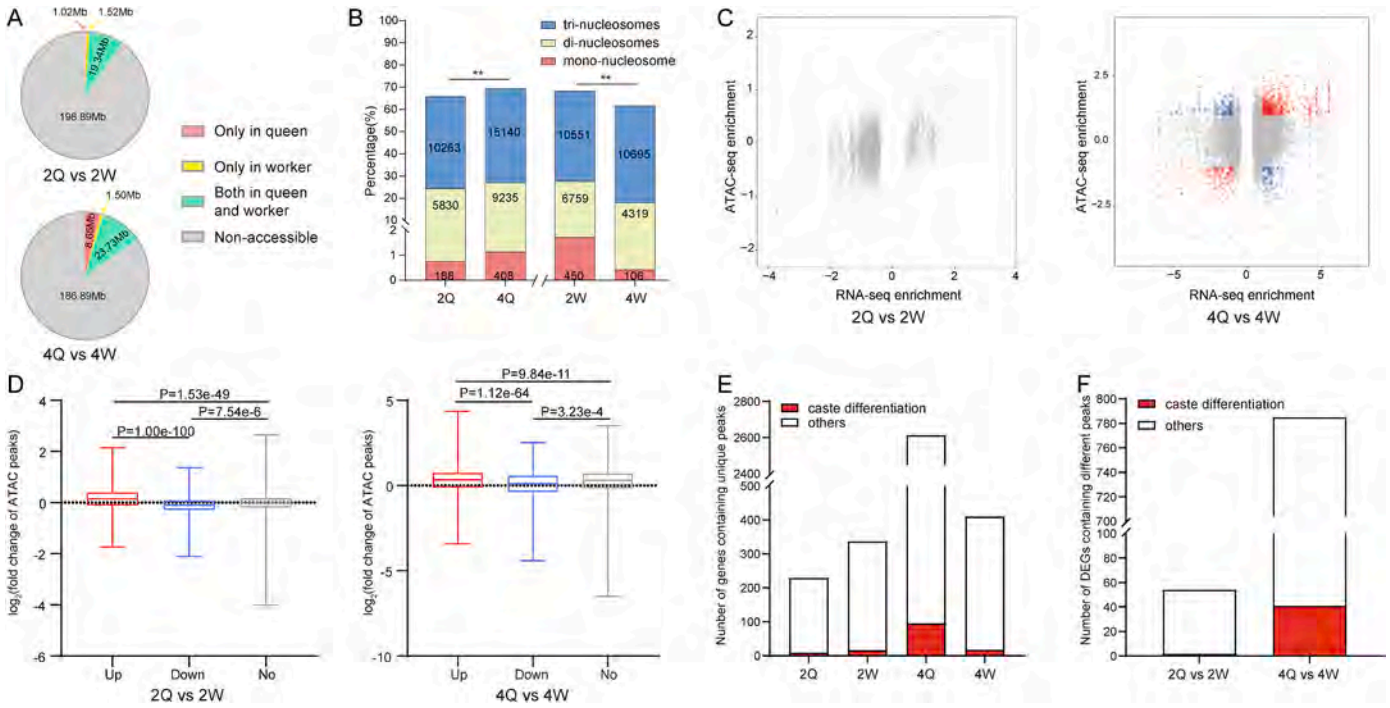

Fig. 2. ATAC-seq analysis of queen and worker larvae. (**A**) Pie chart of comparison of accessible regions in 2Q and 2W, and 4Q and 4W. (**B**) Histogram of the proportions of different nucleosomes between queen and worker larvae. The bars are the ratios of mononucleosome, di-nucleosomes and tri-nucleosomes in 2Q,2W,4Q and 4W. The numbers inside the bars are the accurate number of three nucleosome types in each group. "**" indicates a significant difference ($p < 0.01$, chi-square test) (**C**) Scatter plot of the difference in significant ATAC-seq enrichment between queen and worker (y-axis) against the Log_2FC of transcript expression between queen and worker (x-axis). (**D**) Box plots comparing fold changes of ATAC peaks between protein coding genes in comparisons of 2Q and 2W, and 4Q and 4W, respectively. Test by Kruskal-Wallis H test. (**E**) Number of genes containing unique ATAC-seq peaks in queen or worker larvae, and (**F**) 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.

omics were mainly involved in the propanoate metabolism, pyruvate metabolism and insect hormone biosynthesis pathway and oxidoreductase activities (Fig. S7).

We selected 58 key DEGs that have previously been associated with honeybee caste differentiation (Table S8). More of these genes were differentially regulated by at least one epigenomic system in 4 day comparisons compared to 2 day comparisons (Fig. 4C and D). These suggest that there is a small divergence in gene expression between queen and workers at an early developmental stage that is not widely reinforced by differential genomic regulation. At the 4 day larval stage the differences in genes expression between workers and queens were more profound, and many more of the differences are reinforced by at least one form of genomic regulation.

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

3.6. Gene expression differences associated with multiple epigenetic modifications

In 2 day comparison, genes containing significant differences of three epigenetic modifications between queen and worker larvae rarely overlapped (Fig. S8A), but these notably increased in 4 day comparison (Fig. S8B). The proportion of DEGs that were associated with at least one other epigenomic difference was lower in 2 day comparison (51.42%, Fig. 4F) than the 4 day comparison (76.15%, Fig. 4G). Moreover, the proportion of DEGs associated with more than one epigenomic difference was also higher in the 4 day than 2 day comparisons (Fig. 4F and G). 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 associated with honeybee caste differentiation (Fig. 4H and I), a higher proportion of these key DEGs were differentially regulated by at least 2 types of epigenetic modifications (Fig. 4I) compared to total DEGs (Fig. 4G), 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. 4I), perhaps suggesting that gene expression is more directly regulated by chromatin accessibility and histone modification and less directly influenced by chromosome structure.

