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Epigenetics in a honeybee hive

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Tese apresentada à Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo para obtenção do título de Doutor em Ciências.

Área de Concentração: Biologia Celular e Molecular

Orientador: Prof. Dr. Klaus Hartmann Hartfelder

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Resumo

CARDOSO-JÚNIOR, C. A. M. **Epigenética em coméias de abelhas.** 2020. 133f. Tese (Doutorado em Biologia Celular e Molecular) – Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, 2020.

Mecanismos epigenéticos desempenham um papel importante na expressão gênica alterando a estrutura da cromatina sem alterar a sequência do DNA. Os mecanismos melhores estudados são a metilação do DNA, modificações pós-traducionais nas histonas e RNAs não-codificantes. Este trabalho teve como objetivo explorar as funções desses mecanismos epigenéticos em diversos processos do ciclo de vida de abelhas adultas da espécie *Apis mellifera*. Primeiramente, estudamos o papel da metilação do DNA na longevidade de operárias e rainhas. Neste contexto, nós determinamos os efeitos de estímulos sociais, como feromônios e variações demográficas sazonais em colmeias de abelhas, na expressão de genes codificadores de enzimas que promovem modificações epigenéticas no DNA, RNA e histonas. Finalmente, investigamos como o gene codificador da DNA metiltransferase 3 (DNMT3), uma enzima chave na reprogramação da metilação do DNA, é regulada durante a maior transição da vida das operárias, nomeadamente, a transição entre o cuidado da cria e o forrageamento. Nossas análises da expressão dos genes das Dnmts e ensaios funcionais de suas atividades enzimáticas mostraram que a metilação do DNA está associada a longevidade de abelhas operárias, provavelmente envolvendo a regulação da vitelogenina, uma proteína que controla as taxas de maturação comportamental desta casta. Além disso, fatores ambientais (ex: feromônio da rainha e exposição à larvas ou adultos jovens) regulam a expressão de genes que codificam modificadores epigenéticos do DNA, RNA e histonas. Esses dados sugerem que reprogramações epigenéticas controlam a expressão gênica, permitindo adaptação a novos ambientes sociais. Uma segunda parte importante deste projeto gerou dados de metilomas através do sequenciamento de bissulfito para comparar genes diferencialmente metilados nos cérebros e ovários de operárias sujeitas a contextos sociais distintos. Interessantemente, e ao contrário do esperado, esses resultados revelaram poucas alterações na metilação do DNA em resposta a um novo contexto social, apesar de alterações significativas na expressão dos genes *Dnmt*. Além disso, os padrões de metilação em ovários e cérebros são quase idênticos, apesar das diferenças funcionais nesses tecidos, indicando também que é improvável que a metilação do DNA regule a expressão gênica em abelhas. Isso nos leva a concluir que a maquinaria de metilação do DNA possivelmente possuem outras funções, as quais não são diretamente associadas à metilação do DNA. De acordo com essa hipótese, análises *in silico* e dados de microscopia confocal mostraram a localização citoplasmática da proteína DNMT3, a qual foi encontrada predominantemente associada a vesículas lipídicas de células do corpo gorduroso.

Finalmente, descobrimos que o microRNA de abelhas *ame-miR-29b* é um regulador autêntico da expressão de *Dnmt3*, e essa função é conservada evolutivamente entre abelhas e mamíferos, incluindo humanos. Em conclusão, este estudo revelou um grau considerável e inesperado de complexidade nos papéis dos mecanismos epigenéticos e sua regulação da expressão gênica na vida social das abelhas.

Palavras chaves: Epigenética. Metilação do DNA. Envelhecimento. Abelhas. *Apis mellifera*.

Abstract

CARDOSO-JÚNIOR, C. A. M. **Epigenetics in a honeybee hive.** 2020. 133f. Thesis (Ph.D. in Cell and Molecular Biology) – Ribeirão Preto School of Medicine, University of São Paulo, Ribeirão Preto, 2020.

Epigenetic mechanisms play a major role in gene expression, altering the chromatin structure without changing the DNA sequence. The best studied epigenetic mechanisms are DNA methylation, histone post-translational modifications and non-coding RNAs. This work aimed to explore the functions of these epigenetic mechanisms in the context of several processes in the adult life cycle of the honeybee, *Apis mellifera*. Firstly, we studied the role of DNA methylation in the longevity of workers and queens. In this context, we determined the effects of social stimuli, such as the queen pheromone and the seasonal demographic variation in the beehive, on the expression of genes that code for enzymes that promote epigenetic alterations on the DNA, RNA and histones. Finally, we investigated how the gene encoding the DNA methyltransferase 3 (DNMT3), a key enzyme for the reprogramming of DNA methylation, is regulated during the major behavioural transition in a worker bee's life, namely, the transition from brood care to foraging. Our analyses of *Dnmt* genes expression and functional assays of their enzymatic activity showed that DNA methylation is associated with longevity in honeybee workers. This likely involves the regulation of vitellogenin, a protein that controls behavioural maturation rates in this caste. Moreover, environmental factors (e.g., queen pheromone and exposure to larvae or young adults) regulate the expression of genes that code for epigenetic modifiers of DNA, RNA and histones. These data suggest that epigenetic reprogramming controls gene expression, allowing adaptation to new social environments. In a second major project we generated methylome data by bisulfite sequencing for comparisons between differentially methylated genes in the brains and ovaries of workers subjected to distinct social contexts. Strikingly, and contrary to expectation, these results revealed only very few changes in DNA methylation in response to a new social context, despite significant alterations in the expression of *Dnmt* genes. Furthermore, the methylome patterns in ovaries and brains are almost identical, despite the functional differences for these tissues, thus also indicating that DNA methylation is unlikely to regulate honeybee gene expression. This led us to conclude that the DNA methylation machinery possibly displays other functions that are not directly associated with DNA methylation. In line with this hypothesis, *in silico* analyses and confocal microscopy data showed that a cytoplasmic localization of the DNMT3 protein is predominantly found associated with lipid vesicles. Finally, we found that the honeybee microRNA *ame-miR-29b* is a *bona-fide* regulator of *Dnmt3* gene expression, and this function is evolutionary conserved between honeybees and mammals, including humans. In conclusion, this study revealed a

considerable and unexpected degree of complexity in the roles of epigenetic mechanisms and their regulation of gene expression in a honeybee's social life.

Key words: Epigenetics. Aging. DNA methylation. Honey bee. *Apis mellifera*.

SUMMARY

1. Introduction

The term "Epigenetics" was firstly coined by the biologist Conrad H. Waddington in 1942. He defined epigenetics as changes in the phenotype without alterations in the genotype. Nowadays, the concept of epigenetics comprises a group of molecular mechanisms that act on the inheritance of gene expression patterns by adapting the chromatin structure [1]. Chromatin can be interpreted as the physiological part of our genetic information, which include the double-stranded DNA and its associated packaging proteins. Depending on the degree of association between the DNA, histones, and other chromatin-modifier proteins, the chromatin state can vary from high to low compaction, denoted as heterochromatin and euchromatin, respectively [2]. Heterochromatin regions are transcriptionally inactive regions where the nucleosomes, which are composed by different types of histones, are tightly associated with the DNA, blocking the access of proteins, especially transcription factors, that will regulate the transcription of specific genes [3]. Euchromatin regions, on the other hand, are transcriptionally active because of the low association between the regulatory regions of the DNA and nucleosomes [4].

Epigenetic modifications consist of a broad range of molecular programs that act at several levels of gene expression, such as DNA methylation, histone post-translational modifications, histone variants, RNA methylation, microRNAs and other non-coding RNAs, just to mention the major players [5]. These epigenetic mechanisms mostly control gene activity by altering the chromatin structure. Some epigenetic mechanisms, however, such as non-coding RNAs (e.g., microRNAs, piwiRNAs) act at posttranscriptional stages of gene expression [6]. Other epigenetic events, e.g., histone phosphorylation and histone variants, are not directly linked to gene expression, but act, for example, by maintaining the genomic architecture, such as genomic stability and chromosome segregation during cell division cycles (revised in [1,5]).

In this study I focused on classical epigenetic mechanisms and the role(s) they play in several social traits of the honeybee. Specifically, the roles of DNA methylation, histone modifier genes, and microRNAs were studied in different tissues and castes subjected to different social contexts that influence ageing, reproductive and behavioural processes. The main hypothesis of this work is that the high degree in honeybees makes, at least in part, use of the complex and efficient epigenetic mechanisms to flexibly regulate gene expression. Thus, epigenetic events were predicted to be associated with important aspects of the social lifestyle of honeybees, including pheromonal regulation of behavioural maturation of workers, as well as the remarkable differences of reproductive and longevity traits between queens and workers. In addition, we also studied the mechanisms that

control the expression of the DNA methyltransferase 3 (DNMT3) gene and its protein, given its postulated importance in regulating *de novo* DNA methylation.

In the following subsections, I will present an overview on these epigenetic mechanisms and how they canonically control gene expression in social insects or in other biological models. Detailed information of each of the studied mechanisms is provided in the following sections of this introduction. The experiments and results obtained in this thesis will then be presented in the form of five chapters, followed by a general conclusion summarizing the main take home messages of this study. Finally, a list of peer-reviewed articles published during the development of this work, or manuscripts under review, as well as publication not directly linked to epigenetics mechanisms in honeybees are presented as attachments to this thesis.

1.1 DNA methylation and honeybees

The DNA methylation is the best studied epigenetic mark [1]. This epigenetic mark refers to an inheritable and reversible chemical modification placed on the carbon 5 of cytosines in the context of CpG dinucleotides. DNA methylation is catalysed by DNA methyltransferase (Dnmt) enzymes, which transfer a methyl group from S-adenosylmethyonine to a cytosine [7]. Methionine is an essential amino acid precursor of S-adenosylmethyonine, so the uptake of methyl-donor molecules occurs strictly during feeding [8]. The DNMT1 is considered DNA methylation enzyme, as it promotes DNA methylation during replication, by coping the methylation pattern from the DNA mother strands to the newly synthetized DNA strands. In contrast, DNMT3 is responsible for creating new patterns of methylation due to its *de novo* catalytic activity [7]. DNMT2, also called Trdmt1, was initially classified as a DNA methyltransferase based on sequence similarity, but this enzyme is in fact an RNA methyltransferase, primarily targeting tRNAs [9].

The DNMT enzymes, and consequently DNA methylation, play essential roles during the development of several organisms [10,11]. For example, the knockout of either DNMT1, DNMT3A, or DNMT3B is lethal in several biological models, including human embryonic stem cells, mouse, and zebrafish [12–15]. On the other hand, the knockout of the DNMT2 did not cause deleterious phenotypes in *Drosophila melanogaster* [16], yet it did so in zebrafish, indicating species-specific activity [17]. The varying results obtained in DNMTs knockout experiments can be explained by the diverse functions of each of these enzymes. Whereas DNMT1 and DNMT3 are strongly associated with the generation and maintenance of DNA methylation [7], DNMT2 acts on tRNA substrates [9]. Under stress conditions it can also affect the biogenesis of microRNAs by regulating the cleavage of methylated-tRNA [18,19].

Epigenetic studies showed that the DNMTs are not sequence-specific as they are able to methylate the whole genome [1,7]. Mechanistically, however, we still do not fully understand how the DNMTs are delivered to specific genomic loci [20]. Therefore, depending on where the methylation mark is deposited, it will influence a diverse range of biological mechanisms, including the control of gene expression, genomic stability, repression of mobility elements of DNA, and also the X chromosome inactivation [1,21]. For example, the methylation at sites in promoter regions is known to repress gene expression by reducing the affinity between DNA and transcription factors, and the methylation of transposable elements is known to repress their activity. Regions with high densities of CpG nucleotides, called CpG islands, are often differentially methylated between normal and carcinogenic cells, indicating that this epigenetic mark might be of importance for several pathologies [22,23].

Much less understood is the methylation within gene bodies (intragenic methylation), and it has intrigued scientists since its discovery. Gene body methylation occurs in exons and introns, and is phylogenetically widespread among animals and plants [24,25], but there is no consensus yet on its role. In plants, for instance, it does not regulate gene expression, it is not a request for viability, and also does not affect transcriptional elongation [26–28]. In mammals, it has been shown that gene body methylation may influence gene expression, yet its underlying mechanistic aspects still remain unclear [29]. In the highly eusocial honeybees, gene body methylation at been suggested to regulate gene expression through alternative splicing [30].

The roles of DNA methylation in invertebrates is still poorly understood because the main genetic models, the fruit fly *D. melanogaster* and the nematode *Caenorhabditis elegans*, lack the principal components to epigenetically modify their DNA [31]. For this reason, social insects emerged as models for epigenetic studies, because they present an active epigenetic system and a fascinating repertory of social behaviour [32,33]. Honeybees were used to explore the genomic functions of gene body methylation because in its genome, methylation is found exclusively at gene bodies [34], thus facilitating the acquisition of data without the confounding effects from other methylated genomic compartments.

Honeybees are worldwide spread insects that are excellent to study DNA methylation and its roles on fascinating features of a social lifestyle, such as the presence of different castes (queens and workers), a haplodiploid system for sex determination, and complex social behaviours [33,35]. Furthermore, honeybee methylomes are much less complex than the ones of mammals, as only ~1% of the CpGs are methylated [34,36]. Methylation in honeybees is enriched at highly expressed genes, including house-keeping genes [34,37].

In *A. mellifera,* as in all Hymenoptera, sex is determined by a haplodiploid system, whereby unfertilized eggs give rise to males and fertilized eggs develop into female individuals. Honeybee societies are characterised by an elevated degree of social organization marked by the reproductive division of labour among its castes in the female sex, workers and queens. The developmental pathways underlying caste development are differentially activated by the diet received by the larvae. Queen-destined larvae are solely fed royal jelly, a secretion of the hypopharyngeal and mandibular glands in the head of workers that is rich in protein, lipid and carbohydrates [38]. On the other hand, worker-destined larvae receive worker jelly, a similar head gland secretion, yet less rich in carbohydrates, during the first three days of larval development. Subsequently they are fed a mixed diet consisting of worker jelly, pollen and honey [39,40].

Remarkable differences regarding reproductive, behavioural, physiological, and morphological aspects distinguish the two castes [39]. For example, queens are highly reproductive females that can lay up to 2000 eggs per day [39–41], while workers are facultatively sterile females that only lay unfertilized eggs in the absence of the queen [39,42]. Queens, through their mandibular gland pheromone, maintain the reproductive monopoly of a colony by supressing ovary activation in workers [42,43]. Interestingly, the knockdown of DNMT3 function by RNA interference in worker larvae resulted in a queen-like phenotype, thus restoring the biological effects of the royal jelly diet [44]. Differences were also found when contrasting the methylomes of queens and workers in both larval and adult stages [34,36]. Thus, DNA methylation is thought to play a major role in the development and function of the honeybee castes [32,34,36,44,45].

Another interesting aspect of eusociality is the absence of the "reproductive *vs.* longevity" trade-off, which is a hallmark in practically all solitarily living Bilateria. This trade-off predicts that the high energetic costs associated with reproduction result in a decline in life span [46,47]. Social insects are an exception to this rule, as for instance in the honeybee, the highly reproductive queens live almost 20 times more than non-reproductive workers. Ageing in honeybee workers is also fascinating, because it is associated with behavioural maturation, meaning that young worker specialize in in-hive activities, such as cleaning and attending and feeding the brood, whereas older workers perform more risky tasks outside the colony, including foraging and colony defence [39,48]. Interestingly, both behavioural maturation and ageing in honeybee workers can be delayed, accelerated, or even reversed, depending on the needs of the colony [48,49]. Behavioural maturation and ageing in workers is determined by intrinsic and extrinsic factors, including social clues, such as pheromones, that affect their behaviour by regulating gene expression, [50–52]. Importantly, DNA methylation patterns were found to be associated with the division of labour in honeybee workers [53], and these patterns were shown to be reversible depending on the task exhibited by the workers. This suggests that the dynamic

methylation of DNA plays an important role in the behavioural maturation and ageing in honeybee workers [53]. Yet, whether this dynamic in epigenetic marks is also associated with the chronological ageing and not only the behavioural maturation of honeybee workers remained to be elucidated.

These studies that showed altered patterns of DNA methylation comparing individuals of the different castes and worker bees displaying different social tasks were essentially correlational studies, and information from functional studies is still limited. A study showed that the RNA interference-mediated *Dnmt3*-knockdown affected the expression of 17% of the honeybee genes [30]. Nonetheless, a reanalysis of the data published in this study showed that only a very small fraction of the honeybee transcriptome is in fact likely to be regulated by the DNMT3 activity [54]. The problem of a clear functional role for gene body methylation in honeybees, as well as in other social insects, became apparent in a study that compared the methylomes of reproductive and non-reproductive clonal raider ants, a species that reproduce asexually and, therefore, allows to design experiments without the confounding effects of genetic variants [55]. This study showed that the link between DNA methylation and social function is actually in this ant [55], and possibly so also in other social insects, contradicting to a certain extent the previous reports for honeybees [36]. A possible explanation for these contrasting results may be that DNA methylation is in fact genotype-specific in these social insects [55,56]. Thus, the role of gene body methylation in the honeybee and, more broadly, in other social insect genomes, requires further studies.

1.2. Histone post-translational modifications

Histones play essential roles in the life of eukaryotes by packing the large strands of DNA into the small nucleus in a functional fashion. This involves the winding of double-stranded DNA around nucleosomes. Nucleosomes are a multiproteic complexes composed of two molecules of each of the following histones: H2A, H2B, H3 and H4, while H1 forms a bridge between two neighbouring nucleosomes, allowing the strong compaction of chromatin [57]. Covalent post-translational modifications can occur on the unfolded N-terminal tails of nucleosome proteins, and these can have a major role in gene expression regulation [58]. Epigenetic modifications of histones regulate gene expression by promoting either local chromatin remodelling (e.g. cis-regulatory events) or signalling to promote distant epigenetic regulation (e.g. trans-regulatory events) [59]. Both, cis and trans-regulatory events are determined by a serious of epigenetic phenomena, including the actual epigenetic mark, the modified residue on histone tails and the cross-talk with other epigenetic events (e.g. DNA methylation, non-coding RNAs), constituting thus an epigenetic code that integrates several epigenetic events to regulate gene expression

How histone post-translational modifications (HPTMs) can affect nucleosome positioning and, in consequence transcription, was extensively studied in the context of the formation and expansion of heterochromatin in *D. melanogaster*. It was shown that gene expression is regulated by HPTMs, explaining the long-known phenomenon of "position-effect variegation". This phenomenon was first described by Hermann J. Muller in the white-mottled-4 (w^{m4}) Drosophila mutant [60]. This mutant showed eye colour variegation due to the juxtaposition of genes for eye pigmentation next to heterochromatin domains by chromosomal rearrangement or transposition, resulting in gene silencing. Heterochromatin extension occurs via propagation of epigenetic elements along chromosomes [3].

Histone methylation occurs in lysine and arginine residues in the H3 tails and also in related proteins, for instance SU(VAR)3-9 and HP1 [3,61]. It causes transcription activation or repression, depending on the amino acid modified and the degree of modification, which can be found at three degrees, as mono-, di-, or three-methylation. Each type of histone methylation results in a restructured chromatin in either direction, i.e. from euchromatin to heterochromatin and *vice versa*. Histone methylation is also influenced by the neighbouring epigenetic marks on DNA and adjacent nucleosomes. With such an elaborated system of epigenetic information, the existence of an epigenetic code has been proposed that coordinates gene expression. Therefore, chromatin remodelling is likely the result of a crosstalk between several histone and other epigenetic marks, including DNA methylation, non-coding RNAs and activation of RNA Polymerase II.

Because of the complex association between DNA and methylated histones, this epigenetic mark did not receive much attention when it was discovered [62]. By that time, histone acetylation received more attention because this epigenetic mark is directly linked to activation of gene expression. Acetylation neutralizes positive charges on the histone tails. This reduces the affinity between the DNA and nucleosomes, leading to chromatin opening and consequently facilitating transcription [1,58]. The direct link between gene transcription and this epigenetic modification led to the discovery of the machineries involved in reversibly modifications of histone residues. These can be classified as writers and erasers, or in other terms histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively. HATs are responsible for adding acetyl groups to lysine residues on histone tails, whereas HDACs remove these groups [63,64]. Later another class of epigenetic modifier proteins was found, the readers, which bind to modified residues and promote chromatin remodelling. Interestingly, a class of well-characterized NAD^+-dependent HDACs enzymes called Sirtuins is known to play a major role in the longevity of several animal model organisms, including *D. melanogaster* and *C. elegans* [65]. Studies in honeybees on Sirtuins and HPTMs in general are still scarce (but see [66,67]). However, the identification of several histone marks already suggests that the

epigenetic regulatory role of chromatin structure is conserved in honeybees [68]. Notably, histone acetylation was recently shown to affect female caste bias in larval development of honeybee queens and workers [69].

Although histone methylation and acetylation are the better characterized HPTMs, these are not the only events. Rather, histones can be modified by a broad range of epigenetic modifications that all affect gene expression, including phosphorylation, ubiquitination, and sumoylation [58]. For example, histone sumoylation can recruit HDACs that then induce transcriptional repression [70,71]. Sumoylation occurs by the transfer of small ubiquitin-like modifier (SUMO) polypeptides to lysine residues of histones and other substrates, and they play critical roles in several physiological disorders [72]. Interestingly, some epigenetic marks on histones are not directly associated with transcription [59]. For instance, histone ubiquitination and phosphorylation are associated with DNA-related processes in a transcription-independent manner, as they regulate biological processes involved in DNA damage responses, chromosomal integrity, and cell cycle progression [73,74].

This said, HPTMs should represent interesting candidates for controlling gene expression in different contexts of the honeybee's social lifestyle. Nonetheless, little is known yet about the histone modifications that regulate gene activity and the proteins/genes associated with these epigenetic mechanisms in honeybees. In Chapter III of this thesis I report on results concerning the annotation of HPTMs genes and investigations into the role of these genes in the context of the response to queen mandibular pheromone, which plays a major role in regulating worker behavioural maturation, ageing, and reproduction.

1.3. MicroRNAs and other non-coding RNAs

Non-coding RNAs (nc-RNAs) comprise a diverse class of molecules that can be characterized by their function, size, or the biological processes in which they participate. These ncRNAs include piwi-RNAs, small-interference RNAs, small nucleolar RNAs, centrosome and telomerase small RNAs, ribosomal RNAs, and long ncRNAs [75]. A well-characterized ncRNA is the long-ncRNA Xist. Xist promotes the X-chromosomal dose compensation in mammals by inactivating one of the X-chromosomes in female tissues [76]. However, the probably best known nc-RNAs are the ribosomal RNAs that are indispensable for life maintenance in all the major branches of life, Eukaryota, Archaea and Bacteria (revised in [77]). Similarly well-known are tRNAs, which are key molecules for translating mRNA information into functional proteins. Recent findings, however, showed that the function of tRNAs is not restricted to translation, as they can also be signalling molecules. For instance, epigenetic modification of tRNA nucleotides (e.g., RNA methylation) was shown to be relevant in

several cellular process, including epigenetic memory, biogenesis of microRNAs, response to stress, translation stability, bacterial drug-resistance, and also ageing [18,19,78–81].

Although RNA methylation has not been studied in honeybees so far, other epigenetic mechanisms associated with ncRNAs are better understood. MicroRNAs, a class of short ncRNAs with an average of ~22 nucleotides in length, have revolutionized our understanding of molecular biology since their discovery in 1993 in the worm *C. elegans* [82,83]. MicroRNAs act in the cytoplasm by targeting the 3' untranslated region of mRNAs. This disrupts translation or induces mRNA cleavage, leading to transcriptional silencing. MicroRNAs are transcribed as pri-miRNA, which have a hairpin structure and are recognized by the Drosha and spliceosome proteins, converting pri-miRNAs into pre-miRNAs. After exportation to the cytoplasm by the exportin-5 protein, pre-miRNAs are recognized by Dicer or Argonaut proteins that transform them into mature, functional microRNAs, which are able to bind to their mRNA targets. Nonetheless, the short length of microRNAs makes them to a certain degree promiscuous, i.e., they may target a more or less broad range of different mRNA populations (revised in [6]).

MicroRNAs play important roles in honeybees. MicroRNAs are differentially expressed between queen and worker-destined larvae [84]. Plant microRNAs present in the food of queen and worker larvae were shown to be functional against caste-specific mRNAs of these larvae, revealing an interesting regulatory module involving both plant and bee-derived RNA molecules interacting in the caste determination process [85]. MicroRNAs are also likely to play a role on the regulation of ovary activation in adult workers [86], which are suppressed by the queen and larval pheromones [43,87,88].

Honeybee microRNAs are intensively studied, including by research groups in Ribeirão Preto [86,89,90], and using bioinformatics tools, we set a focus on the *ame-miR-29b*, an evolutionary ancient microRNA from the family 29 that canonically regulates the expression of *Dnmts* in mammals. Using correlational and functional approaches, we found evidence for an interaction between *ame-miR-29b* and *Dnmt3* (see Chapter IV). This link actually also motivated our search for other epigenetic events that might control *Dnmt3* expression underlying ageing in honeybee workers.

Aims

2. Aims

The general aim of this project was to shed light on the role of epigenetic modification, especially DNA methylation, in the social context of honeybees. For this, four specific aims were defined:

2.1 Determine whether DNA methylation plays a role in the chronological aging of honeybee workers:

2.1.1 Quantify the expression of DNMTs encoding genes in different body compartments of honeybee workers with different ages;

2.1.2. Determine the pharmacological effects of RG108, a DNMT inhibitor, on: (i) survival rates, (ii) expression of aging-associated genes, (iii) modulation of juvenile hormone levels;

2.1.3. Determine the relationship of aging regulators (e.g, vitellogenin, TOR) and the expression of DNMTs encoding genes expression;

2.2 Quantify the transcription levels of DNMT genes and histone modifiers genes in workers subjected to different social environments, such as the presence/absence of young workers, brood, or the queen;

2.3 Investigate the regulation of *Dnmt3* gene expression and its protein:

2.3.1 Quantify the mRNA levels of *Dnmt3* in honeybee nurses and foragers from different colonies;

2.3.2 Identify and validate whether and how the *ame-miR-29b* regulates the expression of *Dnmt3;*

2.3.3 Immunolocalize the DNMT3 protein in the fat bodies of nurses and foragers by immunofluorescence laser confocal microscopy;

2.3.4 Determine the subcellular localization of DNMT3 protein by immunogold labelling in the fat body of forager bees;

2.3.4 Perform *in silico* analyses on the DNMT3 amino acid sequence and its predicted three-dimensional structure;

2.4 Determine the effects of social manipulation in the expression and methylation patterns of genes in worker honeybees:

2.4.1 Perform whole genome bisulfite sequencing on brain and ovary samples of workers from queenright and queenless colonies;

2.4.2. Assess the effects of the queen's presence/absence on the methylome of worker brains and ovaries;

2.4.3 Compare the methylomes of individuals from different colonies to determine possible colony effects on the methylation patterns;

2.4.4 Identify and compare differently methylated regions between brains and ovaries;

2.4.5 Quantify the expression of differentially methylated genes with respect to social status, colony and tissue.

Results, Discussion and Material and Methods

3. Results, Discussion and Material and Methods

Chapter I

3.1 Chapter I

This chapter refers to the following article:

Title: DNA methylation affects the lifespan of honey bee (*Apis mellifera* L.) workers – evidences for a regulatory module that involves vitellogenin expression but is independent of juvenile hormone function

Authors: Carlos A. M. Cardoso-Júnior, Karina R. Guidugli-Lazzarini, Klaus H. Hartfelder

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DNA methylation affects the lifespan of honey bee (*Apis mellifera* L.) workers – Evidence for a regulatory module that involves vitellogenin expression but is independent of juvenile hormone function

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ABSTRACT

The canonic regulatory module for lifespan of honey bee (*Apis mellifera*) workers involves a mutual repressor relationship between juvenile hormone (JH) and vitellogenin (Vg). Compared to vertebrates, however, little is known about a possible role of epigenetic factors. The full genomic repertoire of DNA methyltransferases (DNMTs) makes the honey bee an attractive emergent model for studying the role of epigenetics in the aging process of invertebrates, and especially so in social insects. We first quantified the transcript levels of the four DNMTs encoding genes in the head thorax and abdomens of workers of different age, showing that *dnmt1a* and *dnmt3* expression is up-regulated in abdomens of old workers, whereas *dnmt1b* and *dnmt2* are down-regulated in heads of old workers. Pharmacological genome demethylation by RG108 treatment caused an increase in worker lifespan. