Lineage and parent-of-origin effects in DNA methylation of honey bees (*Apis mellifera*) revealed by reciprocal crosses and whole-genome bisulfite sequencing

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Abstract

Parent-of-origin methylation arises when the methylation patterns of a particular allele is dependent on the parent it was inherited from. Previous work in honey bees has shown evidence of parent-of-origin specific expression, yet the mechanisms regulating such pattern remains unknown in honey bees. In mammals and plants, DNA methylation is known to regulate parent-of-origin effects such as genomic imprinting. Here, we utilize genotyping of reciprocal European and Africanized honey bee crosses to study genome-wide allele-specific methylation patterns in sterile and reproductive individuals. Our data confirms the presence of allele-specific methylation in honey bees in lineage-specific contexts but also importantly, though to a lesser degree, parent-of-origin contexts. We show that the majority of allele-specific methylation occur due to lineage rather than parent-of-origin factors, regardless of reproductive state. Interestingly, genes affected by allele-specific DNA methylation often exhibit both lineage and parent-of-origin effects, indicating that they are particularly labile in terms of DNA methylation patterns. Additionally, we re-analyzed our previous study on parent-of-origin specific expression in honey bees and found little association with parent-of-origin specific methylation. These results indicate strong genetic background effects on allelic DNA methylation, and suggest that while parent-of-origin effects are manifested in both DNA methylation and gene expression, they are not directly associated with each other.

Keywords

Epigenetics, intragenomic conflict, honey bees, social insects, DNA methylation
Significance Statement

Preferential expression of one parental allele over the other has been long observed in mammals and plants, and recently, in honey bees. Complexities of honey bee societies may provide a fertile ground for parent-of-origin specific gene expression to resolve intragenomic conflict. Here, we examined DNA methylation in honey bees as a potential molecular mechanism for driving parent-of-origin expression, using whole-genome bisulfite sequencing of reciprocal crosses between genetically divergent strains. We present data consistent with parent-of-origin specific DNA methylation, however we did not detect a direct link between parent-of-origin specific expression and methylation. Moreover, the strongest factor of allele-specific methylation was the effect of genetic background. Our findings begin to resolve the complex relationship between allele-specific methylation and expression in insect genomes and the factors driving these phenomena.

Introduction

Parent-specific expression occurs when the expression of an allele varies depending on whether it was inherited from the mother (matrigene) or father (patrigene) and can even be associated with complete silencing of expression of one of the parental alleles (Haig 2000; Wilkins and Haig 2003; Wolf and Hager 2006; Ferguson-Smith 2011). Several theories have been proposed to explain the causes and evolutionary origins of parent-specific expression (e.g., (Patten, et al. 2014). Among them, Haig’s kinship theory of intragenomic conflict hypothesizes that parent-specific expression is due to maternally and paternally inherited genes being under different selection pressures (Haig 2000; Pegoraro, et al. 2017). In cases where a female produces offspring with multiple males, selection may favor matrigene expression profiles that promote
equal distribution of resources among siblings while patrigene expression profiles would support processes that prioritize individual fitness (Haig 2000; Pegoraro, et al. 2017). Support for this theory has been provided in mammalian and plant species, where matrigene expression tends to be associated with impeded growth while patrigene expression stimulates it (Haig 2000; Wilkins and Haig 2003). The kinship theory of intragenomic conflict is applicable to social insects because the myriad of differences in matrigene and patrigene relatedness found in individuals in social insect colonies (Queller 2003). Honey bees are a particularly dramatic example in which intragenomic conflict is thought to play an important role in shaping social behaviors, since honey bees are haplodiploid, and the single reproductive female queen mates with 10 or more males, giving rise to offspring with significant variation in their matrigene-patrigene relatedness (Queller 2003; Kocher, et al. 2015; Galbraith, Kocher, et al. 2016; Pegoraro, et al. 2017).

Transcriptomic studies in social insects have provided support for the kinship theory of intragenomic conflict (Bonasio, et al. 2012; Lonsdale, et al. 2017). Kocher et al. (2015) showed largely consistent patterns of parent-specific expression across developmental stages, behavioral states, and tissues, though these findings may have been confounded by lineage effects (Gibson, et al. 2015). The kinship theory of intragenomic conflict predicts that patrigenes should stimulate worker reproduction during queenless conditions (Haig 1992; Queller 2003). Oldroyd et al (Oldroyd, et al. 2014) and Galbraith et al (Galbraith, Kocher, et al. 2016) demonstrated that worker ovary size (a trait that is positively correlated with worker ovary activation rates under queenless conditions) and worker ovary activation are correlated with paternal genotype in two different studies (Oldroyd, et al. 2014; Galbraith, Kocher, et al. 2016). Galbraith et al. (Galbraith, Kocher, et al. 2016) further demonstrated that patrigenes were preferentially
upregulated in reproductive tissues of reproductive workers versus sterile workers in reciprocal crosses between Africanized and European honey bee stocks.

While these observations support intragenomic conflict and parent-of-origin effects, the identities of molecular mechanisms behind these phenomena in social insects remain unclear (Kocher, et al. 2015; Galbraith, Kocher, et al. 2016; Galbraith, Yi, et al. 2016). In mammalian and plant species, epigenetic regulation primarily in the form of DNA methylation is thought to mediate parent-specific expression and parent-specific silencing of alleles (Reik and Walter 2001; Bird 2002; Queller 2003; Law and Jacobsen 2010). Intriguingly, the honey bee also possesses a fully functional DNA methylation system (Wang, et al. 2006; Lyko, et al. 2010). However, unlike in mammalian and plant species, DNA methylation in honey bees is found primarily in gene bodies and coding regions, and thus may not have the same function (Elango, et al. 2009; Lyko, et al. 2010; Zeng and Yi 2010; Galbraith, et al. 2015). Gene body methylation has been shown to be positively correlated with gene expression in insects (Lyko, et al. 2010; Zeng and Yi 2010; Wang, et al. 2013; Galbraith, et al. 2015; Wu, et al. 2020), which would suggest that allele-specific methylation and expression would also share the same direction of association. A previous study in the hymenopteran wasp *Nasonia vitripennis* utilizing reciprocal crosses found strong lineage-specific effects where the hybrid offspring recapitulated the methylation levels of nearly all of the differentially methylated genes between their parents (Wang, et al. 2016). Despite the near complete transmission of lineage-specific methylation patterns to the hybrid offspring and strong correlations between allele-specific methylation and expression, there was no evidence of parent-of-origin effects on DNA methylation (Wang, et al. 2016).
Here, we utilized samples collected from a previous study of reciprocal crosses between two divergent strains of honey bees (Galbraith, Kocher, et al. 2016) to survey genome-wide DNA methylation using whole-genome bisulfite sequencing (WGBS). For these studies, sterile and reproductive honey bee workers were generated from reciprocal crosses of European and Africanized honey bee stocks. These samples allow us to evaluate allele-specific DNA methylation patterns across the genome and determine if these are associated with parent, lineage or reproductive state differences, taking advantage of the lineage-based single nucleotide polymorphisms (SNPs) present in these two stocks. By uncoupling of parent and lineage effects, and pairing nucleotide-resolution DNA methylation data with gene expression data from the same experiment, we uncover underlying genetic and parent-specific effects on DNA methylation and determine if these are associated with behavioral and physiological variation in worker honey bees.

**Materials and methods**

**Biological Sample Collection**

Rearing and collection of honey bee samples were described in (Galbraith, Kocher, et al. 2016). We obtained a total of 8 sterile and 8 reproductive workers that were equally sourced from genetic blocks A and B and European\textsubscript{mother} x Africanized\textsubscript{father} and Africanized\textsubscript{mother} x European\textsubscript{father} crosses (one of each cross per block, and thus 2 sterile and 2 reproductive workers from each cross). Our samples were collected from the same colonies as those used for transcriptomic analysis in Galbraith et al. 2016 and were the full-sisters of the workers used in that study (same mother and same father). Genomic DNA was extracted from the reproductive...
tissues (ovaries and abdominal fat bodies) of each individual, as in Galbraith et al. 2016. The DNA was collected from each individual for bisulfite sequencing library preparation using a Gentra Puregene Tissue kit (Qiagen, Cat. No. 158667).

Whole-genome Bisulfite Library Preparation and Sequencing

The libraries were made with an Illumina sequencer compatible protocol (Urich, et al. 2015). The extracted DNA was fragmented using the Covaris S-series focused ultrasonicator (Covaris Cat. No. S220) using the “500bp-target peak size” protocol. The fragmented DNA was then size selected (200bp-600bp) with the SPRI bead-based size selection protocol (Urich, et al. 2015). The DNA End repair step was then performed where DNA fragments containing damaged or incompatible 5’- and/or 3’- protruding ends are converted to 5’-phosphorylated, blunt-ended DNA. Blunt-ended DNA fragments were ligated with 3’ –dAMP overhangs and methylated adapters.