3.7. Effects of a key candidate gene on caste differentiation

We selected 6 key genes [vitellogenin (*VG*) (Zhang et al., 2022), hexamerin 70a (*Hex70a*) (Cameron et al., 2013), lethal(2)essential for life (*L(2)EFL*) (Garcia et al., 2009), probable cytochrome P450 6a13 (*P450-6a13*) (Mao et al., 2015), juvenile hormone acid O-methyltransferase (*JHAMT*) (Bomtorin et al., 2014) and heat shock protein 90 (*HSP90*) (Evans and Wheeler, 2000)] that have been shown involved in caste differentiation, and 2 genes [4CL and L-lactate dehydrogenase (*LLD*, Loc411188)] which we suspected as key genes involved in caste differentiation also. The 4CL is involved in *p*-coumaric acid synthesis in honeybee larval diets and may be involved in honeybee caste

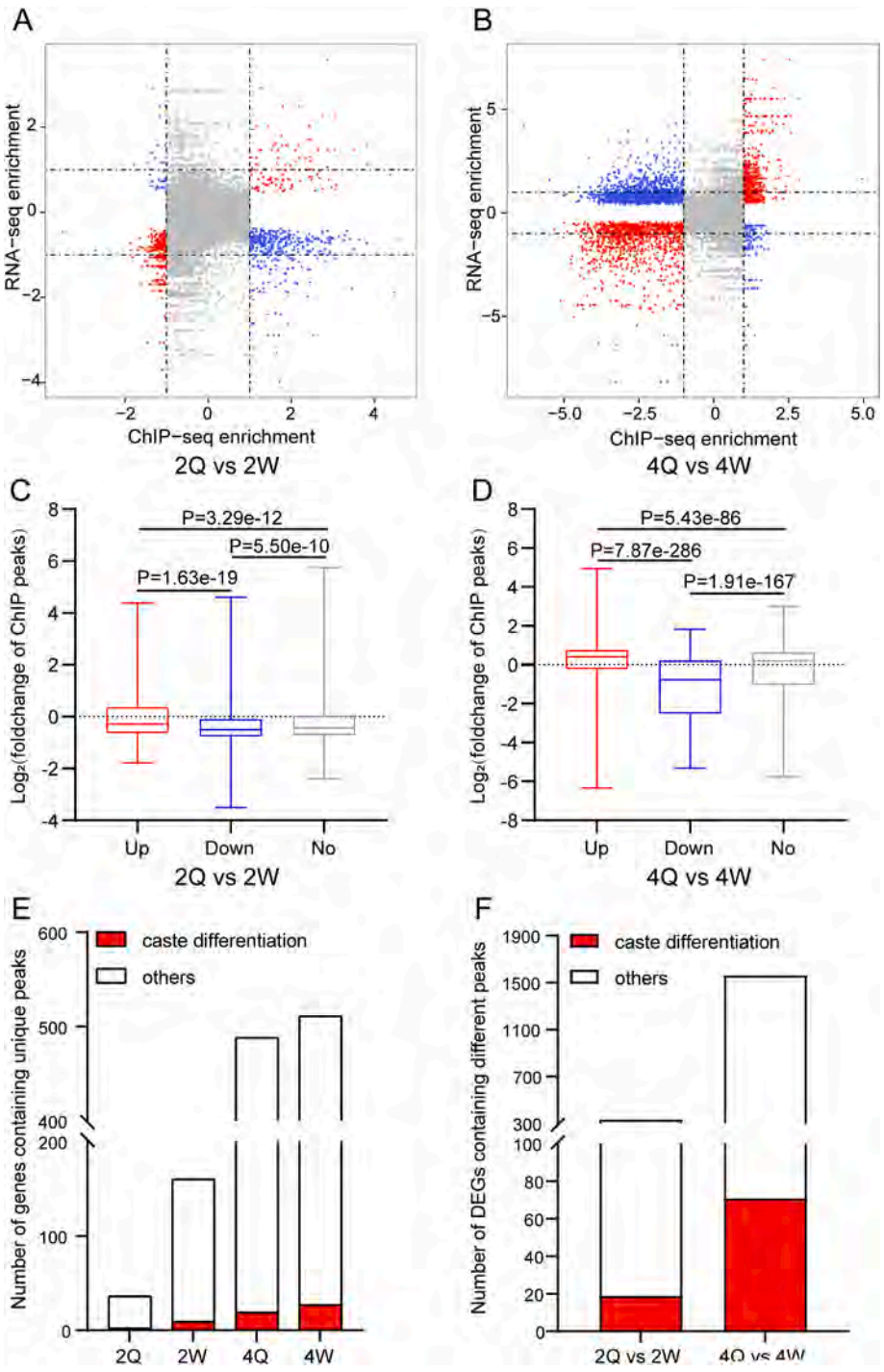

Fig. 3. ChIP-seq analysis of queen and worker larvae. (**A** and **B**) Scatter plots of the significant differences in ChIP-seq enrichment between queen worker larvae samples (x-axis) against the Log_2FC of transcript expression between queen worker larvae samples (y-axis). (**C** and **D**) Box plot comparing fold change of ChIP peaks between protein coding gene in 2Q/2W and 4Q/4W, respectively. Test by Kruskal-Wallis H test. (**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.

differentiation (Stuible et al., 2000; Cukovica et al., 2001; Mao et al., 2015; Islam et al., 2019). Our results showed a clear trend that all 8 genes had greater differences in epigenomic regulation in 4Q/4W comparison than the 2Q/2W comparison (Fig. 5A and B and Fig. S6).

4CL and *LLD* differed in all four omics assays in our 4Q/4W comparison. We did an interaction analysis on the four-omic overlapped 72 DEGs from 4 day comparison, and two key DEGs (4CL and *LLD)* had the largest node in the gene interaction network. These two key genes linked to 36 genes which were significantly different in at least one omic, in which seven genes are known to be important for caste differentiation such as *HSP90* (Evans and Wheeler, 2000), *Loc408567* (Hasegawa et al., 2009) and *Loc412541* (Li et al., 2010) (Fig. 5C). Reducing the expression of these two genes with RNAi (Fig. 5D) resulted in a decrease in body weight (Fig. 5E) and body size (Fig. 5F) of newly-emerged queens compared to the control. Reduced expression of 4CL resulted in fewer ovarioles in the adult queen ovary when compared to controls (Fig. 5G; *t*-test, T = 16.86, $P = 1.3e\text{-}5$).

4. Discussion

Here we used a multiomics approach to obtain different perspectives on the divergent epigenomic profiles of worker and queen bees. The methods we used (Hi-C, ATAC-seq, ChIP-Seq and RNA-Seq) explored different types of epigenomic differences between the worker and queen

