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Transgenerational accumulation of methylome changes discovered in commercially reared honey bee (*Apis mellifera*) queens

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A B S T R A C T

Whether a female honey bee (*Apis mellifera*) develops into a worker or a queen depends on her nutrition during development, which changes the epigenome to alter the developmental trajectory. Beekeepers typically exploit this developmental plasticity to produce queen bee by transplanting worker larvae into queen cells to be reared as queens, thus redirecting a worker developmental pathway to a queen developmental pathway. We studied the consequences of this manipulation for the queen phenotype and methylome over four generations. Queens reared from worker larvae consistently had fewer ovarioles than queens reared from eggs. Over four generations the methylomes of lines of queens reared from eggs and worker larvae diverged, accumulating increasing differences in exons of genes related to caste differentiation, growth and immunity. We discuss the consequences of these cryptic changes to the honey bee epigenome for the health and viability of honey bee stocks.

1. Introduction

Epigenomics is revealing how genomic developmental systems are themselves sensitive to the developmental environment (Cavalli and Heard, 2019). A consequence of this is the possibility of developmental stressors to rewrite the epigenome with profound, and potentially enduring consequences for animal development (Burggren, 2015; Cavalli and Heard, 2019). The western honey bee (*Apis mellifera*) presents a dramatic natural example of developmental plasticity that is epigenomically regulated. The nutritional environment during development selectively changes methylation of the bee genome which establishes the very different worker and queen phenotypes (He et al., 2017; Kucharski et al., 2008). This provides a natural system for study of how the epigenome can be affected by developmental stress. Here we studied how a current developmental stress routinely applied in contemporary agriculture influenced the honey bee queen epigenome over both long and short timescales.

An interaction of developmental systems with the environment has long been assumed, but it was also a common conception that genomics mechanisms shaping development were themselves isolated from environmental influences, with stressors subverting an ideal genomic developmental pattern. Epigenomics has overturned this view and highlighted how numerous epigenomic systems are directly sensitive to the environment (Burggren, 2015). Indeed, this can be a vital aspect of their functionality (He et al., 2017; Jung-Hoffmann, 1966; Maleszka, 2008), but it can also result in dysfunction (Cavalli and Heard, 2019).

Classic studies with the honey bee have shown how the sensitivity of epigenomic systems to the environment can be an essential mechanism of developmental plasticity (Kucharski et al., 2008; Lyko et al., 2010; Maleszka, 2008). There are two very distinct developmental outcomes for female honey bees: large reproductive queen bee and small sterile worker bee (Evans and Wheeler, 2001; Hartfelder K, 1998; Jung-Hoffmann, 1966). These different castes are key to the success of the honey bee eusocial and colony lifestyle, but there are no genetic differences between worker and queen bee despite the major morphological differences between them (Evans and Wheeler, 2001; Hartfelder K, 1998; Jung-Hoffmann, 1966). The two castes develop in different nutritional environments. Queen-destined larvae are fed far more richer food (royal jelly) than worker-destined larvae, and the developmental pathways for workers and queens diverge during early larval

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development (Jung-Hoffmann, 1966; Maleszka, 2008).

The honey bee methylome is sensitive to the nutrition of the development larvae so that the early nutritional environment establishes the larva on either a worker or queen developmental pathway (Maleszka, 2008). Experimental manipulations of DNA methylation early in larval development can switch worker-destined larvae to a queen developmental pathway, revealing the key role of changes in the DNA methylome in the natural phenotypic plasticity of the honey bee (Kucharski et al., 2008; Shi et al., 2013).

Environmental stressors can also disrupt the epigenome leading to developmental dysfunction, However. This is increasingly being recognized as an important component of many diseases (Cavalli and Heard, 2019; Pembrey et al., 2014). An emerging concern is the possibility for stress-induced changes in the epigenome to be passed on to offspring (Cavalli and Heard, 2019; Skvortsova et al., 2018). Until recently this was considered highly unlikely, but more and more cases are emerging. These include numerous examples from humans of transgenerational inheritance of epigenomic changes induced by smoking, nutritional stresses and toxins (Pembrey et al., 2014). Inherited epigenomic changes resulting from environmental stress on the parent have now been linked to pathologies and phenotypic changes in plants, worms, flies, fish, birds, rodents, pigs, and humans (Nilsson et al., 2018) (Anway et al., 2005; Dias and Ressler, 2014; Nilsson et al., 2018). For example, if male rats were exposed to the endocrine disruptor vinclozolin during embryonic gonadal sex determination their fertility and behavior was affected, as was the methylation state of their sperm such that the changes persisted over four generations (Anway et al., 2005). Male mice maintained on a high fat diet for three generations accumulate changes in epigenetic systems regulating lipogenesis altering susceptibility to obesity (Li et al., 2012).

The honey bee provides a fortuitous natural system to explore how the epigenome might respond to sustained developmental stress. In the natural process of queen development the queen lays an egg in an especially large queen cell made by the workers (Wei et al., 2019). The workers fill the cell with royal jelly proving the hatchling with abundant rich food. By contrast, in contemporary commercial beekeeping, most queens are raised by artificially transplanting young worker larvae from worker cells into artificial queen cells, which the workers then provision with royal jelly to produce a queen (Büchler et al., 2013; Doolittle, 1888). A consequence of this manipulative queen rearing method is that larvae begin development on a worker-destined trajectory and later switch to a queen-destined developmental trajectory. Queens reared from older worker larvae are smaller, lighter, have smaller ovaries and show changes in the methylation of many genes important for caste differentiation when compared to queens reared from honey bee eggs, (which more closely matches the natural process of queen production) (He et al., 2017; Woyke, 1971). These differences influence colony growth and performance (Rangel et al., 2012), and deteriorating queen quality is increasingly being recognized as an important factor in the recent declines in honey bee health (Brodschneider et al., 2019).

Here we examined the consequences of rearing queens from worker larvae for repeated generations. We found not only that this influenced the phenotype and epigenome of the adult queens, but that repeated manipulations across successive generations caused an accumulation of changes to the honey bee methylome, affecting particularly genes involved the differentiation of worker and queen phenotypes. We argue such epigenomic attrition of developmental systems might be contributing to a decline in quality of honey bee stocks.

2. Materials and methods

2.1. Animals

The Western honey bee, *Apis mellifera*, was used throughout this study. Honey bee colonies were maintained at the Honeybee Research Institute, Jiangxi Agricultural University, Nanchang, China (28.46μN, 115.49μE), according to standard beekeeping techniques. All experiments were performed in accordance with the guidelines from the Animal Care and Use Committee of Jiangxi Agricultural University, China.

