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

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Running title: Accumulated epigenetic changes in queen rearing

Competing interests

The authors declare no competing interest.

Abstract: 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 bees 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.

Keywords: DNA Methylation, Epigenome, caste differentiation, social insect, sociogenomics, genome, differential methylation

Introduction

The emerging Science of epigenomics is changing our understanding of development, and the degree of genomic sensitivity to the environment (Berger *et al.,* 2009; *Cavalli & Heard,* 2019; *Holliday & Pugh,* 1975; Norouzitallab *et al.,* 2019; Schrey *et al.,* 2016). Previously it had been assumed that the genomically determined developmental program was reasonably fixed, even if it could be vulnerable to subversion by environmental stressors. Epigenomics is revealing how genomic developmental systems are themselves sensitive to the environment (*Cavalli & Heard,* 2019). A consequence of this is the possibility of developmental stressors to rewrite the epigenome with profound, and potentially transgenerational consequences for animal development (Burggren 2015; *Cavalli & Heard,* 2019). Here we explored this possibility with the western honey bee (*Apis mellifera*). We applied to developing honey bee queens an environmental manipulation commonly used in commercial queen rearing. 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.

Epigenomics refers to the many molecular systems that regulate the activity and function of the genome (Berger *et al.,* 2009). Epigenomic systems include DNA methylation (*Holliday & Pugh,* 1975), histone modification (Turner, 1998) and chromatin structure (Yaniv, 2014), among others (Jodar *et al.,* 2013; Mattick, 2009; Schaefer *et al.,* 2010). These systems interact with the genome and with each other in complex ways to regulate the pattern of gene expression, which protein sequences are

produced from genomics sequences, and thereby mechanisms of development (Aristizabal *et al.,* 2019; Dunham *et al.,* 2012; Weinhold, 2006).

An interaction of developmental systems with the environment has long been assumed, but it was also a common conception that the genomics mechanisms regulating the developmental pathways were isolated from environmental influences. Epigenomics has overturned this view and highlighted how numerous epigenomic systems are 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 & 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 bees and small sterile worker bees (Evans & Wheeler, 2001; Hartfelder, 1998; Jung-Hoffmann, 1966). These different castes are key to the success of the honey bees eusocial and colonies lifestyle, but there are no genetic differences between worker and queen bees despite the major morphological differences between them (Evans & Wheeler, 2001; Hartfelder, 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 development (Jung-Hoffmann, 1966; Maleszka, 2008). DNA methylation is one of the most important genetic epigenetic mechanisms (Jung-Hoffmann, 1966; Maleszka, 2008), and unusually for insects the honeybee has

a functional DNA methylation system (Wang *et al.,* 2006). 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).

The sensitivity of the epigenome to environmental stressors can also lead to developmental dysfunction, however. This is increasingly being recognized as an important component of many diseases (*Cavalli & 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 & Heard,* 2019; Pembrey *et al.,* 2014). 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).

It is now clear that epigenomic insults can be inherited from parent to offspring in many species, and in some cases the inherited epigenomic change can persist for more than one generation (Anway *et al.,* 2005; Dias & 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