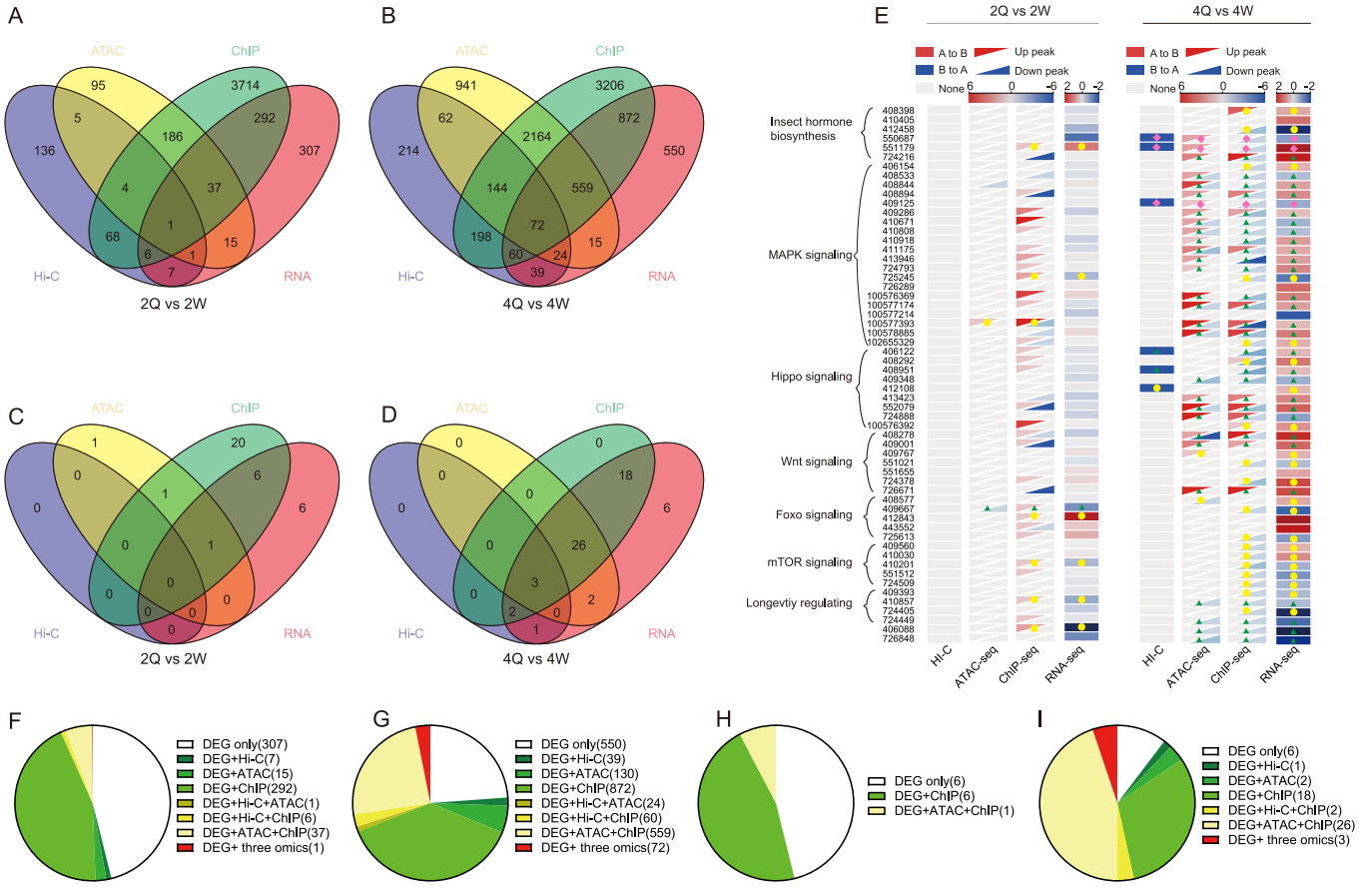

Fig. 4. Multiomics analysis between queen and worker larvae. Venn diagram showing the overlap in DEGs identified by different omics methods in 2Q/2W comparisons (**A**) and 4Q/4W comparisons (**B**). (**C**) We selected 58 genes that have been reported to be associated with the caste differentiation in the honey bee. The figure shows which epigenomic modifications of these 58 DEGs differed in 2Q/2W comparisons. (**D**) The 58 DEGs and their epigenetic modofications differed in 4Q/4W comparisons. More of these genes were differentially regulated by at least one epigenomic system in 4 day comparisons compared to 2 day comparison. (**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 omic analyses. Green triangle means there were significant differences observed in three omic comparisons. Pink square means there were significant differences observed in four omic comparisons. The locations of the yellow circle, green triangle, and pink square indicate differences in these omics between queen and worker larvae. (**F, G**) Pie chart of number of genes that differ in epigenetic regulation in comparisons of 2Q/2W (**F**) and 4Q/4W (**G**). (**H, I**) Pie chart of number of 58 key genes that differ in epigenetic regulation in comparisons of 2Q/2W (**H**) and 4Q/4W (**I**).

castes. Our results clearly showed that all these four omics were involved in honeybee caste differentiation. Thousands of DEGs were identified between worker and queen larvae (Fig. S9), which are similar to previous studies (He et al., 2019). We found differences in 3D chromosome structure and dramatically different proportions of accessible chromatin between queen and worker larvae (Figs. 1 and 2), including 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. Moreover, many DEGs that were enriched for key pathways such as TOR, IRS, Notch, Hippo etc (Patel et al., 2007; Mutti et al., 2011; Chen et al., 2017) (see Figs. S2C–S2F) were related to these distinct A/B switch areas, TAD boundaries and loops (Fig. 1F-I, Fig. S2A). Therefore, our findings suggest that differences in chromosome structure are involved in honeybee caste differentiation, possibly via regulating gene expression, as has been suggested previously (Rodríguez-Carballo et al., 2017; Wang et al., 2018, 2021b; Sikorska and Sexton, 2020; Eres and Gilad, 2021).Our ChIP-seq results revealed a clear difference in histone modification (H3K27ac) between workers and queens, which were consistent with Hurd's study that H3K27ac participates in the regulation of honeybee caste differentiation (Wojciechowski et al., 2018). Our ATAC-seq results found differences of unique peaks and significantly different peaks between queen and workers (Fig. 2A and C), which were associated with thousands of DEGs (Fig. 2E and F). The ratio of mononucleosome, di-nucleosomes and tri-nucleosomes in queens dramatically increased with age while decreased in workers (Fig. 2B), indicating a different transcription activity between queen and workers during their larval development.

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 (Winston, 1991). 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 S7). These results indicate that the development of queen-worker dimorphism involves distinct patterns of accessible chromatin. In addition, we found that ATF1 was the most abundant transcription factor in all samples. ATF1 is an important transcription factor involved in many biological processes such as the production of adenosine triphosphate, mitochondrial respiration, DNA synthesis and repair, and general cell growth (Bleckmann et al., 2002; Ghosh et al., 2002; Jin and O'Neill, 2010; Jin and O'Neill, 2014; Nickels et al., 2022). Therefore, further investigations should explore the possible biological functions of ATF1 in honeybee caste differentiation.