2.2. Queen rearing and sampling

Our queen rearing strategy is summarized in Fig. 1. Our initial founding queen (Fig. 1) was a standard commercially available queen instrumentally inseminated with semen from a single unrelated drone. She was restricted for 6 h (10 a.m. - 4 p.m.) to a plastic honey bee frame to lay eggs in worker cells. This frame was designed such that the plastic base of each cell holding the egg or larva could be transferred to plastic honey bee queen cells (Pan et al., 2013). 20–30 eggs or larvae were transferred to queen cells at 4pm on the 2nd, 4th, and 5th day after laying. Thus, three types of daughter queen groups were established.

G1E were generation 1 queens reared from eggs transferred to queen cells on the 2nd day after laying. G1L1 were G1 queens reared from one-day old larvae transferred to queen cells on the 4th day after laying. G1L2 were G1 queens reared from two-day old larvae transferred to queen cells on the 5th day after laying. The queen cells were placed in racks in two queenless honey bee colony to be tended by workers, fed royal jelly and reared as queens. In each generation, half of each queen rearing group was assigned to each queenless colony.

Of the G1 queens, three queen cells of each group were selected randomly on the 14th day after laying, and were each placed in a small queenless hive to emerge and mate naturally. The remaining G1 queen cells were numbered, the length of each queen cell was measured and then they were placed in a dark incubator (35 °C, 80%) to emerge. From the 15th day post laying queen cells were checked every 2 h for queen emergence, and hourly after the first G1 queen emerged. The four queens in each group to emerge were taken for methylation analysis. These were immediately flash frozen in liquid nitrogen when collected after emergence and stored in a −80 °C refrigerator.

Remaining queens were sampled to measure ovariole number. These queens were transferred to queen cages, which were placed in queenless colonies for 4–5 days where they could be fed and tended by workers through the cage, since the ovaries of 4–5 day-old queens are easier to stain and count than newly emerged queens (Berger et al., 2016; Patricio and Cruz-Landim, 2002). When 4–5 days old these queens were flash frozen in liquid nitrogen and stored in a −80 °C freezer. To score ovariole number we created paraffin sections of the stained and dissected ovary (Gan et al., 2012). We counted the number of ovarioles in the left ovary by identifying slides in which the ovarioles were very clear and counting slides until we found at least two giving exactly the same number of ovarioles(fiji-win64 software, Fig. 2B).

We used two-way ANOVA to investigate the effects of queen type and generation on ovariole number, and Fisher's PLSD test to analyze differences between queen types within each generation. The number of queens in each sample group varied and was affected by queen larvae and queenless colony survival. We sampled 3–11 queens for ovariole analysis in each group (Fig. 2).

To rear the second generation queens (G2) we selected one of the mated and laying G1 queens from each group (G1E, G1L1 and G1L2). Each G1 queen was restricted for 6 h (10 a.m.-4 pm) to a plastic worker honey bee frame for laying. We then created three different types of G2 queens as for G1.

Eggs from the G1E queen were transferred as eggs to queen cells on the 2nd day post laying to create the G2E group. Eggs from the G1L1 queens were transferred to queen cells on the 4th day after laying to create the G2L1 group. Eggs from the G1L2 queens were transferred to queen cells on the 5th day after laying to create the G2L2 group (Fig. 1). Queen cells of the G2 groups were treated the same way as the G1 queen cells. The emerging G2 queens were reared and sampled as for the G1 groups.

We repeated this process to create the 3rd and 4th generation queen groups: G3E, G3L1, G3L2 and G4E, G4L1 and G4L2. Three types of

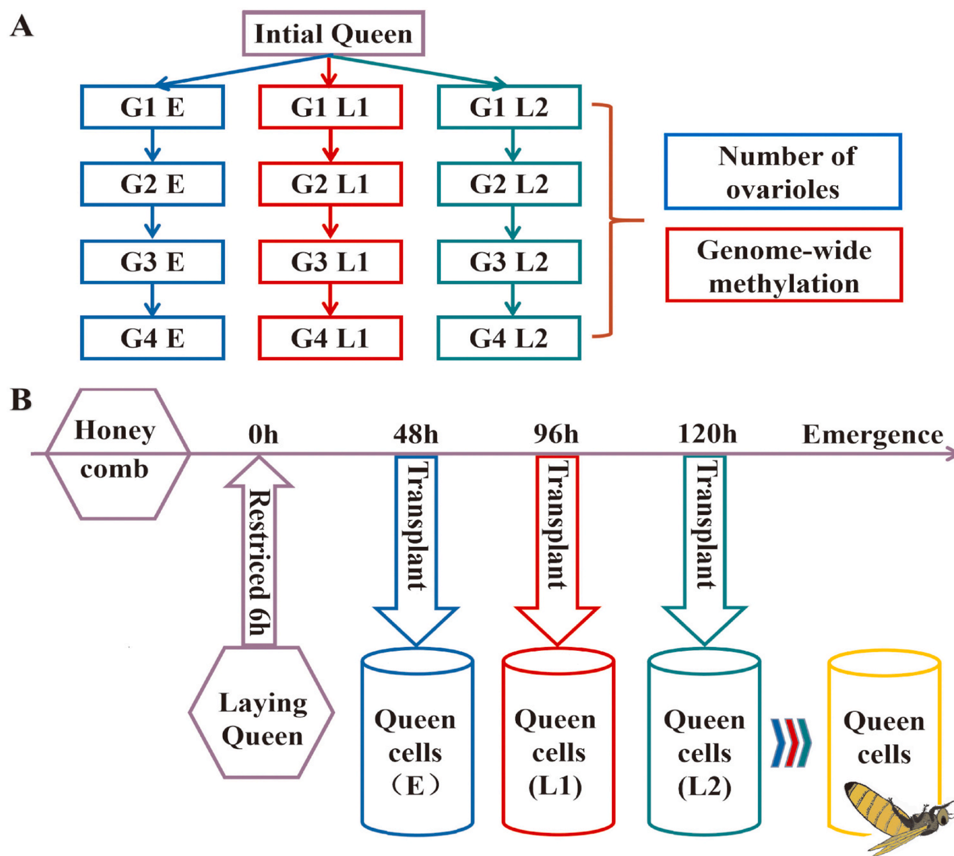

Fig. 1. Experimental design. (A) Lineage of queens and measurements. (B) Timing of the grafting and brood transfers. In the first generation (G1) a queen artificially inseminated with the semen of a single drone was caged on a plastic frame for 6 h to obtain eggs of a known age. Some of the eggs (E) were transferred to queen cells after 48 h, while other eggs were left for 96 h and 120 h and transferred after they reached the first (L1) or second (L2) larval instar respectively. The queens obtained were in turn caged on a plastic frame for 6 h and their brood was again grafted onto queen cells after 48 h, 96 h and 120 h and so on for the next three consecutive generations (generations G2 - G4). At each generation queens that were not used to raise the next generation of eggs were killed, the number of ovarioles was counted and the DNA genome-wide level of methylation from the brain, thorax and ovarioles was measured.

queens were sampled in each generation for methylation and ovariole analysis. 12 queen groups were sampled in total.