114 the methylation state of their sperm such that the changes persisted over four
115 generations (Anway *et al.,* 2005). Male mice maintained on a high fat diet for three
116 generations accumulate changes in epigenetic systems regulating lopgenesis altering
117 susceptibility to obesity (Li *et al.,* 2012).
118
119 The issue of persistent transgenerational inheritance of environmental stressors is of
120 concern because most modern animal and plant production for the food industry
121 involves rearing species in semi-artificial environments and with modified diets. If
122 these environmental changes impact the epigenome, then there is the potential for
123 cryptic epigenomic modification an animal or plant stocks without any genetic
124 changes or conventional inbreeding.
125
126 The honey bee provides a fortuitous model organism to explore this possibility.
127 Studies disagree on whether the honey bee has been 'domesticated' in the
128 conventional since few genetic differences can be detected between commercial bee
129 stocks and wild honey bees (Chapman *et al.,* 2008; Harpur *et al.,* 2012; Schiff and
130 Sheppard, 1996). A long established commercial process of honey bee queen rearing
131 is variable and quite artificial however (Chapman *et al.,* 2008; Harpur *et al.,* 2012;
132 Schiff & Sheppard, 1996). In the natural queen rearing process the queen lays an egg
133 in an especially large queen cell made by the workers (Wei *et al.,* 2019). The workers
134 fill the cell with royal jelly proving the larvae on hatching with abundant rich food.
135 By contrast, in commercial beekeeping, most queens are raised by artificially
136 transplanting young worker larvae from worker cells into artificial queen cells, which
137 the workers then provision with royal jelly to produce a queen (Büchler *et al.,* 2013;
138 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 phenotype. Recent studies have shown that queens reared from older 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 (Gray, 2019).

Here we examined the consequences of rearing queens from worker larvae for repeated generations. We found an accumulation of epigenomic changes with this rearing method, which might be contributing to a decline in quality of honey bee stocks.

Materials and methods

The Western honeybee, *Apis mellifera*, was used throughout this study. Honeybee colonies were maintained at the Honeybee Research Institute, Jiangxi Agricultural University, Nanchang, China (28.46uN, 115.49uE), 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.

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 6h (10 am - 4 pm) to a plastic honeybee 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 honeybee 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 6 queens in each group to emerge were taken for methylation analysis. These were immediately flash frozen

187 in liquid nitrogen when collected after emergence and stored in a -80 ℃ refrigerator.
188 All remaining queens to emerge were sampled to measure ovariole number.
189
190 These queens were transferred to queen cages, which were placed in queenless
191 colonies for 4-5 days where they could be fed and tended by workers through the cage.
192 The ovaries of 4-5 day-old queens are easier to stain and count than newly emerged
193 queens (Berger *et al.,* 2016; Patricio & Cruz-Landim, 2002). When 4-5 days old these
194 queens were flash frozen in liquid nitrogen and stored in a -80 ℃ freezer. To score
195 ovariole number we created paraffin sections of the stained and dissected ovary (Gan
196 *et al.,* 2012). We counted the number of ovarioles in the left ovary by identifying
197 slides in which the ovarioles were very clear and counting slides until we found at
198 least two giving exactly the same number of ovarioles（fiji-win64 software, Fig. S1).
199 We used two-way ANOVA to investigate the effects of queen type and generation on
200 ovariole number, and one way ANOVA to analyse differences between queen types
201 within each generation. The number of queens in each sample group varied and was
202 affected by queen larvae and queenless colony surviv*al.,* We sampled 3-11 queens for
203 ovariole analysis in each group (Fig. 2).
204
205 To rear the second generation queens (G2) we selected one of the mated and laying
206 G1 queens from each group (G1E, G1L1 and G1L2). Each G1 queen was restricted
207 for 6 h (10 am-4 pm) to a plastic worker honeybee frame for laying. We then created
208 three different types of G2 queens as for G1.
209
210 Eggs from the G1E queen were transferred as eggs to queen cells on the 2nd day post
211 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 queens were sampled in each generation for methylation and ovariole anlaysis 12 queen groups were sampled in tot*al.,*

The preparation process of paraffin section of queen bee 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 hours. For dehydration and fixing we used an Automatic dehydrator (Leica, TP1020), which implemented the following processes on the samples. Ovaries were dehydrated in a graded ethanol series (70% - 100%). Ovaries were then cleared using xylene. Then, samples were 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 hours. 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), and then

mounting 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).

Genome wide methylation analysis

Queens sampled for methylation analysis were flash frozen in liquid nitrogen when collected after emergence and stored in a -80 ℃ refrigerator. Of the six queens sampled for methylation anlaysis, three or four queens were used for genome-wide methylation testing. The brain, ovary and thorax 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 meythylation analysis. Of these, only one sample did not meet our quality control requirements for genomic sequencing, consequently we lost one G2L2 sample.