All three epigenetic modifications we explored (histone modification, chromatin accessibility and chromosome structure) had positive

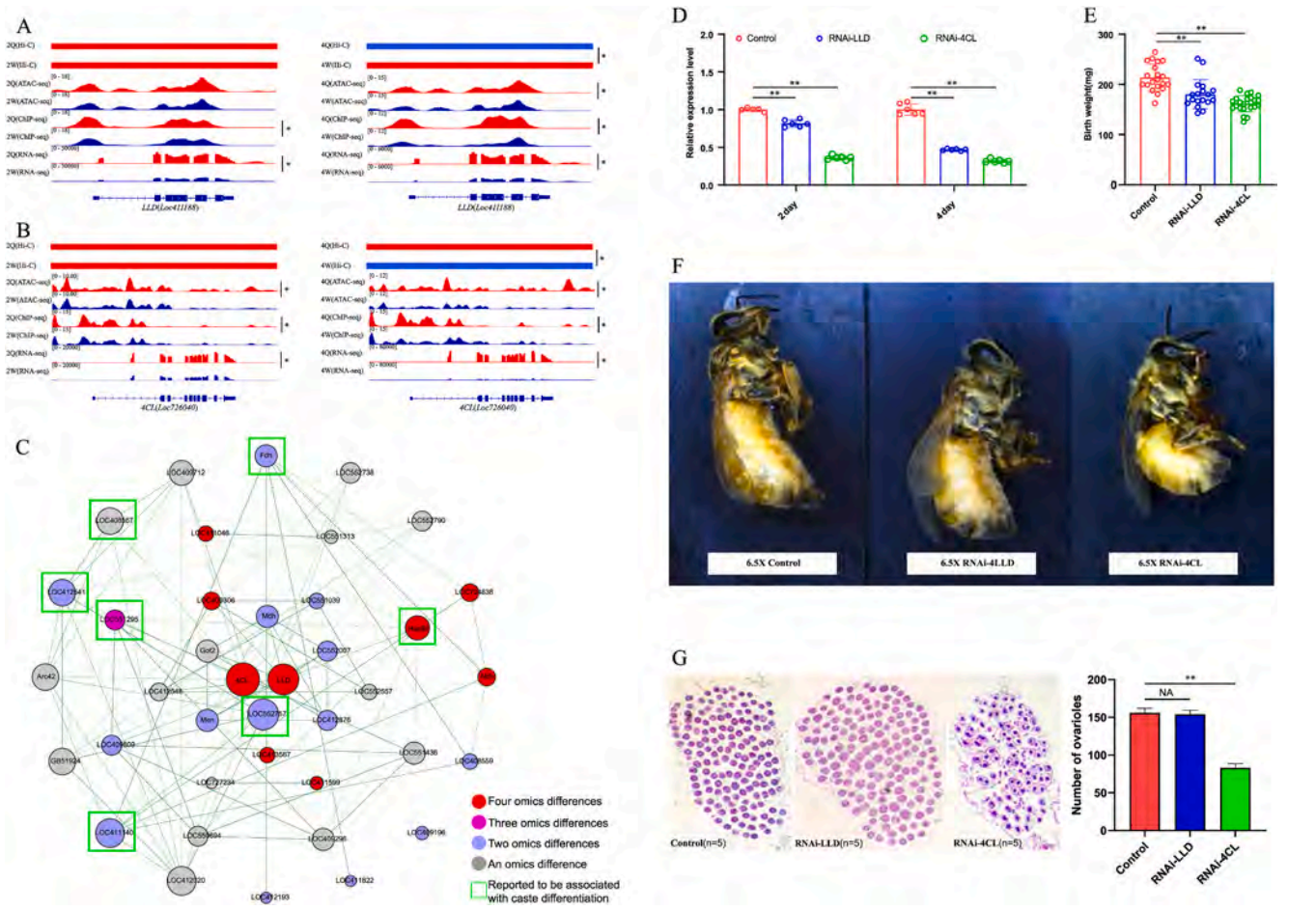

Fig. 5. RNAi verification of the *LLD* and *4CL*gene. (**A**) Example showing the correlation of Hi-C, ATAC signal, ChIP signal and RNA-seq reads in 2Q/2W (left) and 4Q/4W (right) for the *LLD* gene. In Hi-C data, red bars represent A compartments, whereas blue ones represent B compartments. The red waves are read coverage of queens in ATAC signal, ChIP signal and RNA-seq, while the blue ones are that of workers. Data in "[]" are the scales of read counts. "*" indicates that there is a difference between two groups. Same to B. (**B**) Example showing the correlation of Hi-C, ATAC signal, ChIP signal and RNA-seq reads in 2Q/2W (left) and 4Q/4W (right) for the 4CL gene. (**C**) Network analysis of two key genes, 4CL and *LLD*. Red circles indicate that genes were different in all four omics; Pink circles indicate that genes were different in three omics; Purple circles indicate that genes were different in two omics, and grey indicate that genes were different in an omic. Green squares indicate genes that have been reported to be associated with honeybee caste differentiation. (**D**) Gene expression in siRNA fed and control larvae. "**" indicate a significant difference ($p < 0.01$ *t*-test). (**E**) Weight of newly-emerged queens, from control (feeding the siRNA-NC), RNAi-*LLD* (feeding the siRNA-*LLD*) and RNAi-4CL groups (feeding the siRNA-4CL). Bars are the mean ± SE. "**" indicate a significant difference ($p < 0.01$ Mann-Whitney *U* test). (**F**) Photo of queen after feeding siRNA reagent, left is the control group, middle is the RNAi-*LLD* group and right is the RNAi-4CL group. (**G**) Section of the newly-emerged queen's left ovaries, left is the control group, middle is the RNAi-*LLD* group and right is the RNAi-4CL group. "n" is the number of samples. Bar graphs represent statistical plots of ovarian number in the three groups. Bars are the mean ± SEM. "**" indicate a significant difference ($p < 0.01$ *t*-test).

correlations with gene expression (Fig. 1D, E, 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 (Ikegami et al., 2009; Wang et al., 2009; Bannister and Kouzarides, 2011; Buitrago et al., 2021; Lu et al., 2021). Our data suggest that gene expression differences underlying caste differentiation are regulated by an interacting system of epigenetic modifications rather than dominated by a single epigenetic modification (Fig. 4) revealing very different epigenomic landscapes for developing worker and queen bees which develop during the course of larval development.

Moreover, the differences in chromosome structure, chromatin accessibility and histone modification between workers and queens at the early developmental stage (see Fig. 1E-I, Fig. 2A-F, Fig. 3A, E, 3F), were not very pronounced and they had little association with DEGs (Fig. 4A) or key DEGs known to be involved in the 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 (Figs. 4 and 5). This pattern was even more pronounced for our 58 key DEGs that have previously been associated with caste differentiation (Fig. 5E and F). The implication is the development of a layering of epigenomic control of gene expression differences during larval development as the phenotypes diverge. This might serve to stabilise critical functional differences in gene expression between the worker and queen phenotypes.