2.3. Paraffin section of the queen ovary

Briefly, queens from each group were thawed to room temperature and both ovaries dissected from the abdomen. Ovaries were fixed in 4% paraformaldehyde fix solution (BBI Life Sciences) for 12 h. For dehydration and fixing we used an Automatic dehydrator (Leica, TP1020). Ovaries were dehydrated in a graded ethanol series (70%–100%). Ovaries were then cleared using xylene and samples then placed in a 1:1 absolute ethanol/xylene mixture for 30min, then changed to xylene for 10min, followed by fresh xylene for 5min. They were transferred to a 1:1 xylene paraffin mixture for 30min, and then paraffin wax for more than 2 h.

Ovaries were embedded and blocked in paraffin wax using a Heated Paraffin Embedding Station (Leica). 5–7 (m sections were cut using a Leica RM 2245 microtome. Sections were placed on histological slides (Autostainer XL), stained with HE Staining Kit (BOSTER AR1180), mounted with neutral balsam mounting medium (BBI Life Sciences) and covered with a coverslip. The slides were then imaged and photographed using a 100x transmission light microscope (OLYMPUS, DP80) (Fig. 2B).

2.4. Genome wide methylation analysis

Queens sampled for methylation analysis were flash frozen in liquid nitrogen when collected after emergence and stored in a −80 °C refrigerator. Four queen bee were sampled in each group. The brain, thorax and ovary of each queen was dissected over ice as one sample. Tissues from each queen were pooled for genomic DNA extraction. In total, 12 queen groups were sampled for methylation analysis. Of these, samples that did not meet our quality control requirements for genomic sequencing were deleted. Nine out of 48 samples were unsuitable. One sample from the queen groups G1E, G1L1, G2E, G2L1, G3E, G3L1, and G3L2 were unsuitable, and two samples from the queen group G2L2 were unsuitable. (Details shown in Supplementary Table S1).

2.5. Genomic DNA extraction and quantification

Genomic DNA was extracted using the StarSpin Animal DNA Kit (GenStar). Genomic DNA degradation and contamination was assessed by running the DNA on agarose gels. DNA purity was assessed using a NanoPhotometer® (IMPLEN, CA, USA). DNA concentration was measured using a Qubit® DNA Assay Kit (Life Technologies, CA, USA) and a Qubit® 2.0 Flurometer (Life Technologies, CA, USA). DNA samples were then sent for whole-genome bisulfite sequencing analysis by the Novogene Bioinformatics Technology Co., Ltd/www.novogene.cn using the method summarized below.

2.6. Library preparation and quantification

Then these DNA fragments were treated twice with bisulfite using EZ DNA Methylation-GoldTM Kit (Zymo Research), before the resulting single-strand DNA fragments were PCR amplificated using KAPA HiFi HotStart Uracil + ReadyMix (2X).

100 ng genomic DNA was spiked with 0.5 ng lambda DNA and fragmented by sonication to 200–300 bp using a Covaris S220 DNA Sequencing/gene analyzer. These DNA fragments were treated with bisulfite using EZ DNA Methylation -GoldTM Kit (Zymo Research, CA, USA). Bisulfite converted DNA were processed by the Accel-NGS Methyl -Seq DNA Library Kit to create dual-indexed Methyl-Seq libraries. All libraries were amplified in a 9-cycle indexing PCR reaction. Library DNA concentration was quantified by a Qubit® 2.0 Flurometer (Life Technologies, CA, USA) and quantitative PCR. The insert size was assayed on an Agilent Bioanalyzer 2100 system.

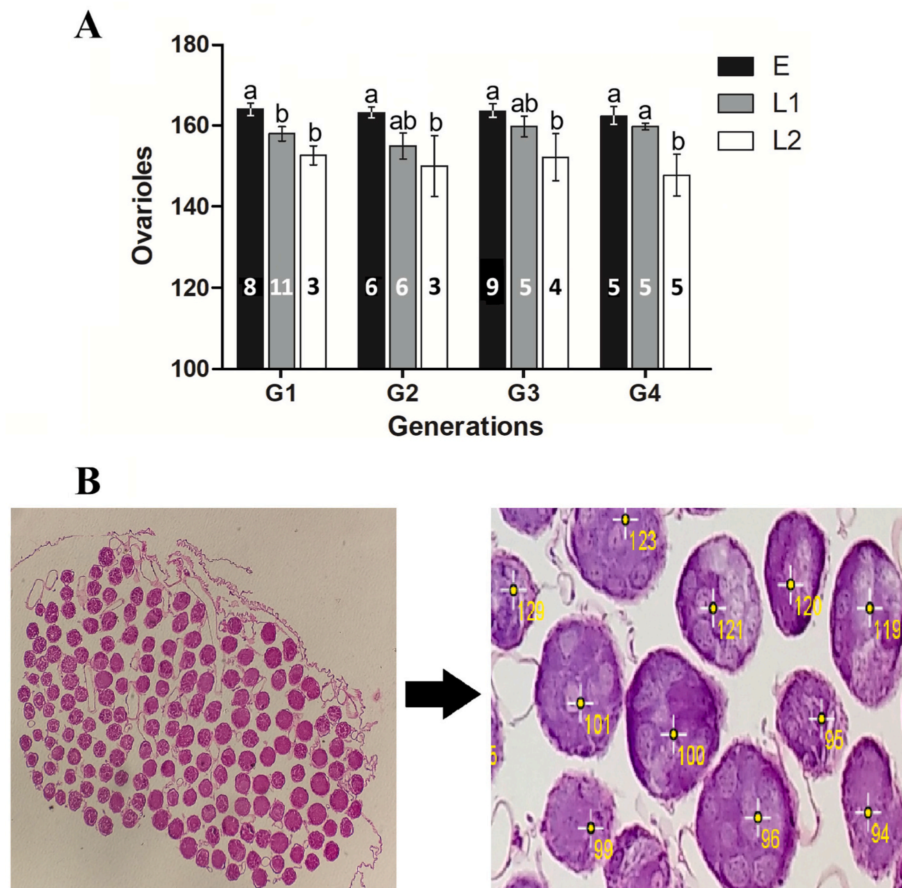

Fig. 2. (A) Number of ovarioles in E, L1 and L2 queens in generations G1 - G4. Bars show mean ± SEM. Sample size was shown in each bar. One-way ANOVA was performed on each generation. Different letters above bars indicated significantly difference within each generation. (B) Specimen ovariole section illustrating ovariole counting methods (see Methods).

2.7. Data analysis

The library preparations were sequenced on an Illumina Hiseq XTen and 125 bp to 150 bp paired end-reads were generated. Image analysis and base identification were performed with Illumina CASAVA pipeline.

2.8. Quality control

FastQC (fastqc_v0.11.5) was used to perform basic statistics on the quality of the raw reads. Read sequences produced by the Illumina pipeline in FASTQ format were pre-processed through Trimmomatic (Trimmomatic-0.36) software use the parameter (SLIDINGWINDOW:4:15, LEADING:3, TRAILING:3, ILLUMINACLIP: adapter.fa:2, 30:10, MINLEN:36). Reads that passed all of these filtering steps were counted as clean reads and all subsequent analyses were performed on these. Finally, we used FastQC to perform basic statistics on the quality of the clean reads data.

2.9. Reference data preparation before analysis

Before the analysis, we prepared the reference data for *Apis mellifera*, including the reference sequence (as a fasta file), the annotation file in gtf format, the GO annotation file, a description of genes in the *Apis mellifera* genome (downloaded from NCBI) and the gene region file (also from NCBI, in BED format).

2.10. Mapping reads to the reference genome

Bismark software (version 0.16.3) was used to perform alignments of bisulfite-treated reads to a reference genome (Amel_HAv3.1 (GCF_003254395.2)) (Krueger and Andrews, 2011). For alignment of the library reads to the reference genome, the reference genome and library reads were firstly transformed into bisulfite-converted versions of the sequences (C-to-T and G-to-A) and then assigned to a digital index using bowtie2, so that the index information included data on the sequences, their origin, and the experiment (Langmead and Salzberg, 2012). Sequence reads from the bisulphite-sequenced samples were aligned to fully bisulfite-converted versions (C-to-T and G-to-A converted) versions of the genome in a directional manner. Sequence reads that produced a unique best alignment from the two alignment processes (original top and bottom strand) were then compared to the normal genomic sequence and the methylation state of all cytosine positions in the read was thus inferred. Reads that aligned to the same regions of the genome were regarded as duplicates. The sequencing depth and coverage were calculated assessing number of overlapping reads relative to number of duplicate reads.

2.11. Estimating methylation level

To identify the level of methylation at each site, we modeled the count of methylated cytosines (mC) at a site as a binomial (Bin) random variable with methylation rate r: mC ~ Bin (mC + umC*r) (http://www.stat.yale.edu/Courses/1997-98/101/binom.htm).

In order to calculate the methylation level of a sequence, we divided the sequence into multiple bins, of 10 kb. The sum of methylated and unmethylated read counts in each bin were calculated. Methylation level (ML) for each bin or C site shows the fraction of methylated Cs, and is defined as: ML (C) = (reads mC)/(reads (mC) + reads (C)).

Calculated ML was further corrected with the bisulfite non-conversion rate according to previous studies (Lister et al., 2013). Given the bisulfite non-conversion rate r, the corrected ML was estimated as: ML (corrected) = (ML - r)/(1-r).

2.12. Differential methylation analysis

Differentially methylated regions (DMRs) were identified using the DSS software (v 2.28.0) (Feng et al., 2014). DSS is an R library performing differential analysis for count-based sequencing data. It detects differentially methylated loci or regions (DML/DMRs) from bisulfite sequencing (BS-seq). The core of DSS is a new dispersion shrinkage method for estimating the dispersion parameter from Gamma-Poisson or Beta-Binomial distributions. DMRs were identified using the parameters: smoothing = TRUE, smoothing.span = 200, delta = 0, p.threshold = 1e-05, minlen = 50, minCG = 3, dis.merge = 100, pct.sig = 0.5 (Feng et al., 2014; Park and Wu, 2016; Wu et al., 2015). The final list of DMRs were assigned genomic positions, or overlapped between comparisons. We defined the genes related to DMRs as genes whose gene body region (from TSS to TES) or promoter region (upstream 2 kb from the TSS) overlapped with the DMRs.

2.13. GO and KEGG enrichment analysis of DMR-related genes (DMGs)

A DMR related gene is defined as a gene within which the DMR is located. If there is differential methylation in any region of the gene body, the gene will be considered as the differentially methylated gene (DMG). Gene Ontology (GO) enrichment analysis of genes related to DMRs was implemented by the GOseq R package (Young et al., 2010), in which gene length bias was corrected. GO terms with corrected P-value less than 0.05 were considered significantly enriched among DMR-related genes. The KEGG (Kanehisa et al., 2008) database related genes to high-level functions and utilities of a biological system, (http://www.genome.jp/kegg/). We used KOBAS software (Mao et al., 2005) to test the statistical enrichment of DMR related genes to different KEGG pathways.

3. Results

3.1. DNA methylation sequence quality

From our four generations of queens of each rearing type (Fig. 1) we also assessed the methylation status of the genome with bisulphite sequencing. From each sample the average number of clean reads was 38, 751, 257, with 10.2 G of clean base sequences (Table S1). The average phred scores Q30% (Ewing and Green, 1998) was 92.4% (Table S1). The average bisulfite conversion rate was 99.7% (Table S1). The average site coverage rate was 25.06 (Table S2), indicating that there was acceptable sequencing quality (NIH roadmap epigenomics project, http://www.roadmapepigenomics.org/protocols). The pearson correlation coefficient among biological replicates of each experimental group were all $\geq$0.97 (Table S3), indicating good repeatability among the biological replicates of each group.

3.2. Effect of queen rearing method on queen morphology and the methylome

Ovariole number differed significantly between queens of different rearing types (Fig. 2, Two-way ANOVA, $F = 18.869$, $DF = 2$, $P < 0.001$), but no effect of generation (G1 - G4, Fig. 2) on ovariole number (Two-way ANOVA, $F = 0.321$, $DF = 3$, $P = 0.809$), and no interaction (Two-way ANOVA, $F = 0.326$, $DF = 6$, $P = 0.921$). Consistently ovariole number was reduced in L2 queens compared to E queens, with L1 queens intermediate between these groups (Fig. 2).

When comparing queens from different rearing types we noted an increase in the number of DMGs with each generation of rearing. In each generation we compared DMGs between L1 with E queens. The number of DMGs increased with each generation of rearing (Fig. 3A, Table S4). We observed a similar phenomenon when we compared L2 with E queens in each generation (Fig. 3A, Table S4).

To account for any possible effect of season or time on number of DMGs in our study we analyzed the number of DMGs in successive generations of each queen rearing type. For E queens, numbers of DMGs were extremely stable when we compared G2 with G1, G3 with G2 and G4 with G3 (Fig. 3F, Table S5). By contrast when we examined L1 and L2 queens we observed the numbers of DMGs increased in each comparison of successive generations (Fig. 3F).

With each successive generation the relatedness of queens between our rearing groups decreased. This could also cause the number of DMGs to increase each generation. To explore this possibility, we analyzed the number of DMGs shared by comparisons of different rearing groups within each generation and unique to comparisons of different rearing groups within each generation (Fig. 3B–E), and unique to and shared between comparisons of successive generations (Fig. 3G–I). Our data show that DMGs increased with successive generations of comparison (Fig. 3A–E, Fig. S1). But DMGs were consistently greater for L2-E comparisons than L1-E comparisons (Fig. 3A–E). Further when comparing DMGs between successive generations of E queens (Fig. 3G) the number was very stable, suggesting that a decline in relatedness did not increase DMG number much. But when comparing L1 and E queens or L2 and E queens the number of DMGs increased with each generation (Fig. 3 F, H and I). This suggests that for L1 and L2 queens' methylation differences accumulated with each generation of repeating L1 or L2 rearing.

When considering the lists of DMGs in each generation in more detail, we focused on genes previously identified as related to reproduction or longevity (Corona and Robinson, 2006; He et al., 2017; Yin et al., 2018), immunity (Barribeau et al., 2015; Boutin et al., 2015; He et al., 2017), metabolism (He et al., 2017), and DMGs identified in our own data as related to these groups from KEGG pathway analyses. Some genes may be related to multiple functions, but we only counted them once. Our classification criteria was: reproduction or longevity > immunity > metabolism. We used this functional annotation of target genes to numbers of DMGs in different functional groups in our different comparisons (Fig. 4). We saw an increasing number of genes involved in key caste differentiation processes (body development, immunity and reproduction/longevity) differentially methylated between different rearing types with each generation of rearing (Fig. 4 and Table S6).

Consistently in the DMG gene lists comparing our queen groups (Table S7) we noted 106 genes with functions that have been related to caste differentiation (Beltran et al., 2007; Buttstedt et al., 2016; Guan et al., 2013a,b; Marshall et al., 2019; Tian et al., 2018), body development and metabolism (Bull et al., 2012; Davis et al., 2002; Evans et al., 2006; Mao et al., 2017; Miller et al., 2012; Parker et al., 2012; Shi et al., 2011; Zufelato et al., 2004), and gene regulatory pathways related to caste differentiation (Amdam, 2011; Barchuk et al., 2007; Foret et al., 2012; He et al., 2017; Yin et al., 2018). Of these, we analyzed 40 genes that appeared most consistently in our DMG lists across generations (Fig. 3 and Table S8). From G1 – G4 there was an increase in both the number of DMGs (Fig. 5), and the ratio of methylation differences between the compared sequences (Fig. 5).

From the 40 genes in Fig. 5, we selected two genes from gene regulatory pathways already implicated in the epigenomic mechanism of queen/worker differentiation (Foret et al., 2012). The *Cat* gene is involved in FoxO pathway and longevity (Klichko et al., 2004). *S6k1* is involved in mTOR signaling pathway and TGF-beta pathway (Chen et al., 2012; Foret et al., 2012). For these two genes we examined where in the gene sequence changes in methylation occurred (Fig. 6 and Table S9). We focused on exons (Lyko et al., 2010), and limited Fig. 6 to displaying only exons in which significant DMRs occurred. We observed accumulating changes in the amount of methylation at specific sites in our two genes from G1 - G4.

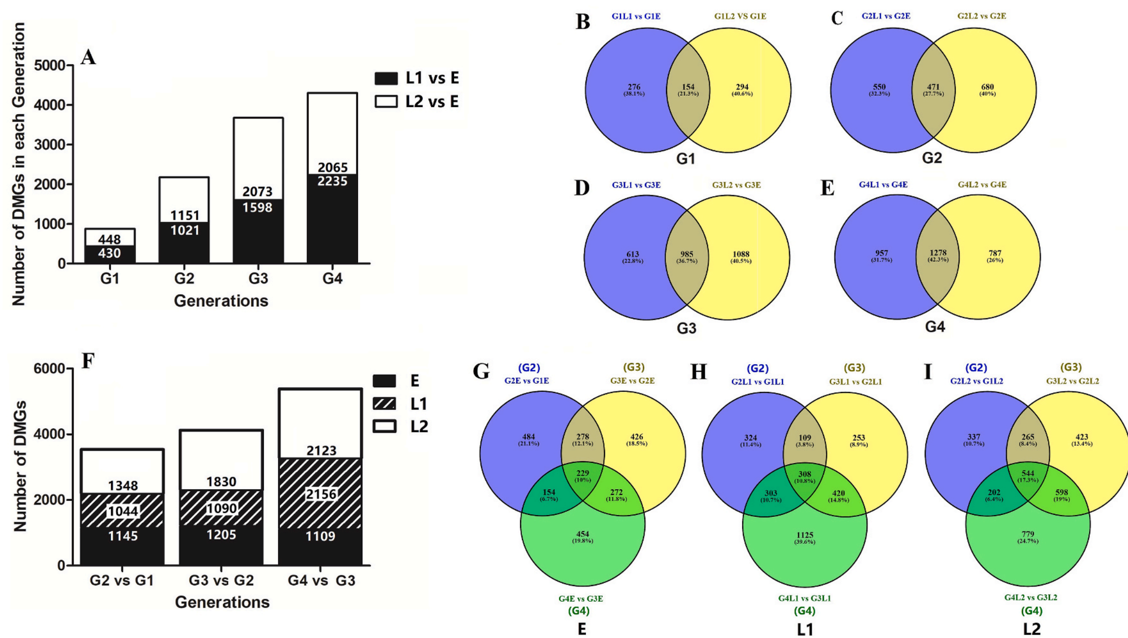

Fig. 3. (A) Summary of numbers of differentially methylated genes (DMGs) comparing L1 with E (black), and L2 with E (white) in generations G1 - G4. The number of DMGs in each comparison was written into each bar (Summary of DMG IDs in Table S4). (B–E) Venn diagrams of the numbers of DMGs comparing L1 with E, and L2 with E in generations G1 - G4. (F) Numbers of DMGs comparing each queen rearing type across successive generations. The three bars showed DMGs in comparisons of G2 with G1, G3 with G2, and G4 with G3. Within each stacked bar we showed the number of DMGs for each of the three different queen rearing groups (for example, comparing G2E with G1E, G2L1 with G1L1, and G2L2 with G1L2). The number of DMGs was shown in each bar. Summary of DMG IDs in Table S5. (G–I) Venn diagrams of the numbers of DMGs comparing GnE with Gn-1E (G), GnL1 with Gn-1L1 (H), and GnL2 with Gn-1L2 (I) (n = 2, 3, or 4).

4. Discussion

The development of both the worker and queen honey bee castes is dependent on differential methylation of the bee genome (Barchuk et al., 2007; Kucharski et al., 2008; Lyko et al., 2010; Maleszka, 2008; Maleszka et al., 2014). This epigenomic "developmental switch" allows workers to control which eggs develop as future queens for their colonies by controlling the nutrition of larvae (Jung-Hoffmann, 1966). But here we show this epigenomic developmental system is itself compromised by the developmental stress inherent in contemporary apicultural methods of queen rearing.

In commercial queen rearing it is common to transplant larvae up to 3-days old from worker cells into queen cells where they will be subsequently provisioned as queens. This practice has been in very widespread use in apiculture since 1888 (Doolittle, 1888). 2-day old worker larvae transplanted to queen cells could be successfully raised as queens, but there were consequences from this developmental manipulation for the queen phenotype. We consistently found that queen ovariole number was lower in L1 and L2 queens compared with E queens (Fig. 2A). The number of ovarioles determines how many eggs can be produced and matured by the queen. This difference would be expected to have consequences for colony growth and function since the queen is the sole reproductive in a honey bee colony, and the mother of the entire worker population. Indeed, Rangel et al. (2012) reported slower growth of bee colonies headed by queens reared from older worker larvae. Our findings confirmed earlier studies reporting an effect of queen rearing type on queen reproductive organs (Woyke, 1971).

We found many differences in the methylome of queens of different rearing types, but for the first time we tracked how these differences changed if rearing types repeated for successive generations (Figs. 3–6). We found that methylation differences between the different rearing types increased with each successive generation (Figs. 3–6). In effect, we observed a progressive divergence in the methylome of our queen rearing types as we sustained the different methods of queen rearing.

Our analyses focused on pathways and genes that have previously been related to the process of caste differentiation in honey bees (He et al., 2017) such that after four generations of rearing the methylation differences between L1 or L2 queens and E queens were far greater than after one generation (Figs. 4–6).

Confounded with our inter-generational sampling is the passage of time, change in season at the time of sampling and reduced genetic relatedness of between our rearing groups, and so we must consider how each of these might have contributed to the increase in DMGs across generations we report. When we compared L1 with E or L2 with E within our four generations, we saw an increasing number of DMGs with each generation of rearing (Fig. 3A), but when we measured DMGs between mother and daughter E queens across our four generations the number of DMGs was very stable and did not increase (Fig. 3F and G). This result shows that for E queens (the condition that most closely matches the evolved and natural method of queen rearing) the number of DMGs did not increase significantly with each generation of sampling. For E queens we did not see the confounds of reduced genetic relatedness, time or season increasing the number of DMGs significantly in successive intergenerational comparisons (Fig. 3F and G).

For both L1 of L2 queen groups, the number of DMGs in successive mother/daughter comparisons increased with each generation of rearing (Fig. 3F, H, and I) indicating an increase of DMGs with each generation. The number of DMGs between E queens and L2 queens increased from 448 in generation 1 to 2065 in generation 4 (Fig. 3A). Many of these changes were associated with genes with functional characterizations linked to caste determination (Fig. 4B). It seems unlikely that season, time or reduced relatedness would have increased DMG number in our L2 and L1 groups but not our E group. Hence we feel our data includes a signature of repeated rearing of queens from larvae increasing gene methylation differences with each generation.

Our G1 queens were all daughters of one queen mated with the sperm of single drone (Fig. 1), consequently in G1 our queens were all full sisters. In each subsequent generation, however, queens mated naturally with the local population of drones to create the next generation of queens. While we could not prevent relatedness between our

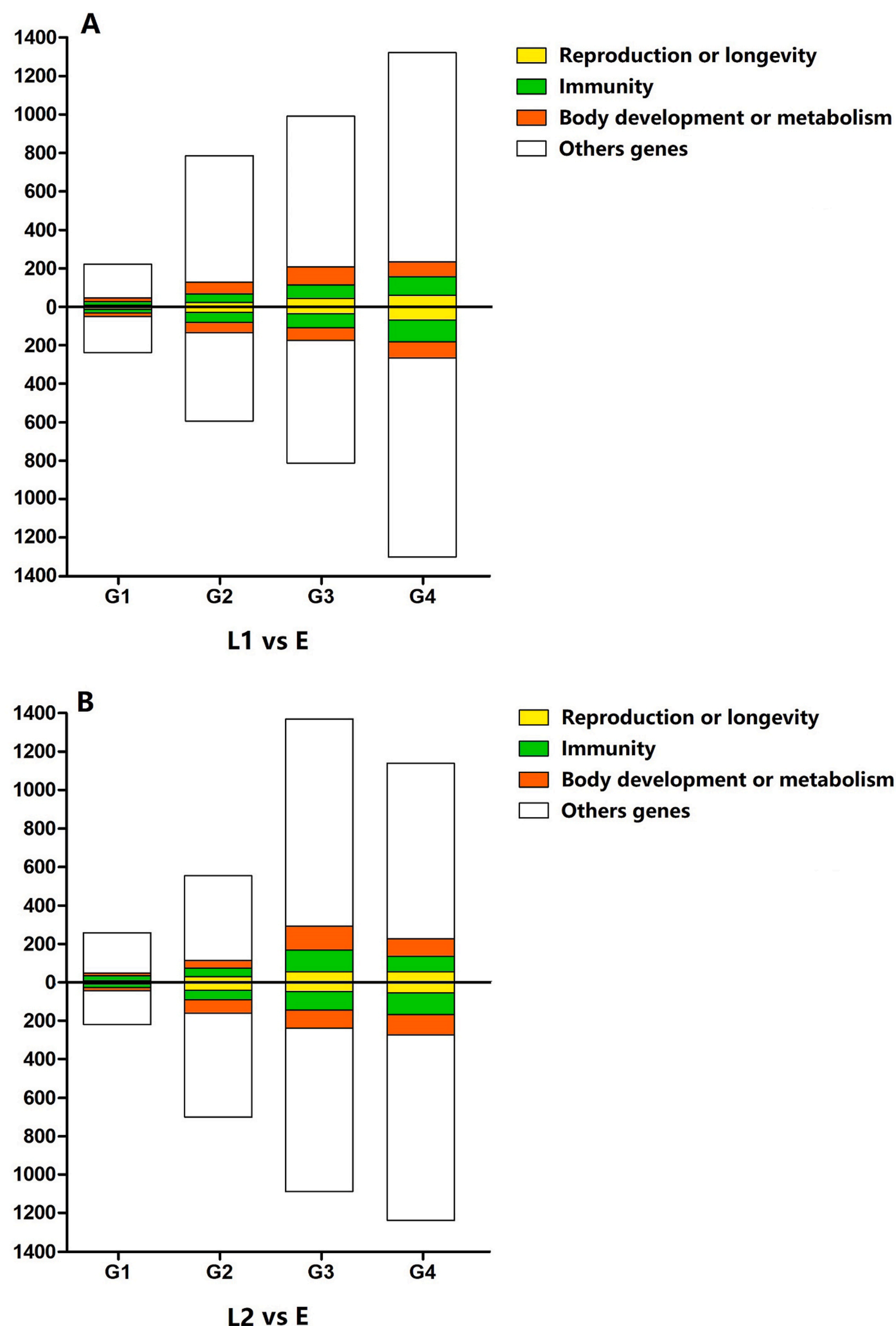

Fig. 4. Number of differentially methylated genes in different functional classes within each generation, when comparing L1 with E (A) and L2 with E (B).

rearing groups decreasing with each generation, this strategy should have prevented our developmental lines from diverging into distinct genetic lines by repeated introgression with the same genetic background.

There are about 70,000 methylated cytosine sites in the *Apis mellifera* genome. Most of these are CpG dinucleotides in exons (Lyko et al., 2010). In L1 and L2 queen groups methylation changes accumulated in exons. In insects methylation of exons has been related to functional changes in gene expression, and/or may mediate splice variation (Cingolani et al., 2013; Foret et al., 2009, 2012; Li-Byarlay et al., 2013; Wojciechowski et al., 2014).

If we consider the L1 and L2 queen rearing types as experiencing a form of developmental stress, then we report an accumulation of methylation changes with sustained stress across generations. Similar findings have been reported for nematodes and rodents where rearing an organism under stress for repeated generations induced more methylation changes than rearing under stress for a single generation (Li et al., 2012; Remy, 2010).

Burggren (2015) in a review of this general phenomenon highlights how epigenetic changes should be recognized as graded time related changes that can both “wash in” and “wash out” of the genome over time. As examples, rearing mice on a fatty diet across three generations has been shown to “wash in” epigenetic changes (DNA methylation, modification in histones) contributing to obesity susceptibility (Li et al., 2012). In nematodes, repeated exposure to an odour across 4 generations resulted in what had originally been an induced behavioral change to this odour to become a stable inherited behavioral change (genomic imprinting) (Remy, 2010). The distinction between acquired and inherited characteristics is not absolute (Burggren, 2015; Furrow and Feldman, 2014; Robinson and Barron, 2017).

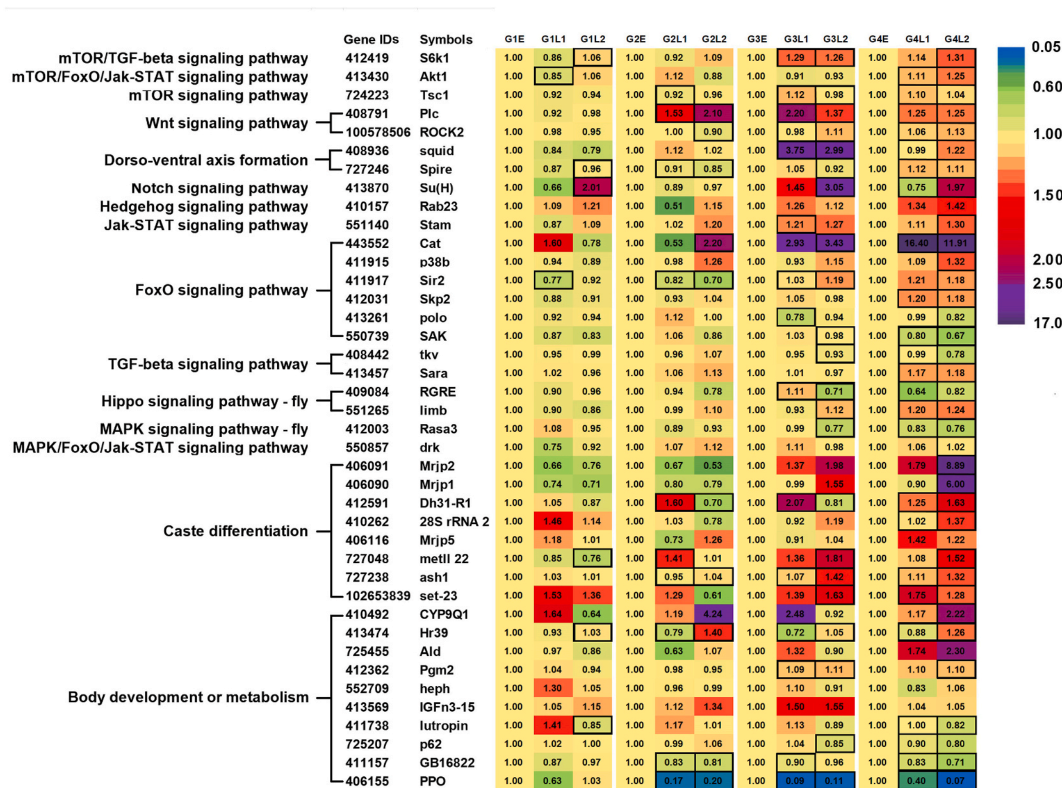

Fig. 5. 40 focal genes were selected for their known functions in caste differentiation, body development or metabolism of the honey bee. For these 40 genes we compared within each generation the relative methylation level of L1 and L2 queens with E queens for each gene. Methylation level was calculated by comparing the proportions of methylated reads at each site in all exons of a gene for each sample group. Relative methylation level (shown by color of each box) was then calculated as the ratio of methylation levels for each comparison (within a generation, L1 with E or L2 with E). Green and blue indicated hypo-methylated genes in groups compared with E. Red and purple indicated hyper-methylated genes, and yellow indicates no difference. The deeper the color, the greater the difference. Black borders indicated that there was at least one exon in this gene that was significantly differentially methylated between the compared groups. For each gene we showed gene functions, gene IDs and gene symbols from left to right. More detailed information of these 40 genes have been provided in Table S8. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

The distinct worker and queen developmental trajectories are dependent on epigenomic regulation, hence the cryptic accumulation of changes to the queen methylome seen here is troubling.

In this study we did not see any progressive change in ovariole number resulting from rearing queens from L2 larvae for four generations, but rearing queens from larvae has been a standard practice in apiculture for decades, and commercially this practice could sustain a developmental stress on a queen stock for many generations. We now recognize that queens reared from worker-destined larvae are of lower quality than queens reared from eggs (Rangel et al., 2012). Our work highlights the more concerning possibility of this practice causing a progressive and cryptic erosion of the epigenetically regulated queen developmental pathway. This could reduce queen quality without any detectable changes in bee genetics or conventional inbreeding.