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 summarised below.

Library preparation and quantification

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). They were then processed by the Accel -NGS Methyl -Seq DNA Library Kit to add a truncated adapter sequence, including an index sequence, to each fragment. 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.

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.

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 pipleline 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.

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).

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 & 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 & 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 produce 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.

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 10kb. 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)).

Differential methylation analysis

Differentially methylated regions (DMRs) were identified using the DSS software. 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 &Wu, 2016; Wu *et al.,* 2015). We defined the genes related to DMRs as genes whose gene body region (from TSS to TES) or promoter region (upstream 2kb from the TSS) overlapped with the DMRs.

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

Gene Ontology (GO) enrichment analysis of genes related to DMRs was implemented by the GOseq R package (Feng *et al.,* 2014; Park & Wu, 2016; Wu *et al.,* 2015), 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 to test the statistical enrichment of DMR related genes to different KEGG pathways.

Results

Effect of queen rearing method on queen morphology and genome methylation

For this study we sampled queens across four generations (Fig. 1). The intention of our queen breeding program was to examine the epigenetic consequences of different methods of queen rearing by establishing lines of queens that differed in their queen rearing method. These were lines in which queens were consistently reared from eggs (E), one-day old larvae (L1) or two-day old larvae (L2).

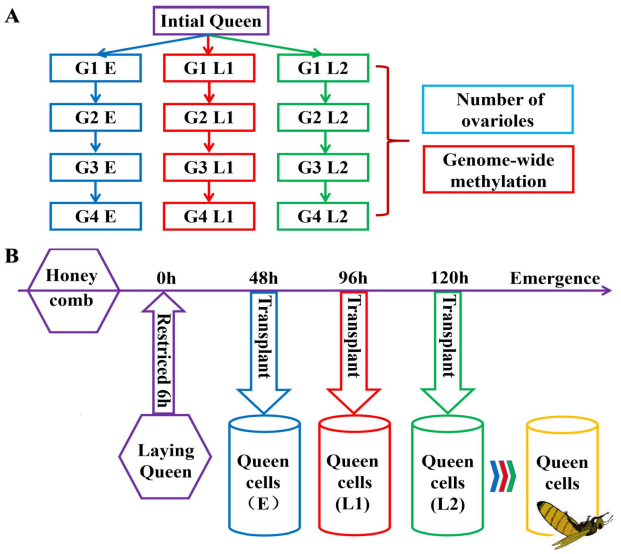

Fig. 1. Experimental design. **(A)** Lineage of queens and measurements. (B**)** Timing of the grafting and brood transfers. A queen artificially inseminated with the semen of a single drone was caged on a plastic frame to obtain eggs of a known age during 6 hours. Some of the eggs ("E") were transferred to queen cells after 48 h, while other eggs were left for 96 h ("L1") and 120h ("L2") and transferred after they reached the first or second larval stage respectively (generation 1 "G1"). 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 and the number of ovarioles was counted and the DNA genome-wide level of methylation from the brain, thorax and ovarioles was measured.

Ovariole number differed significantly between queens of different types (Two-way ANOVA, $F = 18.869$, $DF = 2$, $P < 0.001$), but no effect of generation (G1 - G4, Fig. 1) 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. 2A).