Stable differences in gene expression depend on both the nature of environmental change and the regulatory epigenomic landscape (Herman et al., 2014). The 4 day sample point was beyond the point at which a worker larva can be successfully transformed into adult queens by feeding them with more nutritional diets, suggesting the worker developmental trajectory is canalized at this point (Weaver, 1957, 1966). In the 4 day worker/queen larval comparison a higher proportion of the 58 key DEGs related to caste differentiation differed in two or three different epigenetic modifications (Fig. 5F). Moreover, 8 selected key genes also had more differences in genomic regulation in 4Q/4W comparisons compared to 2Q/2W comparisons (Fig. 5A and B, Fig. S6). From this we infer that gene expression differences that are critical to the formation of the distinct worker and queen phenotypes become

stabilised in development through regulation by multiple degenerate epigenomic systems.

To test our inference that genes of key importance for queen/worker differences are subject to the most extensive epigenomic regulation we selected the candidate DEG *LLD* and 4CL for RNA interference (RNAi) verification. Both genes have been causally linked to caste differentiation and both genes differed between 4W and 4Q in all four omics assays (Fig. 5B). Previous studies have shown that 4CL is involved in *p*-coumaric acid synthesis, and this acid which exists in honeybee larval diets plays an important role in honeybee caste differentiation (Stuible et al., 2000; Cukovica et al., 2001; Mao et al., 2015; Islam et al., 2019). Here RNAi knock down of 4CL expression resulted in newly emerged queens of lower weight, smaller body size and fewer ovariole numbers than controls (Fig. 5C–F). Knocking down of expression of *LLD* resulted in lower weight of newly emerged queens but did not change ovariole counts (Fig. 5C–F). In the interaction network, 4CL and *LLD* were associated with 36 genes, seven of which have been linked to caste differentiation (Fig. 5C). Therefore, RNA interference of 4CL may also have an indirect effect on these caste differentiation related genes. In addition, *ATF1* was also predicted to regulate the expression of 4CL (Fig. S3I, Table S9), again reflecting that this key gene is essential for caste differentiation and can be regulated by various epigenetic modifications. Consequently, these findings confirm the importance of expression of these two genes for caste differentiation, and it is notable that they were 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 (Seeley, 1989). This is achieved through different epigenomic regulation of the bee genome during development. Our results, together with previous studies, demonstrated that various epigenetic modifications are all involved in bee phenotypic plasticity (Kucharski et al., 2008; Shi et al., 2011, 2013, 2015; Guo et al., 2013, 2016; Wojciechowski et al., 2018; Wang et al., 2021b). 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 profiles of the worker and queen are not programs. They are not pre-established to control the running of different developmental paths. Instead the epigenomic profiles diverge during development as the worker and queen phenotypes diverge. In this example, phenotypic plasticity epigenomic mechanisms are possibly part of the developmental autoshaping process, divering as workers and queens take progressively divergent developmental pathways through Waddington's developmental landscape. Epigenetic modifications also play an important role in the determination of caste differentiation in ants, wasps and other social insects (Weiner et al., 2013; Simola et al., 2016; Marshall et al., 2019). As a classical social insect, our findings in honeybees serve as a model of epigenetic landscape in the caste system for other Hymenoptera species. Consequently, our study has a potential contribution not only to the field of honey bee caste development, but also to the area of other social insects' phenotypic plasticity driven by environmental factors.

Author contributions

Z.J.Z., and X.J.H., designed the research; Y.Z. and M.J.J. performed experiments; Z.J.Z., A.B.B., X.J.H, Q.H., X.B.W., L.Z.Z., Z.L.W. and W.Y. Y. provided guidance for data; Y.Z., Z. L., and X.J.H. analyzed data; Y.Z., X.J.H., A.B.B. and Z.J.Z wrote the paper. The authors declare no competing interest. All authors were involved in the preparation of the final manuscript.

Funding

This work was supported by the National Natural Science Foundation of China (32172790, 32160815, 31872432), Major Discipline Academic and Technical Leaders Training Program of Jiangxi Province (20204BCJL23041) and the Earmarked Fund for China Agriculture Research System (CARS-44-KXJ15).

Ethics approval and consent to participate

Ethics approval was not applicable.

Consent for publication

Not applicable.

Declaration of competing interest

The authors declare that they have no competing interests.

Data availability

No data was used for the research described in the article.

Acknowledgement

We thank Mr. Xiaofeng He, Pengfei Yu and Han Li for help in data analysis.

Appendix ASupplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2023.103929.

