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ORIGINAL ARTICLE

The Role of Epigenetics in Insects in Changing Environments

A role for DNA methylation in bumblebee morphogenesis hints at female-specific developmental erasure

Ben J. Hunt[1,2] | Mirko Pegoraro[2,3] | Hollie Marshall[2] | Eamonn B. Mallon[2]

[1]Centre for Ecology and Conservation, University of Exeter, Cornwall, UK

[2]Department of Genetics and Genome Biology, University of Leicester, Leicester, UK

[3]School of Biological and Environmental Sciences, Liverpool John Moores University, Liverpool, UK

Correspondence
Hollie Marshall, Department of Genetics and Genome Biology, University of Leicester, University Rd, Leicester, UK.
Email: hjm32@leicester.ac.uk

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Abstract

Epigenetic mechanisms, such as DNA methylation, are crucial factors in animal development. In some mammals, almost all DNA methylation is erased during embryo development and re-established in a sex- and cell-specific manner. This erasure and re-establishment is thought to primarily be a vertebrate-specific trait. Insects are particularly interesting in terms of development as many species often undergo remarkable morphological changes en route to maturity, that is, morphogenesis. However, little is known about the role of epigenetic mechanisms in this process across species. We have used whole-genome bisulfite sequencing to track genome-wide DNA methylation changes through the development of an economically and environmentally important pollinator species, the bumblebee *Bombus terrestris (Hymenoptera:Apidae Linnaeus)*. We find overall levels of DNA methylation vary throughout development, and we find developmentally relevant differentially methylated genes throughout. Intriguingly, we have identified a depletion of DNA methylation in ovaries/eggs and an enrichment of highly methylated genes in sperm. We suggest this could represent a sex-specific DNA methylation erasure event. To our knowledge, this is the first suggestion of possible developmental DNA methylation erasure in an insect species. This study lays the required groundwork for functional experimental work to determine if there is a causal nature to the DNA methylation differences identified. Additionally, the application of single-cell methylation sequencing to this system will enable more accurate identification of if or when DNA methylation is erased during development.

KEYWORDS

Bombus, BS-seq, development, methylome, social insects

INTRODUCTION

Epigenetic mechanisms such as DNA methylation, chromatin modifications and non-coding RNA silencing can cause stable and heritable changes in gene expression. They are also crucial factors in animal development, specifically in generating cell-specific gene expression patterns from a single genome (Paulsen & Ferguson-Smith, 2001). The most well researched of these mechanisms is DNA methylation, which most commonly involves the addition of a methyl group to a cytosine nucleotide in animals, primarily in a CpG context. In mammals, almost all DNA methylation is erased during embryo development and re-established in a sex- and cell-specific manner (Seisenberger et al., 2012), aside from any DNA methylation mediating imprinted genes, and this re-programming is thought to primarily be a vertebrate-specific trait (Xu et al., 2019). Although this process is well understood in mammalian model lab species, we know little about how DNA

Ben J. Hunt and Mirko Pegoraro contributed equally to this work.

methylation functions in the development of other clades. Insects are particularly interesting in terms of development as many species often undergo remarkable morphological changes en route to maturity.

The function of DNA methylation across model laboratory species of insects is highly variable (Lewis et al., 2020), with high levels of gene-body DNA methylation generally being linked to the stable expression of housekeeping genes (Provataris et al., 2018). Insects show significantly different DNA methylation profiles compared with mammals, with considerably lower genome-wide levels of DNA methylation, from less than 1% to around 14% depending on the species (Bewick et al., 2016), and generally exhibiting only gene-body DNA methylation (e.g., Glastad et al., 2011; Li-Byarlay, 2016; Provataris et al., 2018; Sarda et al., 2012), although see Lewis et al. (2020). Within insects, there are also notable differences in DNA methylation levels between holometabolous species and hemimetabolous species, with holometabolous insects generally showing lower levels of DNA methylation in the adult stage (Bewick et al., 2016).

Many organisms undergo a process called metamorphosis during development, defined as an extreme change in body form/structure throughout development. Holometabolous insects, such as butterflies, beetles and bees, develop from eggs into larvae, which transform into pupae before reaching a phenotypically different adult stage. Unlike the well-characterised epigenetic dynamics of mammalian development, the processes underpinning insect development remain more elusive. Currently, only a handful of studies have examined DNA methylation dynamics throughout insect development. In the red flour beetle, *Tribolium castaneum (Coleoptera:Tenebrionidae Herbst)*, and the tobacco hornworm, *Manduca sexta (Lepidoptera:Sphingidae Linnaeus)*, DNA methylation has been shown to decrease during embryo development with the levels increasing in the final adult stage (Gegner et al., 2021; Song et al., 2017). For example, *Drosophila melanogaster (Diptera:Drosophilidae Fabricius)* displays little to no DNA methylation in the adult stage and lacks the essential genes involved in DNA methylation establishment and maintenance (DNMT1 and DNMT3); however, DNA methylation was found to be present in slightly elevated levels in the embryo and pupal stages of development (Deshmukh et al., 2018). The primary focus of developmental epigenetics within insects, however, is within the eusocial species.

Social insects display extreme phenotypic differences between castes, for example, workers/queens/drones, although individuals share highly similar and sometimes identical genetic backgrounds. Epigenetic processes are, therefore, thought to be fundamental to many of the phenotypic differences observed. It was recently noted that there is conflicting evidence for a functional role of DNA methylation in caste determination in social insects (Oldroyd & Yagound, 2021), although many of these studies focus solely on final adult stages. Harris et al. (2019) have recently explored the DNA methylome throughout honeybee development, finding fluctuating levels of DNA methylation between developmental stages, with larvae showing the lowest DNA methylation levels and sperm showing the highest. Bonasio et al. (2012) explored developmental DNA methylation in two ant species, finding the lowest DNA methylation levels were in the embryo of one species (*Camponotus floridanus [Hymenoptera:Formicidae Buckley]*) and the larvae of another (*Harpegnathos saltator[Hymenoptera:Formicidae T.C. Jerdon]*).

Evidence exists for a possible role for DNA methylation during metamorphosis in insects, and specifically within social insects, but the extent to which this is conserved between related species remains unknown. Interestingly, even within mammals, the dynamic reprogramming of DNA methylation marks during development differs between species (Beaujean et al., 2004). To better understand the mechanisms driving development and metamorphosis in insects, it is essential that we explore these processes in a variety of independent species. Additionally, DNA methylation in invertebrates has recently attracted attention as a possible mechanism of environmental adaptation. For example, Rahman and Lozier (2023) recently identified numerous differentially methylated genes between a high- and low-altitude population of the bumblebee, *Bombus vosnesenskii (Hymenoptera:Apidae Radoszkowski)*. For DNA methylation to play a role in adaptive processes, it must be directly heritable from parent to offspring. Therefore, determining whether DNA methylation is re-programmed during development will help to establish a potential mechanism (or lack thereof) for heritability of environmentally induced DNA methylation change.

Bumblebees provide an ideal model to explore the role of DNA methylation during development; they possess a functional DNA methylation system (Sadd et al., 2015), which may play a regulatory role in social caste determination (Amarasinghe et al., 2014; Li et al., 2018; Marshall et al., 2019) and are easily reared within the lab (Duchateau & Velthuis, 1988). They are also both economically and environmentally important as a pollinator species (Woodard et al., 2015), with commercial colonies sold for crop pollination (Velthuis & Van Doorn, 2006). *Bombus terrestris* colonies are founded annually by a singly mated queen; she will initially lay diploid eggs resulting in female workers (Bloch, 1999). A competition phase later occurs between queens and workers, where some workers will become reproductive, without mating, and produce their own haploid sons (Alaux et al., 2006). We take advantage of this system to follow whole-genome and gene-level DNA methylation dynamics through female worker somatic tissue (brain), ovary tissue, male offspring larval head tissue, male pupal head tissue, adult male brain tissue and finally sperm (Figure 1). If DNA methylation does play a role in the metamorphosis of *B. terrestris*, we would predict (i) global differences in DNA methylation levels between different developmental stages and (ii) differentially methylated genes involved in developmental processes between developmental stages. Within this study, we do indeed find both global differences in DNA methylation between developmental stages and differentially methylated genes involved in relevant developmental processes.

RESULTS

Genome-wide developmental DNA methylation dynamics

Genome-wide DNA methylation patterns are most different in worker ovaries/eggs and sperm compared with brain/head tissue

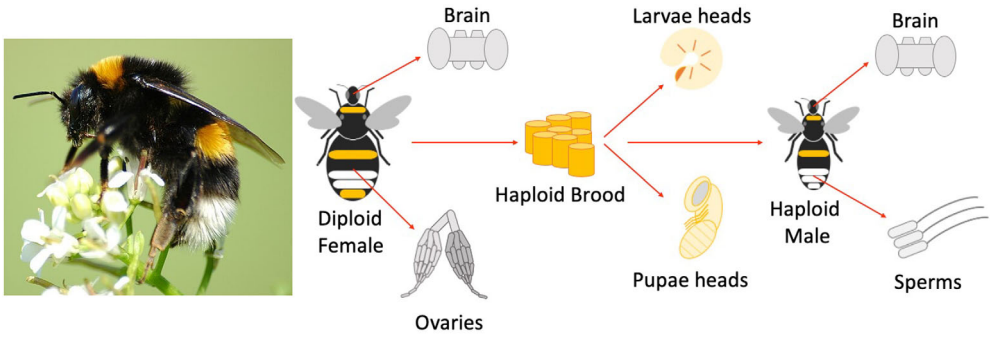

FIGURE 1 Left: study organism, *Bombus terrestris*. Image credit Vera Buhl CC-BY-SA. Right: schematic overview of the samples collected. Specifically, brain and ovary/egg tissue were taken from female workers. Larval heads, pupal heads, brain tissue and sperm were then taken from their male offspring.

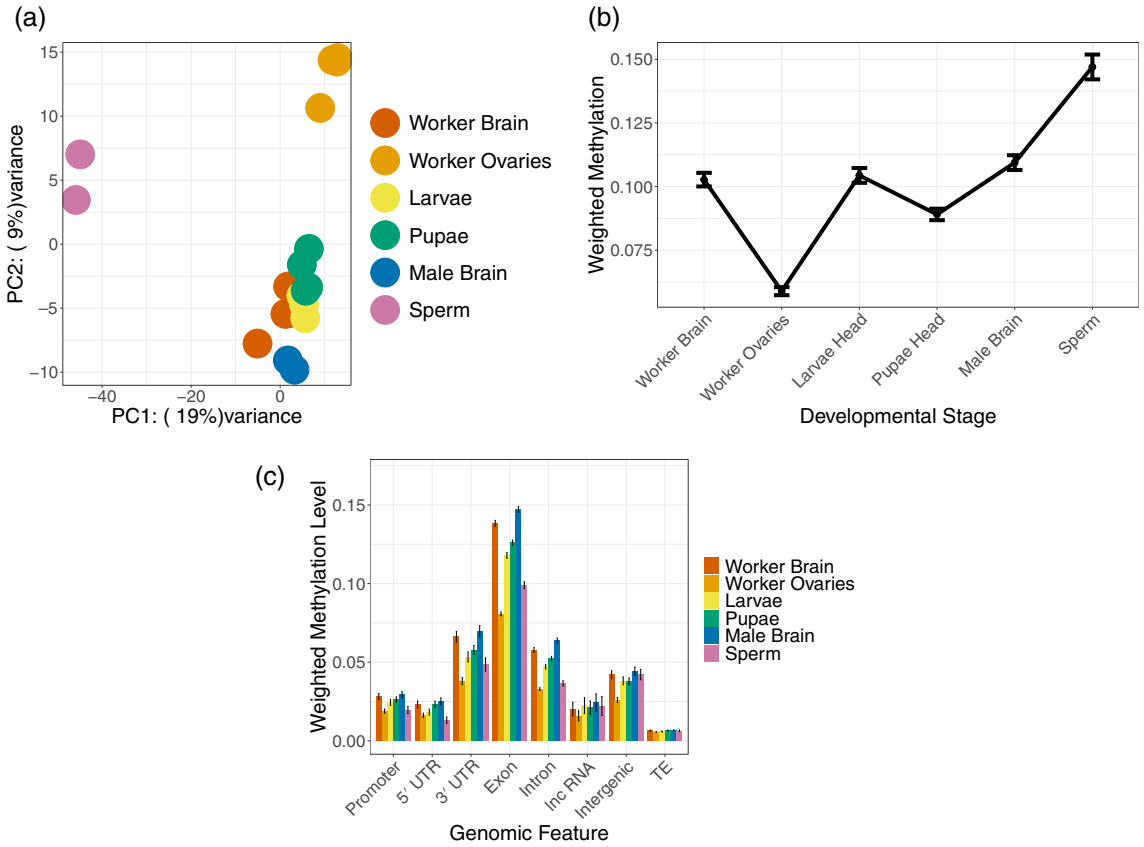

FIGURE 2 (a) Principle component (PC) analysis plot of the methylation level of CpGs which have data in all samples and are classed as methylated in at least one sample via binomial test ($n = 1189$). (b) Gene levels of DNA methylation across developmental stages ($n = 8874$–10,549). Each dot represents the mean methylation level across all genes and replicates per developmental stage. The error bars represent 95% confidence intervals. (c) Mean level of DNA methylation per genomic feature averaged across all replicates per developmental stage. The error bars represent 95% confidence intervals. TE, transposable element. Lnc RNA, long non-coding RNA. UTR, untranslated region.

of adults and developing larvae/pupae (Figure 2a). As DNA methylation is enriched in gene-bodies in bumblebees, we assessed global gene levels across developmental stages. Using a linear mixed effects model, we find all developmental stages show significant differences in gene-level DNA methylation, apart from reproductive worker brains and larvae heads (Figure 2b; Supplementary 1.0.1). Specifically, sperm shows the highest levels of DNA methylation in genes and female the lowest (Figure 2b; Supplementary 1.0.1).

To further explore this pattern genome-wide, we also looked at DNA methylation levels across different genomic features for each developmental stage. We find across stages that DNA methylation is enriched in exons and depleted in promoter and 5′ UTR regions, in line with previous findings in adult somatic tissue (Lewis et al., 2020).

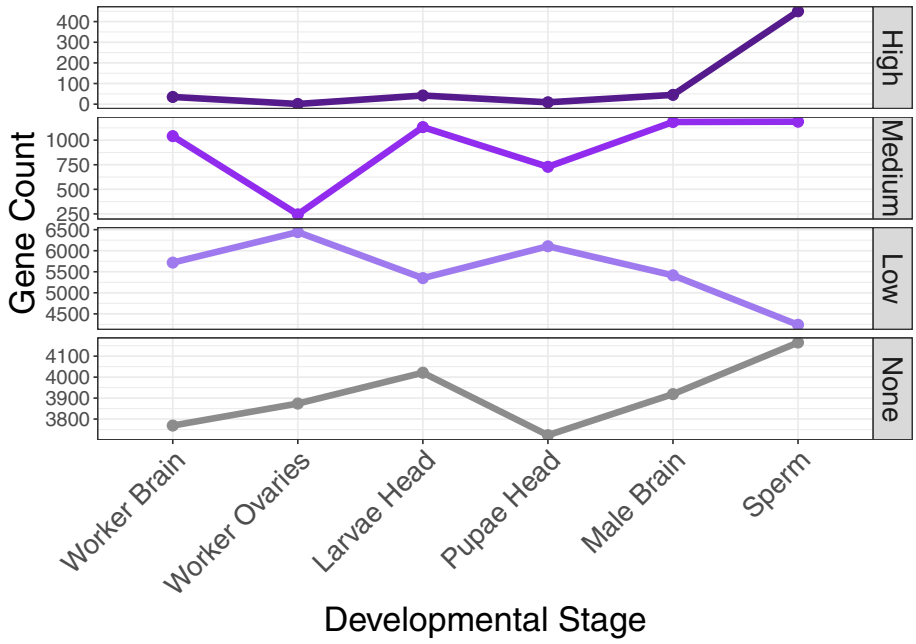

FIGURE 3 Number of genes per developmental stage that are classes as showing high methylation (>0.7), medium methylation (>0.3–0.7), low methylation (>0–0.3) and no methylation.

The depletion of DNA methylation in worker ovaries/eggs observed in Figure 2b is present genome-wide across all genomic features (Supplementary Figure S2).

We also find that the high mean level of DNA methylation in sperm genes is driven by sperm containing substantially more highly methylated genes compared with all other developmental stages (Figure 3). Interestingly, sperm also contains more genes with no DNA methylation compared with all other developmental stages, suggesting a more bimodal pattern of DNA methylation where DNA methylation is specifically targeting or being erased from certain genes (Figure 3). This bimodal distribution in methylation levels in sperm explains why the mean levels of DNA methylation across all exons and introns appear lower (Figure 2c), compared with across genes as a whole (Figure 2b). The lower total number of genes compared with exons and introns allows for outliers to disproportionately affect the mean, see Supplementary 1.0.1 for full summary statistics. Finally, we confirm that the high exon methylation levels that we see are not due to within-gene transposable element (TE) methylation. We find overall lower levels of DNA methylation in TEs compared with genes in *B. terrestris*, as has previously been shown in Lewis et al. (2020) (Figure 2c; Supplementary Figure S2).

To explore the function of highly methylated genes and genes with no DNA methylation in all developmental stages, we carried out a Gene Ontology (GO) enrichment analysis. Highly methylated genes in all developmental stages are enriched for a variety of core cellular processes (Supplementary 1.0.2 and 1.0.3), consistent with a role for DNA methylation in maintaining housekeeping gene function, whereas genes with no DNA methylation across developmental stages are involved in a much larger variety of metabolic and cellular processes (Supplementary 1.0.2 and 1.0.4).

Differential DNA methylation between developmental stages

To examine in which genes DNA methylation changes throughout bumblebee development, we first identified significantly differentially methylated CpGs per sequential comparison. We find the vast majority of significantly differentially methylated CpGs in all comparisons are found within exon regions (Supplementary Figures S3 and S4) and are equally distributed across exons within a gene (Supplementary Figure S5). The number of hypermethylated (i.e., having more methylation) CpGs per comparison mirrors the global DNA methylation trends between developmental stages as seen in Figure 2b,e,g. There are significantly more hypermethylated CpGs in worker brains compared with worker ovaries/eggs (Supplementary Table S1).

To class a gene as differentially methylated between developmental stages, we required the gene to contain an exon which possessed both a significantly differentially methylated CpG and an overall weighted methylation difference of 0.15 (equivalent to 15% overall methylation difference) in the same direction as the CpG, that is, if the CpG is hypermethylated in worker brains compared with ovaries/eggs, then the entire exon must also be hypermethylated in worker brains compared with ovaries/eggs. Again, we find the number of differentially methylated genes mirrors the overall trends in DNA methylation seen in Figure 2b (Table 1). Graphical visualisations of example differentially methylated regions are shown in Supplementary Figures S6–S9. We also checked the overall levels of methylation for genes that are classed as differentially methylated. We find the majority of differentially methylated genes show overall low levels of DNA methylation, that is, have a weighted methylated level greater than 0 and less than or equal to 0.3 (Supplementary Figure S10).

TABLE 1 The number of genes with a hypermethylated exon per developmental stage for all comparisons.

	Worker brain	Worker ovaries	Larvae head	Pupae head	Male brain	Sperm	Chi-squared	Df	*p* value
Worker brain versus worker ovaries	1220	2					1214	1	<0.001*
Worker ovaries versus larvae head		1	285				282.01	1	<0.001*
Larvae head versus pupae head			4	1			1.8	1	0.18
Pupae head versus male brain				4	153		141.41	1	<0.001*
Male brain versus sperm					2	47	41.32	1	<0.001*
Worker brain versus male brain	12				25		4.56	1	0.032*
Worker ovaries versus sperm		0				134	134	1	<0.001*

Note: A chi-squared goodness of fit was carried out per comparison to determine if there are significantly more hypermethylated genes in one developmental stage compared to the other.*<0.05.

Across all comparisons, there are a total of 1516 genes, which change methylation level during development (Supplementary 1.0.5). The majority of these ($n = 962$) are unique to the comparison between worker brains and ovaries/eggs (Supplementary Figure S13). A GO enrichment analysis was then carried out for differentially methylated genes in each comparison, compared against a background set of all methylated genes found within those specific samples. In all comparisons, we find a wide variety of GO terms enriched between developmental stages (Supplementary 1.0.6). Of particular note, we specifically find GO terms involved in neuron development and oogenesis enriched between worker brain and ovary tissue. We also find the terms '*instar larval or pupal development*' (GO:0002165) and '*imaginal disc morphogenesis*' (GO:0007560) enriched between ovaries/eggs and larvae. Between ovaries/eggs and sperm, we find '*spermatid nucleus differentiation*' (GO:0007289) and '*cell fate determination*' (GO:0001709) enriched. Finally, differentially methylated genes throughout developmental comparisons contain GO terms enriched for alternative splicing. See Supplementary 1.0.6 for all enriched GO terms.

VARIABILITY OF DNA METHYLATION ACROSS DEVELOPMENT

To identify genes with the most variable DNA methylation levels throughout development, we calculated the Jensen–Shannon Diversity index per gene per comparison and took the mean index across all comparisons. We find that 827 genes show a considerably higher than average variability in methylation levels (Supplementary 1.0.7; Figure S1). These genes are distributed evenly across the genome (Supplementary Figure S11), with a slightly higher proportion of genes showing a significant Jensen–Shannon divergence (JSD) index on chromosome NC_015774.1 (Supplementary Figure S11).

To explore the function of these genes, we carried out a GO enrichment analysis of the significantly variable genes compared with all genes present in the data set. We find mostly metabolic-related processes enriched; however, there are also some terms enriched related to RNA splicing (Supplementary 1.0.7). We then looked for GO enrichment in the significantly variable genes on chromosome NC_015774.1 compared with all significantly variable genes finding various processes including 'neuron remodelling' (GO:0016322), Supplementary 1.0.7. We find one gene specifically driving the enrichment of this GO term. *Caspase-6* (LOC100652135) controls cell death and the inflammation response (Bartel et al., 2017; Cohen, 1997) and also has annotated GO terms related to lifespan determination. This gene is more highly methylated in head and brain tissue throughout development, compared with gametes (Supplementary S12).

We checked if the highly variable genes are also significantly differentially methylated between stages. We find 24% of variable genes are also differentially methylated ($n = 200$), compared with 17% of non-significantly variable genes (Supplementary Figure S14). The genes that are highly variable and differentially methylated are spread across all developmental comparisons (Supplementary Figure S14). A GO enrichment of these genes compared with all highly variable genes shows enrichment for various processes, including '*mRNA cis splicing, via spliceosome*' (GO:0045292), '*tissue morphogenesis*' (GO:0048792) and '*embryo development*' (GO:0009790) (Supplementary 1.0.8).

DISCUSSION

We tracked genome-wide DNA methylation dynamics throughout bumblebee development, from female worker somatic tissue and reproductive tissue into their male offspring larvae, pupae and adult head/brain tissue and then into the adult male sperm. We find that the levels of DNA methylation vary dramatically in the reproductive tissue/gametes compared with the developing larvae/pupae head and adult brain. Ovaries/eggs show a sharp decrease in DNA methylation levels, whereas sperm shows considerably higher DNA methylation levels. Interestingly, the high levels of DNA methylation in sperm is characterised by a more bimodal pattern of high/no DNA methylation in genes compared with intermediate levels, meaning the overall high levels are driven by a small number of highly methylated genes. We also find differentially methylated genes in sequential

developmental comparisons, most of which are lowly methylated genes, and many are involved in processes relevant to each specific developmental stage/tissue. Finally, we find genes that have the most variable DNA methylation profiles throughout development are primarily enriched for metabolic processes and also have a role in nervous system development and splicing.

Possible female-specific DNA methylation erasure

One of our key findings is that the major global differences in DNA methylation are seen between the somatic tissue across all stages and the reproductive tissue/gametes. DNA methylation is known to be tissue-specific in many species (Pai et al., 2011), and so this is unsurprising. However, we specifically find that ovaries/eggs show a marked decreased in overall DNA methylation, with sperm showing higher levels, driven by a subset of genes. Based on this finding, we hypothesise that DNA methylation profiles may be erased during oogenesis but remain stably transmitted through the male germline. Previous work in honeybees has shown no evidence for DNA methylation erasure during embryogenesis (Yagound et al., 2020). However, Yagound et al. (2020) only examined DNA methylation inheritance from the point of view of transmission of the male's methylome to his daughters. Transmission of the maternal methylome was unexamined. Cardoso-Júnior et al. (2021) do find similar DNA methylation profiles between the brains and ovaries of honeybee queens, contrary to what we find in bumblebees. Additionally, in stark contrast to our findings, Drewell et al. (2014) find that both male and female honeybee gametes show higher methylation compared with male thorax tissue. This could suggest that if developmental erasure is occurring during oogenesis in bumblebees, it is not conserved between the two species. However, a controlled study using identical samples from both species would be needed to confirm maternal methylome persistence or erasure.

The high levels of DNA methylation in sperm is characterised by higher numbers of genes showing high/no methylation rather than intermediate levels. Based on the above hypothesis of paternal methylome transmission, we suggest that this profile in sperm may represent a 'pristine' methylome, that is, a genotype-mediated methylation profile currently uninfluenced by environmental exposures or epigenetic drift. DNA methylation has been strongly linked to the underlying genotype in multiple hymenopteran species (Wang et al., 2016; Yagound et al., 2019), including bumblebees (Marshall et al., 2019). Additionally, Harris et al. (2019) also find evidence for a highly maintained sperm methylome, compared with somatic tissue in honeybees. They suggest the stronger signal of DNA methylation in sperm is present to maintain signatures across generations. We speculate that the sperm methylome may represent the genotype-mediated DNA methylation profile, which then later diversifies by tissue, developmental stage, age, environmental exposure, etc. It may also be that we see more genes with low/medium levels of DNA methylation in head and brain tissue due to a loss of accurate transmission of epigenetic information through mitosis. This loss of epigenetic information over time is documented in other species and is strongly associated with ageing in mammalian systems (Yang et al., 2023). Sequencing of individual samples, with a known genetic background, would allow further testing of this hypothesis.

The idea of female-specific erasure based on our results is just one hypothesis for what we observe. During mammalian gametogenesis, there are waves of methylation and de-methylation in the gametes (Rotondo et al., 2021). Given that DNA methylation can change so rapidly during development in other species, it is possible we captured a specific stage of gamete development in males, that is, only mature sperm. However, this is unlikely to be the case for the female ovary samples as they contain eggs of varying stages of development. If DNA methylation is indeed only transmitted through the paternal lineage in bumblebees, this would have implications for potential imprinting mechanisms (see Pegoraro et al. (2017) for a theoretical review of imprinting in bees) and for a possible role of heritable DNA methylation in environmental adaption (reviewed in Skinner and Nilsson (2021)).

DNA methylation and bumblebee morphogenesis

In addition to global changes in DNA methylation between tissues and developmental stages, we have identified specific differentially methylated genes using sequential comparisons. Although these genes are involved in a multitude of processes, we find developmentally relevant differentially methylated genes throughout, which is suggestive of a functional role for DNA methylation in bumblebee development. To highlight one particular example, genes involved in larval development and imaginal discs are found to be differentially methylated between ovaries (containing developing eggs) and larvae. We find GO terms enriched for imaginal disc pattern formation, morphogenesis and imaginal disc-derived appendage development. Imaginal discs are tissue aggregations in holometabolous insects that develop into various adult structures during metamorphosis (Beira & Paro, 2016). Although we chose to apply a site- and gene-level filter to consider a gene differentially methylated, perhaps also noteworthy may be genes excluded on that basis but with single differentially methylated loci. In the comparison between larvae and pupae, such genes included *patched* (LOC100646247), *smoothened* (LOC100645866), *tramtrack* (LOC100645213) and *split ends* (LOC100652059), which are linked with developmental processes such as compound eye morphogenesis, wing morphogenesis, nervous system development and Wg pathway regulation in larval tissues (Supplementary 1.0.9), and the unfiltered results list showed GO enrichment for terms such as '*anterior/posterior lineage restriction, imaginal disc*' (GO:0048099) and '*Bolwig's organ morphogenesis*' (GO:0001746). Additionally, we find the gene *sirt1* (LOC100650920) is differentially methylated between worker brains and ovaries/eggs and again between ovaries/eggs and larvae. *Sirt1* is a longevity-related gene that has recently been shown to be over-expressed in *B. terrestris* when genome-wide DNA methylation levels are chemically altered (Renard et al., 2023). Given the lifespan differences between female

workers and males, with males being shorter lived (Duchateau & Marien, 1995; Holland & Bourke, 2015), this result is suggestive of DNA methylation being involved in the determination of later lifespan.

Although the relationship between gene methylation and gene expression in invertebrates is uncertain (see below), it is well established in mammals that methylation changes in a single CpG is sufficient to impact expression (Kitazawa & Kitazawa, 2007; Zhang et al., 2010), and we do not rule out the possibility that the above results may have biological significance. Overall, the identification of differentially methylated genes between developmental stages involved directly in the metamorphic process is suggestive of a functional role for DNA methylation in bumblebee development, although we cannot determine from our data whether it drives some regulatory process for these genes or if it is a consequence of gene regulatory changes occurring within development. Nevertheless, this finding allows for future work to examine the functional role of DNA methylation in development, for example, by RNAi knockdown of DNMTs (e.g., Arsala et al., 2022) or through chemical de-methylation of the genome (e.g., Renard et al., 2023).

We also find that the majority of differentially methylated genes between developmental stages show overall low levels of DNA methylation. Previous work has also found this is the case in DNA methylation differences between bumblebee castes (Marshall et al., 2023). This finding supports the idea that lowly methylated genes are able to promote phenotypic plasticity through generating more variable gene expression (Roberts & Gavery, 2012), whereas highly methylated genes are associated with housekeeping functions and stable gene expression across insects (Provataris et al., 2018). A recent hypothesis for the function of DNA methylation in terms of its response to environmental change has been proposed by Dixon et al. (2018). The 'see-saw' hypothesis suggests that transcriptional plasticity is brought about through lowly methylated genes increasing methylation levels as a group, and highly methylated genes decreasing methylation levels. This could explain why there are higher numbers of genes with intermediate levels of DNA methylation in the developing larvae and adult head tissue compared with the gametes, as this is where higher variability in gene expression is required for development.

Differential DNA methylation has generally not been linked to direct differential gene expression in invertebrates (Dixon & Matz, 2022). However, DNA methylation has been linked to a suite of other processes, which may themselves govern downstream gene expression changes. For example, DNA methylation has been linked to alternative splicing in honeybees (Flores et al., 2012). Additionally, a recent study in a related species of bumblebee found differentially methylated genes between a high-altitude and a low-altitude population were also enriched for splicing GO terms (Rahman & Lozier, 2023), suggesting a role for DNA methylation in alternative splicing. Previous work has established large numbers of differential alternative splicing events throughout bumblebee development (Price et al., 2018). Some of the genes with the most variable DNA methylation in our study are indeed involved in mRNA splicing. DNA methylation changes may therefore be playing some role in mediating developmental plasticity through splicing. Although, the potential causative role of DNA methylation in alternative splicing in insects is debated (discussed in Duncan et al., 2022). Additionally, other epigenetic mechanisms have been associated with insect metamorphosis, including microRNAs (Burggren, 2017) and histone modifications. Specifically, histone acetylation has been implicated in the metamorphic process in a few fruit fly and butterfly species (Carré et al., 2005; Mukherjee et al., 2012; Zhang et al., 2019). The presence of DNA methylation in honeybees and fire ants has also been linked to the presence of histone modifications associated with gene expression (Hunt et al., 2013), as has DNA methylation in the silk moth, which also influences histone acetylation, impacting gene expression (Xu et al., 2021). This highlights the complex interplay between multiple mechanisms. A multi-omics study carried out on the same samples (to account for the effects of genetic variation) is required to disentangle the relative roles of epigenetic processes in mediating bumblebee metamorphosis.

Recent advancements in sequencing technology would allow a more precise look at how DNA methylation may be involved, either directly or indirectly, in mediating bumblebee development. For example, single-cell methylation sequencing would allow cell-specific profiles to be determined. Indeed, a recent paper has developed a method to obtain single-cell methylomes from mouse brain tissue (Liu et al., 2023). This would allow a greater resolution for samples with multiple tissues, that is, disentangling the worker ovary and egg profiles. CUT&Tag methods allow the identification of histone modifications using considerably lower DNA input (Kaya-Okur et al., 2019), making this technology more accessible to small insect tissue samples. Finally, to determine if there is a causative role for DNA methylation in bumblebee development, experimental knockouts using RNAi or specific DNA methylation additional/removal using CRISPR-Cas9 (Vojta et al., 2016) are required. This study lays the groundwork required for these future research avenues.

EXPERIMENTAL PROCEDURES

Husbandry and tissue dissection

Five colonies of *B. terrestris audax (Hymenoptera:Apidae Linnaeus)* (Agralan, UK) were established and maintained in wooden nest boxes under red light at 26°C and 60% humidity on a diet of 50% v/v glucose/fructose apiary solution (Meliose-Roquette, France) and pollen (Percie du set, France) (Amarasinghe et al., 2014). Between 80 and 100 days after establishment, callow females were isolated in perspex boxes (18.5 cm × 12.5 cm × 6.5 cm) for 3 days, at which point a further two callow females were added, with old and new callows marked in different colours to identify them. When the older female assumed a dominant role and began laying eggs, larvae and pupae samples were collected (Figure 1). The reproductive female's brain and ovaries/eggs were also sampled. For male brain and sperm samples, offspring males were collected as callows and kept in groups of 10 in a perspex box for 13–16 days before dissection and extraction

(Baer and Schmid-Hempel, 2000). All samples were collected in a 2-h window between 3 and 5 PM to avoid circadian influences on the methylome.

Reproductive workers and adult males were anaesthetised on ice (4°C) and brains/ovaries dissected in PBS. For sperm collection, the protocol described in Baer and Schmid-Hempel (2000) was followed, collecting sperm in 20 μL PBS from accessory testis excluding the testis tissue. Heads were dissected from larvae stage four and from pupae with eye pigmentation but no body pigmentation. Samples were snap-frozen in liquid nitrogen and stored at −80°C.

Genomic DNA extraction, sequencing and mapping

Twenty libraries were prepared from three colonies, comprising four replicates each of reproductive workers brains, ovarian tissue, larval heads and pupal heads, and two replicates each of male brains and sperm. It is worth noting that ovarian tissue includes the developing eggs. Genomic DNA was extracted using QIamp DNA Micro Kit (QIAGEN) with minor modifications. Prior to the addition of Buffer ATL and Proteinase K, tissues were crushed in liquid nitrogen-chilled Eppendorf tubes using a mini pestle. Carrier RNA was used with Buffer AL. DNA concentration was assessed using Nanodrop and Qubit® dsDNA BR Assay Kit (ThermoFisher Scientific, USA). Whole-genome bisulfite sequencing (WGBS), that is, library preparation, bisulfite conversion and 150 bp paired-end sequencing using a HiSeq 2000 machine (Illumina, Inc.), was performed at the Beijing Genomics Institute. All samples include a 1% lambda spike to assess bisulfite conversion efficiency.

Reads were subject to quality control using Trim Galore 0.6.5 (clip_R1 10 –clip_R2 10). Bismark 0.20.0 (Krueger & Andrews, 2011) was used to align the reads to the Bter_1.0 genome (Refseq accession no. GCF_000214255.1; Sadd et al., 2015) (–score-min L,0,-0.4) and remove PCR artefacts in the CpG context. To address an issue with unreliable calls at certain base positions due to flowcell tile quality, deduplicated BAM files were processed using the methylKit 1.16.1 (Akalin et al., 2012) function *processBismarkAln* to exclude bases with minqual <30. The output of this was reformatted to the Bismark coverage format using R 4.0.3 in RStudio 1.3.1093 and then fed back into Bismark's *coverage2cystosine* module using the option *merge_CpGs* to destrand the data and generate a whole genome CpG methylation call file per sample.

The sequenced samples returned libraries ranging from 24 to 38 million paired end reads with alignment rates from 53% to 80% (Supplementary 1.0.0). After deduplication, this gave an average genome-wide coverage of 14X ±3X. The mean number of methylated reads in a CpG context were 0.71 ± 0.07% (standard deviation), in a CHG context, 0.45 ± 0.03% and in a CHH context, 0.47 ± 0.03%. Methylation levels in the latter two contexts did not exceed the error rate (0.5%) estimated by alignment of the libraries to the lambda spike-in genome. These levels of CpG methylation, 0.2%, are in line with previous work in this species (e.g., Marshall et al., 2019). We also confirmed the genome-wide levels of DNA methylation by calculating the percentage of CpG sites that were classed as methylated via a binomial test, with the probability of success being the lambda conversion rate, finding on average 0.14% of all CpGs are methylated (Supplementary 1.0.0).

DNA METHYLATION ANALYSIS

Differential methylation analysis was performed using the R package methylKit 1.16.1 (Akalin et al., 2012). The destranded calls were imported using the *mincov* parameter to exclude sites with coverage <10 reads and further filtered to exclude sites with coverage higher than the 99.5th percentile. Differential methylation analyses were conducted between worker brain–worker ovaries; worker ovaries–larvae head; larvae head–pupae head; pupae head–male brain; male brain–sperm; worker brain–male brain; worker ovaries–sperm. For each comparison, methylKit's *reorg* function was used to extract the relevant samples and reassign treatment IDs. In comparisons where four replicates per group were available for both groups, sites were required to be represented in at least three replicates per group to be tested. In comparisons where two replicates were available in one or both groups, sites were required to be represented in two replicates per group to be tested. A binomial test was used to make per-loci methylation status calls using a 0.5% error rate estimated from the sequencing lambda spike-in control and only loci identified as methylated in at least one sample were tested for differential methylation using the Chi-squared test, controlling for colony as a covariate and correcting for overdispersion. Loci were considered to be differentially methylated if they show a methylation difference of 10% or greater and an FDR-adjusted q-value of 0.05. Loci were annotated with bedtools *intersect* (Quinlan & Hall, 2010). To then call a gene differentially methylated, we required an exon within a given gene to contain at least one significant differentially methylated CpG in addition to having an overall exon-wide methylation difference of 15%. Differentially methylated regions were visualised using Methylation Plotter (Mallona et al., 2014).

Genome-wide DNA methylation profiles were calculated as the mean weighted methylation level (Schultz et al., 2012) across samples per developmental stage for each genomic feature. Briefly, the weighted methylation level of a region is the number of methylated reads divided by the total number of reads within a given region, normalised by the number of CpG sites. This ensures that sites with low coverage do not disproportionately inflate methylation levels. Differences in overall gene-level DNA methylation were determined using linear mixed effects models implemented from the lme4 R package 1.1–33 where replicate and gene were random factors. Post hoc testing was done with the emmeans R package 1.8.6. Genomic features were classed as showing high methylation (weighted methylation >0.7), medium methylation (>0.3–0.7), low methylation (>0–0.3) or no methylation. Introns, 5′ UTR and 3′ UTR regions were annotated using AGAT 0.8.0 (Dainat, 2021). Putative promoter regions were classed as 500 bp upstream of each gene's 5′ UTR and intergenic regions were all other unannotated regions. To account for transposable elements

(TEs) in this analysis, we carried out de novo annotation of TEs within the genome using EDTA 2.1.0 (Ou et al., 2019).The extent of variability in DNA methylation levels across developmental stages was assessed using the JSD index implemented by the MethylIT 0.3.2.3 R package (Sanchez, 2021). Genes were classed as showing significant variability across developmental states when the JSD index was >0.037 based on the distribution of all indices (Supplementary Figure S1).

GO term enrichment

GO terms for *B. terrestris* were taken from the Hymenoptera Genome Database (Bombus_terrestris_HGD_go_annotation.gaf.gz; Walsh et al., 2022). GO enrichment analysis was carried out using the hypergeometric test with Benjamini–Hochberg (Benjamini & Hochberg, 1995) multiple-testing correction, $q < 0.05$. GO terms from differentially methylated genes between comparisons were tested against a GO term background set made from the GO terms associated with all methylated genes per comparison. Genes were determined as methylated if they had a mean weighted methylation level (taken across all replicates within a developmental stage) greater than the lambda weighted methylation level of 0.05 in either of the developmental stages within each comparison. Genes classed as highly methylated per developmental stage were tested for enrichment against all methylated genes for that developmental stage. Genes classed as showing no methylation per developmental stage were tested against all genes present in the WGBS for that developmental stage. Genes with a significant JSD index were tested against all genes present across all data sets which were methylated in at least one developmental stage. REVIGO (Supek et al., 2011) was used to generate GO descriptions from the GO IDs.

AUTHOR CONTRIBUTIONS

Hollie Marshall: Investigation; writing – original draft; methodology; visualization; writing – review and editing; formal analysis; data curation. **Ben J. Hunt:** Investigation; writing – original draft; methodology; visualization; writing – review and editing; formal analysis; data curation. **Mirko Pegoraro:** Conceptualization; investigation; methodology; writing – review and editing. **Eamonn B. Mallon:** Conceptualization; investigation; funding acquisition; methodology; writing – review and editing; supervision; project administration.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All sequencing data related to this project can be found under NCBI BioProject PRJNA573598. Custom scripts for the genome-wide analysis can be found at https://github.com/MooHoll/bumblebee_developmental_meth.

ORCID

Hollie Marshall https://orcid.org/0000-0002-1860-780X

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Data S1. Supporting Information.

Figure S1. Distribution of Jensen–Shannon diversity (JSD) indices for all genes tested ($n = 9016$). Genes were classes as showing a significant JSD index if they were greater than 1.5× the interquartile range, that is, an outlier on this plot.

Figure S2. Number of features per developmental stage that are classed as showing high methylation (>0.7), medium methylation (>0.3–0.7), low methylation (>0–0.3) and no methylation.

Figure S3. The genomic location of the differentially methylated CpGs identified in each comparison coloured by the hypermethylated developmental stage.

Figure S4. Number of differentially methylated CpGs found within each genomic feature per developmental stage comparison.

Figure S5. Number of differentially methylated CpGs found within the first five exons (a) and introns (b), with exons and introns 6+ represented by *n*. The letters represent each developmental stage, A = reproductive worker brain, B = reproductive ovaries, C = larvae, D = pupae, E = male brain and F = sperm.

Figure S6. Example differentially methylated exons for each comparison, a–c = reproductive brains (green) versus ovaries (purple). d–f = ovaries (green) versus larvae (purple). The first panel shows a boxplot of the proportion of methylation for all CpGs within the given exon. Panel two shows the proportion of methylation of each CpG within the given exon with the shading indicating methylation level. Panel three shows the same information as panel two but as a line plot.

Figure S7. Example differentially methylated exons for each comparison, a–c = larvae (green) versus pupae (purple). d–f = pupae (green) versus male brain (purple). The first panel shows a boxplot of the proportion of methylation for all CpGs within the given exon. Panel two shows the proportion of methylation of each CpG within the given exon with the shading indicating methylation level. Panel three shows the same information as panel two but as a line plot.

Figure S8. Example differentially methylated exons for each comparison, a–c = male brain (green) versus sperm (purple). d–f = reproductive brain (green) versus male brain (purple). The first panel shows a boxplot of the proportion of methylation for all CpGs within the given exon. Panel two shows the proportion of methylation of each CpG within the given exon with the shading indicating methylation level. Panel three shows the same information as panel two but as a line plot.

Figure S9. Example differentially methylated exons for each comparison, a–c = ovaries (green) versus sperm (purple). The first panel shows a boxplot of the proportion of methylation for all CpGs within the given exon. Panel two shows the proportion of methylation of each CpG within the given exon with the shading indicating methylation level. Panel three shows the same information as panel two but as a line plot.

Figure S10. Differential methylation comparison showing the weighted methylation level of the differentially methylated genes. High ≥0.7, medium <0.7 and ≥0.3, low <0.3 and >0, none is 0.

Figure S11. (a) Jensen–Shannon diversity indices for every gene, plotted by chromosome. (b) Proportion of genes per chromosome that show a significant Jensen–Shannon diversity index, that is, have more highly variable DNA methylation states across developmental states compared with most other methylated genes. (c) Weighted

methylation level of the top six most variable genes. The red dot shows the mean across replicates within each developmental stage.

Figure S12. Weighted methylation level of two genes involved in life span. The red dot shows the mean across replicates within each developmental stage. LOC100644593: ras-like protein 1. LOC100652135: caspase-6.

Table S1. The number of hypermethylated CpGs per developmental stage for all comparisons. A Chi-squared goodness of fit was carried out per comparison to determine if there are significantly more hypermethylated CpGs in one developmental stage compared with the other.

Figure S13. Overlap of genes between differential DNA methylation comparisons. The set size indicates the total number of hypermethylated genes; the intersection size shows how many of those are common between sets, as indicated by the connections in the bottom panel. For example, 117 genes hypermethylated in worker brains and larvae compared with ovaries.

Figure S14. (a) Number of genes that are differentially methylated in any comparison and also show high variability across developmental stages. (b) UpSet plot showing in which comparison the number of genes that are both di_erentially methylated and also highly variable are found.

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