The effect of methylation modification on insect caste differentiation is a controversial debate. Libbrecht et al. (2016) suggest that differential methylation in insects is weak or absent. Indeed, DNA methylation is unstable and can be altered by various environmental factors such as nutrition or environmental stress (Kucharski et al., 2008; Cavalli and Heard, 2019). However, knocking down the Dnmt3 gene significantly increased the probability of newly hatched L1 larvae developing into queen bees (Kucharski et al., 2008). Shi et al. (2013) reported a significantly higher global DNA methylation level in queen larvae compared to worker larvae. The present study showed a great number of DMGs detected in the comparisons of G1L1 with G1E (430) and G1L2 with G1E (448) (Fig. 3A). The DMG number was similar to that reported by Lyko et al. (2010), who showed 560 DMGs in the brains of queens and workers. Interestingly, of those 560 DMGs 25 and 29 genes also appeared in the comparisons of G1L1 with G1E and G1L2 with G21E respectively (Table 1). Moreover, the number of overlapping genes increased with generations, which also indicates the cumulative epigenetic effect of each rearing type in successive generations (Table 1). These results together with the previous data support the hypothesis that DNA methylation plays an important role in honey bee queen/worker differentiation.

Guan et al. (2013) showed that the relationship between DNA methylation and gene expression is quite weak in the honey bee. We compared our DMGs from two comparisons (G1L1 with G1E, G1L2 with G1E) to the related DEGs from He et al. (2017) and Yi et al. (2020). Similarly, there were 0 and only 3 overlapping genes between our DMGs and DEGs from the above two studies respectively (Table 2). Our results with previous findings indicate that the honey bee gene expression may not be regulated directly by the modification of methylation. DNA methylation may interact with other regulatory means such as alternative splicing (Foret et al., 2012; Li-Byarlay et al., 2013) and chromatin state to influence gene expression. Clearly, the subject requires further investigation.

In summary, we provide the first evidence of accumulating methylation changes arising from domestic rearing of the honey bee. We draw attention to an important potential mechanism for cryptic genomic change (changes in genomic function that could not be detected as changes in DNA sequence caused by inbreeding, for example) in this important species.

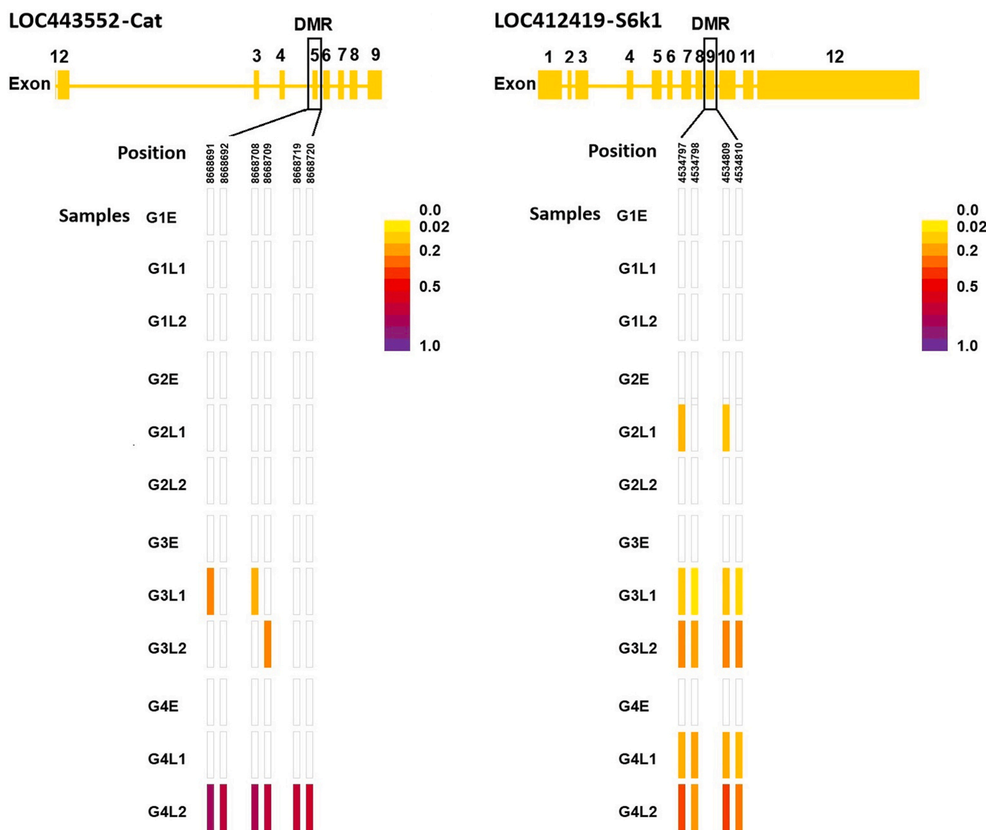

Fig. 6. Two genes (*Cat* and *S6k1*) associated with caste differentiation were analyzed in detail. For each gene we showed the methylation sites within focal exons. The color indicated the level of methylation at each site in all rearing groups across all generations. Yellow, red and purple indicated low, medium and high methylation levels respectively. White indicated no detected methylation. From the top to the bottom and left to right are gene IDs, gene symbols, DMRs, number of all exons, number of sites and samples with methylation level indicated by colors. More detailed information of these two genes has been provided in Table S9. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Comparison between significantly DMGs in queens/workers.

Generations	Comparisons	DMGs in this study	DMGs (Brains in queens/workers) in Lyko et al. (2010)	overlapping genes
G1	L1 vs E	430	560	25
	L2 vs E	448		29
G2	L1 vs E	1021		83
	L2 vs E	1151		98
G3	L1 vs E	1598		146
	L2 vs E	2073		160
G4	L1 vs E	2235		159
	L2 vs E	2065		153

Table 2
Comparison of DEGs with those in other related articles.

Comparisons	DMGs in G1 in this study	He et al. (2017)		Yi et al. (2020)	
		DEGs (3 d queen larvae)	overlapping genes	DEGs (Queens)	overlapping genes
L1 vs E	430	11	0	176	1
L2 vs E	448	75	0	218	2

Author contributions

ZJZ, XJH and WYY designed research. YY and YBL performed research. XJH, ABB and ZLW provided guidance for data. YY analyzed data. ABB and YY wrote the paper.

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Data availability

The raw Illumina sequencing data are accessible through NCBI's database: DNA methylation data of E, L1, L2 in generations (G1 - G4): NCBI Bioproject: PRJNA598779 (SUB6726989).

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

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