References

Agbleke, A.A., Amitai, A., Buenrostro, J.D., Chakrabarti, A., Chu, L., Hansen, A.S., Koenig, K.M., Labade, A.S., Liu, S., Nozaki, T., 2020. Advances in chromatin and chromosome research: perspectives from multiple fields. Mol. Cell 79, 881–901.
Bailey, T.L., Boden, M., Buske, F.A., Frith, M., Grant, C.E., Clementi, L., Ren, J., Li, W.W., Noble, W.S., 2009. Meme SUITE: tools for motif discovery and searching. Nucleic Acids Res. 37, W202–W208.
Bannister, A.J., Kouzarides, T., 2011. Regulation of chromatin by histone modifications. Cell Res. 21, 381–395.
Berdasco, M., Esteller, M., 2010. Aberrant epigenetic landscape in cancer: how cellular identity goes awry. Dev. Cell 19, 698–711.
Bleckmann, S.C., Blendy, J.A., Rudolph, D., Monaghan, A.P., Schmid, W., Schütz, G.n., 2002. Activating transcription factor 1 and CREB are important for cell survival during early mouse development. Mol. Cell Biol. 22, 1919–1925.
Bomtorin, A.D., Mackert, A., Rosa, G.C.C., Moda, L.M., Martins, J.R., Bitondi, M.M.G., Hartfelder, K., Simões, Z.L.P., 2014. Juvenile hormone biosynthesis gene expression in the corpora allata of honey bee (*Apis mellifera L.*) female castes. PLoS One 9, e86923.
Buitrago, D., Labrador, M., Arcon, J.P., Lema, R., Flores, O., Esteve-Codina, A., Blanc, J., Villegas, N., Bellido, D., Gut, M., 2021. Impact of DNA methylation on 3D genome structure. Nat. Commun. 12, 1–17.
Burgess, D.J., 2022. A TAD refined for gene regulation. Nat. Rev. Genet. 1, 1.
Cameron, R.C., Duncan, E.J., Dearden, P.K., 2013. Biased gene expression in early honeybee larval development. BMC Genom. 14, 1–12.
Chen, X., Ma, C., Chen, C., Lu, Q., Shi, W., Liu, Z., Wang, H., Guo, H., 2017. Integration of lncRNA–miRNA–mRNA reveals novel insights into oviposition regulation in honey bees. PeerJ 5, e3881.
Corces, M.R., Trevino, A.E., Hamilton, E.G., Greenside, P.G., Sinnott-Armstrong, N.A., Vesuna, S., Satpathy, A.T., Rubin, A.J., Montine, K.S., Wu, B., 2017. An improved ATAC-seq protocol reduces background and enables interrogation of frozen tissues. Nat. Methods 14, 959–962.
Corona, M., Libbrecht, R., Wheeler, D.E., 2016. Molecular mechanisms of phenotypic plasticity in social insects. Curr. opin. insect sci. 13, 55–60.
Cukovica, D., Ehlting, J., Ziffle, J.A.V., Douglas, C.J., 2001. Structure and evolution of 4-coumarate: coenzyme A ligase (4CL) gene families. Biol. Chem. 382, 645–654.
Duncan, E.J., Gluckman, P.D., Dearden, P.K., 2014. Epigenetics, plasticity, and evolution: how do we link epigenetic change to phenotype? J. Exp. Zool. B Mol. Dev. Evol. 322, 208–220.
Duncan, E.J., Leask, M.P., Dearden, P.K., 2020. Genome architecture facilitates phenotypic plasticity in the honeybee (*Apis mellifera*). Mol. Biol. Evol. 37, 1964–1978.
Durand, N.C., Shamim, M.S., Machol, I., Rao, S.S., Huntley, M.H., Lander, E.S., Aiden, E. L., 2016. Juicer provides a one-click system for analyzing loop-resolution Hi-C experiments. Cell systems 3, 95–98.
Eres, I.E., Gilad, Y., 2021. A TAD skeptic: is 3D genome topology conserved? Trends Genet. 37, 216–223.