We added 10ng of unmethylated lambda phage DNA (Promega, Cat. No. D1501) to the input DNA of each library as a control for bisulfite conversion rates. Bisulfite treatment of genomic DNA was performed using the MethylCode Bisulfite Conversion Kit (Life Technologies, Cat. No. MECOV-50). With this method, non-methylated cytosines are converted to Uracil and read as Thymine (T) when sequenced. Methylated cytosines protected from conversion are still read as cytosine (C). Briefly, purified genomic DNA is treated with CT conversion reagent in a thermocycler for 10 minutes at 98°C, followed by 2.5 hours at 64°C. Low-cycle (4-8) PCR amplification was performed with Kapa HiFi Uracil Hotstart polymerase enzyme (Kapa Biosystems, Cat. No. KK2801) which can tolerate Uracil residues. Libraries were quantified by using Qubit high sensitivity DNA kit (Life Technologies, Cat. No. Q32851) and Agilent High
Sensitivity DNA Bioanalyzer (Agilent Technologies, Cat. No. 5067-4626). The libraries were diluted and loaded onto the Illumina HiSeq X at the Macrogen Laboratory for sequencing using 150bp paired-end reads.

Generating N-masked Genomes

A list of SNPs for each parent from each cross was obtained from a previous study (Galbraith, Kocher, et al. 2016). For each cross, we used stringent criteria to generate a list of informative SNPs. We removed ambiguous SNPs (e.g. heterozygous SNPs in the diploid queen genome and SNPs that would be identical in both drone and queen), SNPs with a Phred score of < 30, and C -> T and T -> C SNPs. We also discarded SNPs with a read depth of < 5 in either the European or Africanized alleles. In addition, only conserved SNPs from both of the crosses in each genetic block were retained, which resulted in 213,056 and 214,504 informative SNPs for genetic blocks A and B, respectively (Supplemental Table 1). Using the resulting list of informative SNPs, we used a custom Python script (DOI: 10.5281/zenodo.3873279) to generate one N-masked genome for each genetic block.

WGBS Data Processing

Raw reads were trimmed for low quality and adaptors using Trim_galore! (Martin 2011) with default parameters. Trimmed reads of each individual were then aligned to their respective N-masked genomes based on genetic block using Bismark and default parameters (Krueger and Andrews 2011). Additionally, reads were aligned to the unmethylated lambda genome (GenBank Accession: J02459.1) to estimate the bisulfite conversion efficiency based on the deamination rate. Following alignment, each aligned read was split into European or Africanized alleles using
SNPSplit (Krueger and Andrews 2016) in paired-end mode based on the previously generated list of informative SNPs for each cross (Supplemental Table 1). Each allele-specific alignment, which we will refer to as one sample, was then deduplicated using Bismark (Krueger and Andrews 2011).

For each sample, we applied the binomial test at each CpG site using the deamination rate as the probability of success and an FDR threshold of < 0.05 (Benjamini and Hochberg 1995) to classify each CpG site as either ‘methylated’ or ‘unmethylated’ (Lyko, et al. 2010; Wang, et al. 2013; Galbraith, et al. 2015). For downstream analyses, we only retained CpG sites that were classified as ‘methylated’ in at least one sample (Huh, et al. 2019).

Whole-genome bisulfite sequencing reads are uploaded on SRA and accessible under BioProject PRJNA608132 and available under the following reviewer link: https://dataview.ncbi.nlm.nih.gov/object/PRJNA608132?reviewer=f5f58epb6cemvuv0i85bdslab3).

**Differential Methylation Analysis**

We used the DSS package (Park and Wu 2016) to identify CpGs that were differentially methylated (referred to as differentially methylated positions, or DMPs). DSS handles variance across biological replicates as well as models read counts from WGBS experiments, while accounting for effects of biological covariates. Specifically, we considered the parent-of-origin effect (paternal or maternal) as well as the lineage effect (Africanized or European), and corrected for multiple testing by FDR threshold of < 0.1. We performed this analysis separately for each genetic block. In addition, a significant site was required to exhibit at least 60% relative
allele-specific methylation bias in both reciprocal crosses (European$_{mother}$ x Africanized$_{father}$ and Africanized$_{mother}$ x European$_{father}$), similarly to how allele-specific expression bias was previously calculated (Kocher, et al. 2015; Galbraith, Kocher, et al. 2016). The relative allele-specific methylation is calculated as the percent of methylation (Schultz, et al. 2012; Galbraith, et al. 2015; Lindsey, et al. 2018) of one allele relative to the total methylation of both alleles. Differentially methylated genes (DMGs) for each independent variable were then defined as genes containing at least one DMP that all showed the same direction of bias (Galbraith, et al. 2015; Kocher, et al. 2015). We classified genes using DMPs for each variable independent of DMPs from other variables, meaning that a gene containing DMPs from multiple variables can be a DMG in multiple categories as long as it satisfied the previous requirement each time (Figure 3).

In addition to analyzing each individual CpG, we assessed allele-specific methylation at the gene body level using a generalized linear model of the binomial family similar to previous studies (Lyko, et al. 2010; Galbraith, et al. 2015). Briefly, the number of methylated and unmethylated reads was used as the response vector and modeled by three categorical variables: lineage (Africanized or European), parent (Paternal or Maternal), and CpG position (Lyko, et al. 2010; Galbraith, et al. 2015). Similar to our CpG level analysis, we applied this model to samples for each genetic block and reproductive status separately.

**RNA-seq Data Processing**

RNA-seq data was previously generated (Galbraith, Kocher, et al. 2016). We re-analyzed this data following the same criteria as in the methylation analysis, so that the results can be directly
compared. Reads (accession no. GSE76164) were trimmed using the same parameters as the WGBS data and aligned to each sample’s respective N-masked genome using HISAT2 with soft clipping disabled (parameter setting: --sp 1000,1000). As with the WGBS data, SNPSplit was used to separate European and Africanized alleles while HTSeq (Anders, et al. 2015) with default parameters was used to count allele-separated reads within coding regions of the genome. The DESeq2 package (Love, et al. 2014) was used to identify differentially expressed genes. Gene expression was quantified based on normalized counts using the “estimateSizeFactors” function from the DESeq2 package. Differential expression was assessed using the same predictors as our differential methylation analysis (parent, and lineage) and the normalized gene counts as the response variable. This analysis was also performed separately for each genetic block. Significance level for genes was at an FDR-adjusted (Benjamini and Hochberg 1995) level of 0.1 and required a 60% relative expression bias per allele similar to the methylation analysis.

Gene Ontology
Gene ontology analysis was performed by uploading BeeBase gene IDs (Elsik, et al. 2014) onto the DAVID bioinformatics Resources 6.8 webpage and using the Functional Annotation tool (Huang da, et al. 2009). GO term enrichment was assessed at a significance level of $P < 0.05$. The background gene list for enrichment analyses was all protein coding genes in the genome.

Results
Lineage and Parent-of-origin effects on DNA methylation
To study allele-specific patterns in DNA methylation, we generated a list of informative SNPs that could be used to reliably differentiate our sequenced reads and assign them to the appropriate parental allele. Since the parents of each genetic block were from different colonies, we assessed variation of DNA methylation for each block separately. Due to the large amount of informative SNPs that are unique to each genetic block, analyzing the genetic blocks separately enabled us to increase the scope of our analysis by surveying a larger portion of the genome as well as giving us an indicator of the robustness of our results (Supplemental Table 1). Within each genetic block, only informative SNPs that were present in both crosses (European\text{mother} \times Africanized\text{father} and Africanized\text{mother} \times European\text{father}) were used for our analyses. In genetic block A, there were 213,056 such SNPs, which allowed us to assign alleles to 48,745 methylated CpGs that were distributed within 5,613 genes. Similarly, there were 214,504 informative SNPs in genetic block B, allowing us to detect 41,764 methylated CpGs within 5,359 genes. In contrast, the number of informative SNPs that were shared between the two blocks was only 27,146, which led to 2,506 methylated CpGs that could be analyzed, yielding little power (Supplemental Figure 1). Consequently, we selected to analyze the two blocks separately to improve our ability to detect differential DNA methylation.

We used a linear model framework to identify individual CpGs that exhibited variation of DNA methylation among samples that is consistent with the effects of parent-of-origin and lineage (see Materials and Methods). The identified CpGs are referred to as differentially methylated positions (DMPs) and are summarized in Table 1 based on their direction of bias. Figure 1 shows some examples of DMPs exhibiting lineage effects and parent-of-origin effects for both sterile and reproductive workers in each of the two blocks.
The strongest among the two factors was the lineage effect in both genetic blocks. In block A, a total of 743 and 1,869 individual CpG sites showed variation consistent with the lineage bias in sterile and reproductive workers, respectively. Similarly, block B had 1,525 and 1,691 individual sites showing lineage based variation in sterile and reproductive workers, respectively (Table 1). Interestingly, we observed greater bias toward European alleles – that is, there were a greater number of European biased CpGs than Africanized biased CpGs. This trend was observed in both blocks and both sterile and reproductive workers, and statistically significant in all cases except the reproductive workers in block A (Table 1; $X^2$ test, $P < 0.05$).

We also found hundreds of DMPs in both reproductive states that showed parent-of-origin effects (Table 1 and Figure 2; Figure 1A,1B,1E, and 1F). For block A, we observed a total of 280 parent-of-origin DMPs in the sterile workers, of which 132 displayed maternal bias and 148 displayed paternal bias (Table 1 and Figure 2). In the reproductive workers, we observed a total of 408 parent-of-origin DMPs, of which 190 DMPs were maternal biased whereas 218 DMPs were paternal biased, which was a significant increase in paternal biased DMPs compared to maternal biased DMPs ($X^2$ test, $P < 0.01$; Table 1 and Figure 2). For block B, there were 208 maternal biased DMPs as opposed to 216 paternal biased DMPs in the sterile workers, and 189 maternal and 188 paternal biased DMPs in the reproductive workers (Table 1 and Figure 2). Interestingly, there were greater numbers of DMPs in reproductive workers compared to sterile workers for all categories in block A ($X^2$ test, $P < 0.05$ for all directions of bias), but there were no significant differences in the numbers of DMPs between reproductive and sterile workers for any of the categories block B.
We found significant overlaps of DMPs between workers of different reproductive states. For example, 69 parent-of-origin DMPs were shared between sterile and reproductive workers in block A while 119 parent-of-origin DMPs were shared between sterile and reproductive workers in block B, both of which were highly significant enrichments compared to a null expectation of no association (Fisher’s exact test, \( P < 0.01 \) for both comparisons). Despite the significant overlaps, a large number of DMPs were specific to each reproductive state. In block A, 211 and 339 parent-of-origin DMPs were specific to sterile and reproductive workers, respectively. Similarly, there were 305 parent-of-origin DMPs specific to sterile workers and 258 parent-of-origin DMPs specific to reproductive workers. Furthermore, 189 sterile-specific and 191 reproductive-specific DMPs are shared across blocks, which is again significant compared to the expectation of no association (Fisher’s exact test, \( P < 0.01 \) for both comparisons). These results point to common, robust factors influencing genome-wide DNA methylation that are independent of the reproductive state and genetic block. Genes containing these DMPs are discussed in more detail in the next section.

**Genes harboring parent-of-origin mediated differential methylation signatures**

To infer functional consequences of differential DNA methylation, we defined differentially methylated genes (DMGs) as genes that contained DMPs exhibiting the same direction of allelic methylation bias (Methods). For example, parent-of-origin DMPs in block A were found across 179 and 230 genes in the sterile and reproductive workers, respectively, and these genes are subsequently referred to as parent-of-origin differentially methylated genes (Table 2). DMGs in other categories were similarly identified (Table 2). The majority of parent-of-origin DMGs in
both reproductive states and genetic blocks contained just a singular DMP (sterile average: 1.21 DMPs; reproductive average: 1.24 DMPs). The genes containing the most DMPs in the sterile workers, GB41581, was found in block B, though it does not have an annotation associated with it (Supplemental Table 2). Block B contained the gene with the most DMPs in reproductive workers (GB44685), though this gene was also unannotated (Supplemental Table 2). Interestingly, protein bicaudal D (GB42781) appears as a maternally biased DMG in both genetic blocks and was previously shown to exhibit both allele-specific methylation and expression in bumble bees (Lonsdale, et al. 2017).