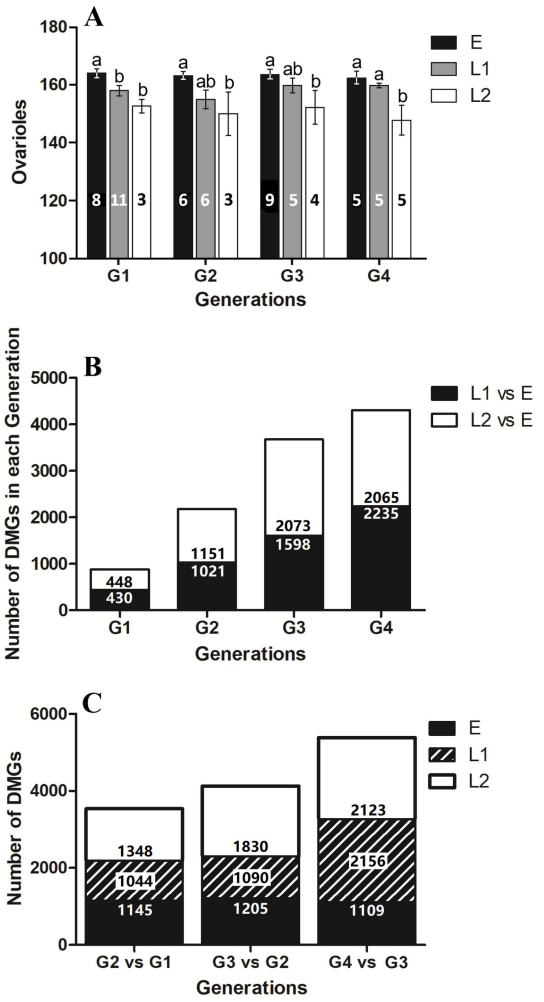

Fig. 2. Summary of morphological and epigenomic differences of queens from the three rearing groups in four different generations. **(A)** Number of ovarioles in E, L1 and L2 queens in generations G1 - G4 (Table S4, Supporting Information). Bars show mean ± SEM. Sample size shown in each bar. One-way ANOVA was performed on each generation. Letters above bars identify groups that did and did not differ significantly within each generation. **(B)** Summary of numbers of differentially methylated genes (DMGs) comparing L1 with E (black), and L2 with E (white) in generations G1 - G4. Number of DMGs in each comparison is written into each bar.

Summary of DMG IDs in Table S5, Supporting Information). **(C)** Numbers of differentially methylated genes (DMGs) comparing for each queen rearing type across successive generations. The three bars show DMGs in comparisons of G2 with G1, G3 with G2, and G4 with G3. Within each stacked bar we show 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). Number of DMGs shown in each bar. Summary of DMG IDs in Table S6, Supporting Information.

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, Supporting Information). The average phred scores Q30% (Ewing & Green, 1998) was 92.4% (Table S1, Supporting Information). The average bisulfite conversion rate was 99.7% (Table S1, Supporting Information). The average site coverage rate was 25.06 (Table S2, Supporting Information), indicating that there was accept equencing quality (NIH roadmap epigenomics project, http://www.roadmapepigenomics.org/protocols). The pearson correlation coefficient among biological replicates of each experimental group were all ≥0.97 (Table S3), indicating good repeatability among the biological replicates of each group. Almost all methylation sites of all samples (98.82%) were the CG type. In our data we detected very few CHH: 1.04% and CHG: 0.14% sites.

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. 2B, Table S5, Supporting Information. We observed a similar phenomenon when we compared L2 with E queens in each generation (Fig. 2B, Table S5, Supporting Information).

399

400 To account for any possible effect of season or time on number of DMGs in our study
401 we analyzed the number of DMGs in successive generations of each queen rearing
402 type. For E queens, numbers of DMGs were extremely stable when we compared G2
403 with G1, G3 with G2 and G4 with G3 (Fig. 2C, Table S6, Supporting Information).
404 By contrast when we examined L1 and L2 queens we observed the numbers of DMGs
405 increased in each comparison of successive generations (Fig. 2C). This suggests that
406 for L1 and L2 queens methylation differences accumulated with each generation of
407 L1 or L2 rearing.

408

409 Consistently in the DEG gene lists comparing our queen groups (Table S7,
410 Supporting Information) we noted 106 genes with functions related to caste
411 differentiation (Beltran *et al.,* 2007; Buttstedt *et al.,* 2016; Guan *et al.,* 2013; Marshall
412 *et al.,* 2019; Tian *et al.,* 2018), body development and metabolism (Bull *et al.,* 2012;
413 Davis *et al.,* 2002; Evans *et al.,* 2006; Mao *et al.,* 2017; Miller *et al.,* 2012; Parker *et*
414 *al.,* 2012; Shi *et al.,* 2011; Zufelato *et al.,* 2004), and gene regulatory pathways related
415 to caste differentiation (Bull *et al.,* 2012; Davis *et al.,* 2002; Evans *et al.,* 2006; Mao
416 *et al.,* 2017; Miller *et al.,* 2012; Parker *et al.,* 2012; Shi *et al.,* 2011; Zufelato *et al.,*
417 2004). Of these, we analysed 40 genes that appeared most consistently in our DEG
418 lists across generations (Fig.3 and Table S8, Supporting Information). From G1 – G4
419 there was an increase in both the number of DEGs (Fig. 3), and the ratio of
420 methylation difference between the compared sequences (Fig. 3).

421

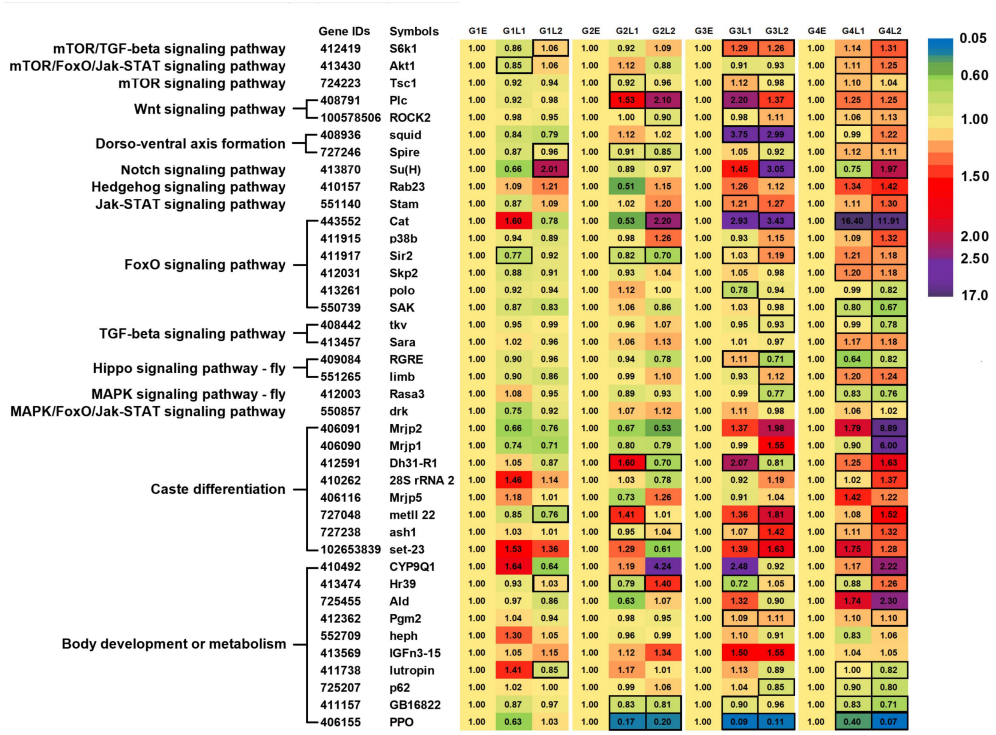

Fig. 3. 40 focal genes were selected for their known functions in caste differentiation, body development or metabolism of bees. For these 40 genes we compared within each generation the relative methylation level of L1 and L2 queens with E queens for all exons within each gene. For each gene, methylation 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 in 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 indicates hypo-methylated genes in groups compared with E. Red and purple indicates hyper-methylated genes, and yellow indicates no difference. The deeper the color, the greater the difference. Black borders indicate that there was at least one exon in this gene that was significantly differentially methylated between the comparison groups. For each gene we show from left to right, gene functions, gene IDs, gene symbols. More detailed information on these 40 genes is given in Table S8, Supporting Information.

From the 40 genes in Fig. 3, we selected 3 genes from gene regulatory pathways already implicated in the epigenetic mechanism of queen / worker differentiation (Foret *et al.,* 2012). The gene *Akt1* is involved in the mTOR signaling pathway, the FoxO pathway and the Jak-STAT signaling pathway (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 (Foret *et al.,* 2012). For these three genes we examined where in the gene sequence changes in methylation occurred (Fig. 4 and Table S9, Supporting Information). We focused on exons (Lyko *et al.,* 2010), and limited Fig. 4 to displaying only exons in which significant DMRs occurred. We observed accumulating changes in amount of methylation at specific sites in our three genes from G1 - G4.

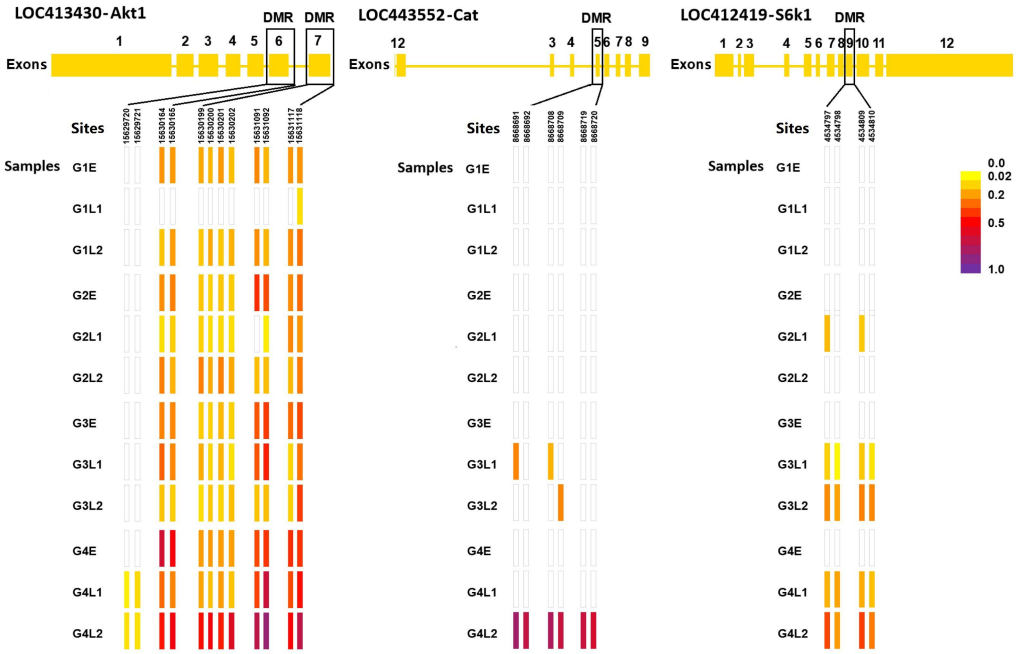

Fig. 4. Three genes (*Akt1*, *Cat* and *S6k1*) associated with caste differentiation were analysed in detail. For each gene we show the methylation sites within focal exons. The color shows the level of methylation at each site in all rearing groups across all generations. Yellow, red, and purple indicates low, medium and high methylation levels respectively. White indicates no methylated detected at this site. From the top to the bottom and left to right are gene IDs, gene symbols, DMRs, number of all exons, number of site, samples with methylation level indicated by color. More detailed information of these 3 genes is given in Table S9, Supporting Information.

Discussion

462 Epigenomic regulation is key to the successful operation of both worker and queen
463 honey bee developmental systems, and to the effective divergence of the two castes
464 (Barchuk *et al.,* 2007; Kucharski *et al.,* 2008; Lyko *et al.,* 2010; Maleszka, 2008;
465 Maleszka *et al.,* 2014). Differential meythyation of the genome is an important part of
466 the developmental divergence (Kucharski *et al.,* 2008; Lyko *et al.,* 2010; Shi *et al.,*
467 2013). The epigenomic systems (particularly DNA methylation) allows the
468 genomically determined developmental system to interact with the nutritional
469 environment to establish whether an egg will develop into a worker or a queen.
470
471 In effect, this epigenomic "developmental switch" allows workers to control which
472 eggs develop as future queens for their colonies by controlling the nutrition of larvae
473 (Jung-Hoffmann, 1966). Contemporary queen rearing interferes with this natural
474 process, however. In commercial queen rearing it is common to transplant larvae up
475 to 3-days old from worker cells into queen cells where they will be subsequently
476 provisioned as queens. This practice has been in very widespread use in apiculture
477 since 1888 (Doolittle, 1888).
478
479 Here we showed that 2-day old worker larvae transplanted to queen cells could be
480 successfully raised as queens, but there were consequences from this developmental
481 manipulation for the queen phenotype. We consistently found that queen ovariole
482 number was lower in L1 and L2 queens compared with E queens (Fig. 2). The number
483 of ovarioles determines how many eggs can be produced and matured by the queen.
484 This difference would be expected to have consequences for colony growth and
485 function since the queen is the sole reproductive in a honey bee colony, and the
486 mother of the entire worker population. Indeed, Rangel *et al* (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).

Recently He *et al* (He *et al.,* 2017) and Yin *et al* found that the differences in size between queens reared from eggs or worker larvae of different ages was correlated with differences in gene expression and gene methylation in many pathways known to be involved in the caste differentiation process in honey bees (He *et al.,* 2017; Yin *et al.,* 2018). Here we also 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. We found that methylation differences between the different rearing types increased with each successive generation (Fig. 3 & Fig. 4). In effect, we observed a progressive divergence in the meyhylome 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 (Fig. 3).

Our data show an accumulation of methylation differences resulting from repeated differences in queen rearing type. We are confident the differences we report are not due to the passage of time or the season at the time of sampling, or genetic divergence between our different developmental 'lines' for the following reasons. First, when we compare L1 with E or L2 with E within our four generations, we saw an increasing number of DMGs with each generation of rearing (Fig. 2B), but when we measured

DMGs between mother and daughter E queens across our four generations the number of DMGs was very stable (Fig. 2C). This suggests that for E queens (the condition that most closely matches the evolved and natural method of queen rearing) DMGs were not influenced by time of sampling or any possible genetic divergence of our rearing lines. This was not the case for L1 of L2 queens. For these groups the number of DMGS in mother / daughter comparisons of both L1 and L2 queens increased with each generation of rearing (Fig. 2C) suggesting an accumulation 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. 2B). Many of these changes were in the genomic pathways that establish the epigenomically regulated queen and worker phenotypes.

We do not consider it likely there was any significant genetic divergence between our rearing lines. 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. This strategy would have prevented our developmental lines from diverging genetically. Hence, we are confident the progressive changes we see in the methylome of L1 and L2 queens indicate an accumulation of methylation differences resulting from this artificial form of queen rearing.

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 (Lyko *et al.,* 2010).

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 plants and rodents where rearing an organism under stress for repeated generations induced more methylation changes than rearing under stress for a single generation (Cropley *et al.,* 2012; Groot *et al.,* 2016; Li *et al.,* 2012). In Arabidopsis persistent stress applied over repeated generations also increased the stability of the induced methylation changes which contributed to the progressive accumulation of methylation changes (Groot *et al.,* 2016). Burggren (Burggren, 2015) reviewed this general highlights how epigenetic changes shoud be recognized as graded time related changes that can both "wash in" and "wash out" over time. Rearing mice on a fatty diet across three generations has been shown to "wash in" epigenetic changes contributing to obesity susceptibility (Burggren, 2015). In nematodes, repeated exposure to an odour across 4 generations resulted in what had originally been an induced behavoural change to this odour to become a stable inherited behjavioural change (Remy, 2010). This illustrates how the distinction between acquired and inherited characteristics is not as complete as we once thought (Burggren, 2015; Furrow & Feldman, 2014; Robinson & Barron, 2017). The epigenome can enhance the evolvabilty of the genome and can facilitate

560 adaptive phenotypic change in an organism (Brown, 2014; Furrow & Feldman, 2014;
561 Schrey *et al.,* 2016).
562
563 In this study we did not see any progressive phenotypic change resulting from rearing
564 queens from L2 larvae for four generations. While all L2 queens had fewer ovarioles
565 than E queens, the number of ovarioles in L2 queens in generation 4 was not less than
566 in generation 1. Even so, the cryptic accumulation of changes to the queen methylome
567 is troubling, even if phenotypic differences were not detectable after just four
568 generations.
569
570 Rearing queens from larvae has been a standard practice in apiculture for decades, and
571 commercially this practice could sustain a developmental stress on a queen stock for
572 many generations. We now recognize that queens reared from worker-destined larvae
573 are of lower quality than queens reared from eggs (Rangel *et al.,* 2012; He *et al.,*
574 2017). Our work highlights the more concerning possibility of the rearing practice
575 causing a progressive and cryptic erosion of the epigenetically regulated queen
576 developmental pathway. This could erode queen quality without any detectable
577 changes in bee genetics or conventional inbreeding. Burggren *et al* (Burggren, 2015)
578 discusses how epigenetic changes can both "wash in" to a genome with sustained
579 stress over time, but they can also "wash out" over time if the stress is removed. Some
580 studies with rodent highlight how accumulated epigenetic changes are not persistent
581 and can fade over generations if the stressor is removed (Li *et al.,* 2012). If this proves
582 true for bees also it would suggest that rearing queens from eggs could be a very
583 effective method to restore and maintain the genomic quality of the queen stock. We

584 urgently need to understand the dynamics with which these cryptic accumulated
585 changes could be washed out of the honey bee genome.
586
587 In summary, we provide the first evidence of accumulating methylation changes
588 arising from domestic rearing of the honey bee. We draw attention to an important
589 potential mechanism for cryptic genomic change in this important species.
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Author contributions

Zhi Jiang Zeng, Xu Jiang He and Wei Yu Yan designed research; Yao Yi and Y.B.L. performed research; Xu Jiang He, Andrew B. Barron and Zi Long Wang provided guidance for data; Yao Yi analyzed data; Andrew B. Barron and Yao Yi wrote the paper.

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). All other data are included in the manuscript, and its Supporting Information file and Supporting Tables (S1-S9). Extra data are available from the corresponding author upon reasonable request.

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Supporting Information

Fig. S1 Paraffin section of queen bee ovary.

Table S1 Raw data quality control statistics.

Table S2 Reads and reference genome alignment results.

Table S3 Pearson correlation coefficient among biological replicates of each group.

Table S4 Number of queen ovarioles (the one side ovary).

Table S5 List of differentially methylated genes comparing L1 and L2 queens with E queens in each generation.

Table S6 List of differentially methylated genes comparing queens of each rearing type across successive generations.

Table S7 List of 106 genes identified by gene ID, and the ratio of methylation level for each comparison (within a generation, L1 : E or L2 : E).

Table S8 The methylation level of 40 focal genes relate to caste differentiation, body development or metabolism in each group.

Table S9 Raw methylation level data at the site on exon in genes *Akt1*, *Cat* and *S6k1*.