Evans, J.D., Wheeler, D.E., 2000. Expression profiles during honeybee caste determination. Genome Biol. 2, 1–6.
Garcia, L., Saraiva Garcia, C.H., Calábria, L.K., Costa Nunes da Cruz, G., Sánchez Puentes, A., Báo, S.N., Fontes, W., Ricart, C.A., Salmen Espindola, F., Valle de Sousa, M., 2009. Proteomic analysis of honey bee brain upon ontogenetic and behavioral development. J. Proteome Res. 8, 1464–1473.
Ghosh, S.K., Gadiparthi, L., Zeng, Z.-Z., Bhanoori, M., Tellez, C., Bar-Eli, M., Rao, G.N., 2002. ATF-1 mediates protease-activated receptor-1 but not receptor tyrosine kinase-induced DNA synthesis in vascular smooth muscle cells. J. Biol. Chem. 277, 21325–21331.
Grant, P.A., 2001. A tale of histone modifications. Genome Biol. 2, 1–6.
Guo, X., Su, S., Geir, S., Li, W., Li, Z., Zhang, S., Chen, S., Chen, R., 2016. Differential expression of miRNAs related to caste differentiation in the honey bee, *Apis mellifera*. Apidologie 47, 495–508.
Guo, X., Su, S., Skogerboe, G., Dai, S., Li, W., Li, Z., Liu, F., Ni, R., Guo, Y., Chen, S., 2013. Recipe for a busy bee: microRNAs in honey bee caste determination. PLoS One 8, e81661.
Hasegawa, M., Asanuma, S., Fujiyuki, T., Kiya, T., Sasaki, T., Endo, D., Morioka, M., Kubo, T., 2009. Differential gene expression in the mandibular glands of queen and worker honeybees, *Apis mellifera L.*: implications for caste-selective aldehyde and fatty acid metabolism. Insect Biochem. Mol. Biol. 39, 661–667.
He, X.J., Barron, A.B., Yang, L., Chen, H., He, Y.Z., Zhang, L.Z., Huang, Q., Wang, Z.L., Wu, X.B., Yan, W.Y., 2022. Extent and complexity of RNA processing in honey bee queen and worker caste development. iScience 25, 104301.
He, X.J., Jiang, W.J., Zhou, M., Barron, A.B., Zeng, Z.J., 2019. A comparison of honeybee (*Apis mellifera*) queen, worker and drone larvae by RNA-Seq. Insect Sci. 26, 499–509.
Heinz, S., Benner, C., Spann, N., Bertolino, E., Lin, Y.C., Laslo, P., Cheng, J.X., Murre, C., Singh, H., Glass, C.K., 2010. Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. Mol. Cell 38, 576–589.
Herman, J.J., Spencer, H.G., Donohue, K., Sultan, S.E., 2014. How stable 'should'epigenetic modifications be? Insights from adaptive plasticity and bet hedging. Evolution 68, 632–643.
Ikegami, K., Ohgane, J., Tanaka, S., Yagi, S., Shiota, K., 2009. Interplay between DNA methylation, histone modification and chromatin remodeling in stem cells and during development. Int. J. Dev. Biol. 53, 203–214.
Islam, M.T., Lee, B.R., Lee, H., Jung, W.J., Bae, D.W., Kim, T.H., 2019. *p*-Coumaric acid induces jasmonic acid-mediated phenolic accumulation and resistance to black rot disease in Brassica napus. Physiol. Mol. Plant Pathol. 106, 270–275.
Jin, X., O'Neill, C., 2010. The presence and activation of two essential transcription factors (cAMP response element-binding protein and cAMP-dependent transcription factor ATF1) in the two-cell mouse embryo. Biol. Reprod. 82, 459–468.
Jin, X., O'Neill, C., 2014. The regulation of the expression and activation of the essential ATF1 transcription factor in the mouse preimplantation embryo. Reproduction 148, 147–157.
Kamakura, M., 2011. Royalactin induces queen differentiation in honeybees. Nature 473, 478–483.
Karlić, R., Chung, H.-R., Lasserre, J., Vlahoviček, K., Vingron, M., 2010. Histone modification levels are predictive for gene expression. PANS (Pest. Artic. News Summ.) 107, 2926–2931.
Kim, D., Langmead, B., Salzberg, S.L., 2015. HISAT: a fast spliced aligner with low memory requirements. Nat. Methods 12, 357–360.
Klemm, S.L., Shipony, Z., Greenleaf, W.J., 2019. Chromatin accessibility and the regulatory epigenome. Nat. Rev. Genet. 20, 207–220.
Krijger, P.H.L., De Laat, W., 2016. Regulation of disease-associated gene expression in the 3D genome. Nat. Rev. Mol. Cell Biol. 17, 771–782.
Kucharski, R., Maleszka, J., Foret, S., Maleszka, R., 2008. Nutritional control of reproductive status in honeybees via DNA methylation. Science 319, 1827–1830.
Li, J., Wu, J., Begna Rundassa, D., Song, F., Zheng, A., Fang, Y., 2010. Differential protein expression in honeybee (Apis mellifera L.) larvae: underlying caste differentiation. PLoS One 5, e13455.
Lieberman-Aiden, E., Van Berkum, N.L., Williams, L., Imakaev, M., Ragoczy, T., Telling, A., Amit, I., Lajoie, B.R., Sabo, P.J., Dorschner, M.O., 2009. Comprehensive mapping of long-range interactions reveals folding principles of the human genome. Science 326, 289–293.
Liu, Y.B., Yi, Y., Abdelmawla, A., Zheng, Y.L., Zeng, Z.J., He, X.J., 2021. Female developmental environment delays development of male honeybee (*Apis mellifera*). BMC Genom. 22, 1–12.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. Methods 25, 402–408.
Lu, J., Huang, Y., Zhang, X., Xu, Y., Nie, S., 2021. Noncoding RNAs involved in DNA methylation and histone methylation, and acetylation in diabetic vascular complications. Pharmacol. Res. 170, 105520.
Luo, L.H., Gribskov, M., Wang, S.F., 2022. Bibliometric Review of ATAC-Seq and its Application in Gene Expression. Bioinform, Brief.
Maleszka, R., 2018. Beyond Royalactin and a master inducer explanation of phenotypic plasticity in honey bees. Commun. Bio. 1, 1–7.
Mao, W., Schuler, M.A., Berenbaum, M.R., 2015. A dietary phytochemical alters caste-associated gene expression in honey bees. Sci. Adv. 1, e1500795.
Marshall, H., Lonsdale, Z.N., Mallon, E.B., 2019. Methylation and gene expression differences between reproductive and sterile bumblebee workers. Evol. Lett. 3, 485–499.
Michener, C.D., Michener, C.D., 1974. The Social Behavior of the Bees: a Comparative Study. Harvard University Press.
Moris, N., Pina, C., Arias, A.M., 2016. Transition states and cell fate decisions in epigenetic landscapes. Nat. Rev. Genet. 17, 693–703.
Mutti, N.S., Dolezal, A.G., Wolschin, F., Mutti, J.S., Gill, K.S., Amdam, G.V., 2011. IRS and TOR nutrient-signaling pathways act via juvenile hormone to influence honey bee caste fate. J. Exp. Biol. 214, 3977–3984.
Nacev, B.A., Jones, K.B., Intlekofer, A.M., Yu, J.S., Allis, C.D., Tap, W.D., Ladanyi, M., Nielsen, T.O., 2020. The epigenomics of sarcoma. Nat. Rev. Cancer 20, 608–623.
Nickels, J.F., Della-Rosa, M.E., Goyeneche, I.M., Charlton, S.J., Sneppen, K., Thon, G., 2022. The transcription factor Atf1 lowers the transition barrier for nucleosome-mediated establishment of heterochromatin. Cell Rep. 39, 110828.
Pan, L.X., Hu, W.W., Cheng, F.P., Hu, X.F., Wang, Z.L., 2022. Transcriptome analysis reveals differentially expressed genes between the ovary and testis of the honey bee Apis mellifera. Apidologie 53, 1–12.
Pan, Q.-Z., Wu, X.-B., Guan, C., Zeng, Z.-J., 2013. A new method of queen rearing without grafting larvae. Am. Bee J. 153, 1279–1280.
Patel, A., Fondrk, M.K., Kaftanoglu, O., Emore, C., Hunt, G., Frederick, K., Amdam, G.V., 2007. The making of a queen: TOR pathway is a key player in diphenic caste development. PLoS One 2, e509.
Rao, S.S., Huntley, M.H., Durand, N.C., Stamenova, E.K., Bochkov, I.D., Robinson, J.T., Sanborn, A.L., Machol, I., Omer, A.D., Lander, E.S., 2014. A 3D map of the human genome at kilobase resolution reveals principles of chromatin looping. Cell 159, 1665–1680.
Rijnkels, M., Kabotyanski, E., Montazer-Torbati, M.B., Beauvais, C.H., Vassetzky, Y., Rosen, J.M., Devinoy, E., 2010. The epigenetic landscape of mammary gland development and functional differentiation. J. Mammary Gland Biol. Neoplasia 15, 85–100.
Robertson, K.D., 2005. DNA methylation and human disease. Nat. Rev. Genet. 6, 597–610.
Rodríguez-Carballo, E., Lopez-Delisle, L., Zhan, Y., Fabre, P.J., Beccari, L., El-Idrissi, I., Huynh, T.H.N., Ozadam, H., Dekker, J., Duboule, D., 2017. The HoxD cluster is a dynamic and resilient TAD boundary controlling the segregation of antagonistic regulatory landscapes. Genes Dev. 31, 2264–2281.
Schlichting, C.D., Wund, M.A., 2014. Phenotypic plasticity and epigenetic marking: an assessment of evidence for genetic accommodation. Evolution 68, 656–672.
Seeley, T.D., 1989. The honey bee colony as a superorganism. Am. Sci. 77, 546–553.
Shi, Y.Y., Huang, Z.Y., Zeng, Z.J., Wang, Z.L., Wu, X.B., Yan, W.Y., 2011. Diet and cell size both affect queen-worker differentiation through DNA methylation in honey bees (Apis mellifera, Apidae). PLoS One 6, e18808.
Shi, Y.Y., Yan, W.Y., Huang, Z.Y., Wang, Z.L., Wu, X.B., Zeng, Z.J., 2013. Genomewide analysis indicates that queen larvae have lower methylation levels in the honey bee (Apis mellifera). Naturwissenschaften 100, 193–197.
Shi, Y.Y., Zheng, H.J., Pan, Q.Z., Wang, Z.L., Zeng, Z.J., 2015. Differentially expressed microRNAs between queen and worker larvae of the honey bee (Apis mellifera). Apidologie 46, 35–45.
Sikorska, N., Sexton, T., 2020. Defining functionally relevant spatial chromatin domains: it is a TAD complicated. J. Mol. Biol. 432, 653–664.
Simola, D.F., Graham, R.J., Brady, C.M., Enzmann, B.L., Desplan, C., Ray, A., Zwiebel, L. J., Bonasio, R., Reinberg, D., Liebig, J., 2016. Epigenetic (re) programming of caste-specific behavior in the ant Camponotus floridanus. Science 351, aac6633.
Skvortsova, K., Iovino, N., Bogdanović, O., 2018. Functions and mechanisms of epigenetic inheritance in animals. Nat. Rev. Mol. Cell Biol. 19, 774–790.
Stabe, H.A., 1930. The rate of growth of worker, drone and queen larvae of the honeybee, *Apis mellifera* linn. J. Econ. Entomol. 23, 447–453.
Stuible, H.-P., Büttner, D., Ehlting, J., Hahlbrock, K., Kombrink, E., 2000. Mutational analysis of 4-coumarate: CoA ligase identifies functionally important amino acids and verifies its close relationship to other adenylate-forming enzymes. FEBS Lett. 467, 117–122.
Szabo, Q., Jost, D., Chang, J.-M., Cattoni, D.I., Papadopoulos, G.L., Bonev, B., Sexton, T., Gurgo, J., Jacquier, C., Nollmann, M., 2018. TADs are 3D structural units of higher-order chromosome organization in Drosophila. Sci. Adv. 4, eaar8082.
Tarbell, E.D., Liu, T., 2019. HMMRATAC: a hidden Markov ModeleR for ATAC-seq. Nucleic Acids Res. 47, e91 e91.
Tsai, K., Cullen, B.R., 2020. Epigenetic and epitranscriptomic regulation of viral replication. Nat. Rev. Microbiol. 18, 559–570.
Verhoeven, K.J., Vonholdt, B.M., Sork, V.L., 2016. Epigenetics in ecology and evolution: what we know and what we need to know. Mol. Ecol. 25, 1631–1638.
Waddington, C., Kacser, H., 1957. The strategy of the genes. A discussion of some aspects of theoretical biology. With an appendix by H. Kacser. Strateg. genes. A Discuss. some Asp. Theor. Biol. With an Append. by H. Kacser.
Waddington, C.H., 1942. Canalization of development and the inheritance of acquired characters. Nature 150, 563–565.
Wang, L., Wuerffel, R., Feldman, S., Khamlichi, A.A., Kenter, A.L., 2009. S region sequence, RNA polymerase II, and histone modifications create chromatin accessibility during class switch recombination. J. Exp. Med. 206, 1817–1830.
Wang, M., Xiao, Y., Li, Y., Wang, X., Qi, S., Wang, Y., Zhao, L., Wang, K., Peng, W., Luo, G.-Z., 2021a. RNA m6A modification functions in larval development and caste differentiation in honeybee (*Apis mellifera*). Cell Rep. 34, 108580.
Wang, Q., Sun, Q., Czajkowsky, D.M., Shao, Z., 2018. Sub-kb Hi-C in D. melanogaster reveals conserved characteristics of TADs between insect and mammalian cells. Nat. Commun. 9, 1–8.
Wang, Y.L., Liu, Y.q., Xu, Q., Xu, Y., Cao, K., Deng, N., Wang, R.M., Zhang, X.Y., Zheng, R.Q., Li, G.L., 2021b. TAD boundary and strength prediction by integrating sequence and epigenetic profile information. Briefings Bioinf. 22, bbab139.
Weaver, N., 1957. Effects of larval age on dimorphic differentiation of the female honey bee. Ann. Entomol. Soc. Am. 50, 283–294.
Weaver, N., 1966. Physiology of caste determination. Annu. Rev. Entomol. 11, 79–102.
Weiner, S.A., Galbraith, D.A., Adams, D.C., Valenzuela, N., Noll, F.B., Grozinger, C.M., Toth, A.L., 2013. A survey of DNA methylation across social insect species, life

stages, and castes reveals abundant and caste-associated methylation in a primitively social wasp. Sci. Nat. 100, 795–799.
West-Eberhard, M.J., 1989. Phenotypic plasticity and the origins of diversity. Annu. Rev. Ecol. Systemat. 20, 249–278.
Whitman, D.W., Agrawal, A.A., 2009. What is phenotypic plasticity and why is it important. Phenotypic plasticity of insects: Mechanisms and consequences 1–63.
Wilson, E.O., 1971. The Insect Societies. Harvard University Press, Cambridge.
Winston, M.L., 1991. The Biology of the Honey Bee. Harvard University Press, Cambridge.
Wojciechowski, M., Lowe, R., Maleszka, J., Conn, D., Maleszka, R., Hurd, P.J., 2018. Phenotypically distinct female castes in honey bees are defined by alternative chromatin states during larval development. Genome Res. 28, 1532–1542.
Wolff, J., Bhardwaj, V., Nothjunge, S., Richard, G., Renschler, G., Gilsbach, R., Manke, T., Backofen, R., Ramírez, F., Grüning, B.A., 2018. Galaxy HiCExplorer: a web server for reproducible Hi-C data analysis, quality control and visualization. Nucleic Acids Res. 46, W11–W16.
Xie, C., Mao, X., Huang, J., Ding, Y., Wu, J., Dong, S., Kong, L., Gao, G., Li, C.-Y., Wei, L., 2011. KOBAS 2.0: a web server for annotation and identification of enriched pathways and diseases. Nucleic Acids Res. 39, W316–W322.
Yi, Y., He, X.J., Barron, A.B., Liu, Y.B., Wang, Z.L., Yan, W.Y., Zeng, Z.J., 2020. Transgenerational accumulation of methylome changes discovered in commercially reared honey bee (*Apis mellifera*) queens. Insect Biochem. Mol. Biol. 127, 103476.
Yu, G., Wang, L.G., He, Q.-Y., 2015. ChIPseeker: an R/Bioconductor package for ChIP peak annotation, comparison and visualization. Bioinformatics 31, 2382–2383.
Zentner, G.E., Henikoff, S., 2013. Regulation of nucleosome dynamics by histone modifications. Nat. Struct. Mol. Biol. 20, 259–266.
Zhang, W., Wang, L., Zhao, Y., Wang, Y., Chen, C., Hu, Y., Zhu, Y., Sun, H., Cheng, Y., Sun, Q., 2022. Single-cell transcriptomic analysis of honeybee brains identifies vitellogenin as caste differentiation-related factor. iScience 25, 104643.
Zhang, Y., Liu, T., Meyer, C.A., Eeckhoute, J., Johnson, D.S., Bernstein, B.E., Nusbaum, C., Myers, R.M., Brown, M., Li, W., 2008. Model-based analysis of ChIP-seq (MACS). Genome Biol. 9, 1–9.
Zhu, L.J., Gazin, C., Lawson, N.D., Lin, S.M., Lapointe, D.S., Green, M.R., 2010. ChIPpeakAnno: a bioconductor package to annotate ChIP-seq and ChIP-chip data. BMC Bioinf. 11, 1–10.