To take advantage of the information provided by the two different genetic blocks, we combined DMGs from both blocks for gene ontology (GO), pathway and comparative analyses. Gene ontology terms enriched in different DMG categories are shown in Supplemental Table 3. GO terms for sterile parent-of-origin DMGs included protein glycosylation, ATP binding functions, and involved in fatty acid degradation pathways (Supplemental Table 3). Reproductive parent-of-origin DMGs were enriched for functions involving intracellular protein transport and mRNA surveillance pathways (Supplemental Table 3).

We observed moderate but significant overlaps between parent-of-origin DMGs of the two reproductive states in both blocks. Thus, these were the genes which showed parent-of-origin effects in both sterile and reproductive workers. Specifically, there were 16 DMGs showing maternal bias (Fisher’s exact test, \( P < 0.01 \)) and 30 DMGs showing paternal bias (Fisher’s exact test, \( P < 0.01 \)) overlapping between sterile and reproductive workers in block A. In block B, there were 45 maternal DMGs (Fisher’s exact test, \( P < 0.01 \)) and 35 paternal DMGs (Fisher’s
exact test, \( P < 0.01 \) overlapping between sterile and reproductive workers. Though none of the overlapping gene sets were enriched for specific GO terms, they nevertheless mirrored the DMP results and reinforce the idea of a common set of genes that are differentially methylated due to parent-of-origin effects.

Interestingly, there was significant overlap between genes showing lineage differential methylation and parent-of-origin differential methylation (Figure 3). We found 46 DMGs exhibiting both lineage and parent-of-origin biases in block A sterile workers (Fisher’s exact test, \( P < 0.01 \)), and 83 DMGs showing both biases in reproductive workers (Fisher’s exact test, \( P < 0.01 \); Figure 3). In block B, sterile workers and reproductive workers had 96 and 83 genes belonging to lineage and parent-of-origin DMGs. Functions of genes that show both types of allele-specific methylation did not deviate from the enriched GO terms of their respective reproductive states, which were generally focused on cell energy metabolism and signal transduction (Supplemental Table 3). Since these genes exhibit both lineage and parent-of-origin differential methylation, they may be particularly labile in terms of allele-specific methylation.

We next examined parent-of-origin DMGs that were unique to sterile and reproductive workers to investigate the relationship between parent-specific methylation and reproductive phenotype. There were a total of 133 sterile-specific DMGs and 184 reproductive-specific DMGs in block A and 266 sterile-specific DMGs and 233 reproductive-specific DMGs in block B. 12 such DMGs were commonly found in sterile workers of both blocks whereas 22 DMGs were common between the reproductive workers (Fisher’s exact test, \( P < 0.05 \) for both comparisons). While
these overlaps were statistically significant, they did not exhibit any significant functional enrichment in our analysis, likely due to the small number. In comparison, DMGs specific to sterile workers in block A were enriched for GO terms associated with protein deubiquitination while reproductive-worker specific DMGs were enriched for functions such as mRNA surveillance pathway and hydrolase activity (Supplemental Table 3). For block B, sterile-specific DMGs were enriched for GO terms related to protein glycosylation and signal transduction whereas reproductive-specific DMGs showed enriched GO terms such as intracellular transport (Supplemental Table 3).

For additional validation of our results, we performed differential methylation analysis of gene body methylation by mirroring previous methods in insects (Lyko, et al. 2010; Galbraith, et al. 2015). These results largely recapitulated our findings from the DMP and DMG analyses, reproducing the same overall allele-specific methylation patterns and overlapping significantly with DMP containing genes (Supplemental Tables 6 & 7).

**Differential allelic methylation is not strongly associated with differential expression**

To explore the link between parent-of-origin expression and methylation, we compared a previously obtained RNA-seq dataset (Galbraith, Kocher, et al. 2016) with our current results. The individuals from the RNA-seq study are sisters of the individuals in the current study. To make the results comparable to the methylation results, we re-analyzed the RNA-seq data using the same analysis pipeline as the current study (Methods). Our results recapitulated trends from the previous study, and while the number of genes in each category was different from the original study (possibly due to the different analysis methods used), they were all subsets of the
genes from Galbraith et al. 2016 (Supplemental Table 4). For both genetic blocks, there was a significantly more patrigene bias compared to matrigene bias as well as bias towards reproductive workers compared to sterile workers (Fisher’s exact test, \( P < 0.01 \) for all comparisons). Interestingly, we found that differentially expressed genes (DEGs) varying due to parent-of-origin and lineage effects were almost exclusive to reproductive workers in both genetic blocks (Supplemental Table 4). Additionally, there were essentially no overlap between allelic DMGs and allelic DEGs in either genetic blocks. In fact, the only overlap we observed were in reproductive workers for the lineage effect in block A and there was a complete lack of overlap for any parent-of-origin genes in both genetic blocks (Supplemental Table 5).

**Discussion**

We used reciprocal crosses between divergent honey bee stocks and whole genome bisulfite sequencing to understand factors that affect DNA methylation at genome-scale. We found a strong lineage effect on DNA methylation (Table 1 and Table 2). Previous studies indicated that genome-wide DNA methylation is highly affected by background genotypes in other species (Jones 2012; Smith and Meissner 2013; Mendizabal, et al. 2014; Yi 2017), including the *Nasonia* jewel wasp (Wang et al 2016) and bumble bee (Marshall, et al. 2019). However, we also found that some of the CpGs in the honey bee genome show variation in methylation consistent with parent-of-origin effects, which was not observed in *Nasonia* (Wang, et al. 2016). As far as we are aware, this is the first time a parent-of-origin effect has been demonstrated in an insect system, and it is unclear why they are present in honey bees and not in *Nasonia*: clearly, additional studies in other insect species are needed. The numbers of DMPs and DMGs showing a parent-of-origin effect were 2-3 fold smaller than those exhibiting lineage effects, indicating that parent-
of-origin effect is not as strong as genetic background effects. Nevertheless, the numbers of genes exhibiting parent-of-origin effects range between 3.2 ~ 9.9 % of genes analyzed, similar ranges as observed in mammals (Luedi, et al. 2007; Ferguson-Smith and Bourchis 2018). We also observed that many genes harbored both parent-of-origin DMPs and lineage-specific DMPs in both blocks (Figure 3). This observation could potentially indicate that some positions or some genes in the honey bee genome tend to be labile in terms of epigenetic modification, and potentially targets of regulation for a many different factors. Future studies using targeted deep sequencing of candidate loci in a broader range of genotypes and samples could more clearly define the methylation patterns at these sites.

Interestingly, we found that, with the exception of the paternal category, there was an increase in both DMP and DMG numbers in the reproductive workers compared to the sterile workers (X² test, P < 0.05 for all comparisons) in block A. This observation mirrored the increase of parent-of-origin effect in reproductive workers at the level of gene expression (Galbraith et al. 2016). However, in block B, this pattern was not observed (except a modest increase in European biased DMPs, Table 1, X² test, P < 0.05). One possibility is that this difference could have arisen due to the different ages of the workers between the two genetic blocks – though all the reproductive workers were confirmed to have activated ovaries, since workers in block A were 4 days older, they were likely more reproductively mature, which could manifest in clearer DNA methylation difference between worker castes. However, this hypothesis needs to be reexamined with larger data set that spans a broader range of ages in the future. Given previous evidence of tissue-specific methylation imprints (Weinstein 2001; Babak, et al. 2015), another possibility may have been the signal from the methylome of the eggs within developed ovarioles which were not
removed prior to DNA extraction and have been shown to have significantly different methylation profiles than adult honey bees (Drewell, et al. 2014).

Previous work on parent-of-origin gene expression supported the prediction that worker ovary activation was associated with biased expression of patrigenes, with a stronger paternal bias in reproductive workers compared to sterile workers (Galbraith, et al. 2015). Our re-analysis of the RNA-seq data recapitulated this finding, though we did not see the same patterns in our DNA methylation analysis. In terms of the link between DNA methylation and gene expression, we observed almost no overlap between parent-specific gene expression and methylation. This could indicate that either DNA methylation does not affect parent-of-origin gene expression, or that the effect of DNA methylation is indirect. Several genes we found exhibiting allele-specific methylation were related to chromatin structure and remodeling (histone-lysine N-methyltransferases, chromatin-remodeling complex ATPase chain Iswi; Supplemental Table 2), which may affect changes in chromatin structure that can affect gene expression levels without methylation changes to the gene (Lachner, et al. 2001; Prantera and Bongiorni 2012). For example, histone-lysine methylation was shown to be implicated in chromosome silencing in two insect species (Prantera and Bongiorni 2012). It is worth noting that studies in insects thus far suggest that differential DNA methylation does not directly correlate with differential gene expression (Galbraith, et al. 2015; Arsenault, et al. 2018; Wu, et al. 2020). Rather, DNA methylation may affect other aspects of gene expression such as gene expression variability or alternative splicing (Huh, et al. 2013; Hunt, et al. 2013; Wang, et al. 2013; Galbraith, et al. 2015; Arsenault, et al. 2018). While our current data set is insufficient for distinguishing these alternative scenarios, our results call for future studies to examine other epigenetic marks,
particularly histone modifications and/or open chromatin features in experiments utilizing greater number of allele-specific SNPs. Such studies can expand the number of sites identified that exhibit allele-specific methylation and will aid in understanding the general principle of genomic imprinting as well as the main role of DNA methylation in insects.

Declarations

The authors declare no competing interests.

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Supplemental information

Supplemental Table 1 – Detailed information on the number of SNPs used, allele-separation statistics, and methylation summary of allele-separated reads.

Supplemental Table 2 – Annotated allele-specific DMG lists, including the number of DMPs found within each gene.

Supplemental Table 3 – Gene ontology results for all gene lists used in the study.

Supplemental File 1 – Contains Supplemental Figure 1, and Supplemental Tables 4 and 5.
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Table 1. Summary of differentially methylated positions based on reproductive state and direction of allele-specific bias.

| Parent-of-origin | Block A | Block B |
|------------------|---------|---------|
|                  | Sterile | Reproductive | Sterile | Reproductive |
| Maternal bias    | 132     | 190     | 208     | 189         |
| Paternal bias    | 148     | 218     | 216     | 188         |
| Africanized bias | 333     | 921     | 696     | 727         |
| European bias    | 410     | 948     | 829     | 964         |
Table 2. Differentially methylated genes (DMGs) categorized by the worker reproductive state and direction of allele-specific bias. Differentially methylated genes contain DMPs that show the same direction of allele-specific bias.

| Parent-of-origin | Block A | | Block B |
|------------------|---------|-----------------|---------|
|                  | Sterile | Reproductive | Sterile | Reproductive |
| Maternal bias    | 82      | 113            | 140     | 127          |
| Paternal bias    | 97      | 117            | 126     | 106          |
| Africanized bias | 165     | 313            | 258     | 259          |
| European bias    | 201     | 314            | 293     | 321          |
Figure 1. Examples of allele-specific methylation bias at the CpG level (DMPs). Each data point represents the fractional methylation level of one sample at the position. A) and B) are DMPs located on scaffolds Group13.4 and Group3.15 at positions 107256 (Gene ID: GB48238) and 474358 (Gene ID: GB47021), respectively, exhibiting parent-of-origin bias (paternal) in the sterile workers. C) shows an example of an Africanized biased DMP on scaffold Group8.12 position 54654 (Gene ID: GB54805) while D) is a European biased DMP located on scaffold Group1.7 position 295042 (Gene ID: GB53211) in sterile workers. In the reproductive workers, panel E) depicts a paternal biased DMP (scaffold Group9.10 position 1931896; Gene ID: GB42829) while F) is a maternal biased DMP (scaffold Group1.41 position 1013225; Gene ID: GB55015). Lineage biased DMPs in reproductive workers are shown in G) (scaffold Group 1.14 position 2081; Gene ID: GB51836) and H) (scaffold Group7.12 position 453285; Gene ID: GB49222), biased towards Africanized and European workers, respectively.
**Figure 2.** Differentially methylated positions (DMPs) for A) workers in genetic block A and B) workers in genetic block B) represented by the relative percentage of Africanized methylation in each cross (Methods). Compared to sterile workers, reproductive workers in genetic block A showed an increased number of DMPs for each category allele-specific bias ($P < 0.05$ for all types). In contrast, this increase was not observed for any category of allele-specific bias in genetic block B. Each colored data point represents a specific type of allele-specific DMP – green is Maternal, blue is Africanized, gold is European, red is Paternal, and gray is not significant.
Figure 3. The numbers of genes identified as a specific category of DMGs in sterile and reproductive workers in A) genetic block A and B) genetic block B. A number of genes were classified as both lineage- and parent-of-origin DMGs. For example, in Block A, 15 genes exhibited both European-biased and paternal-origin biased methylation. *** indicates statistically significant overlap between groups at the $P < 0.001$ using a Fisher’s exact test.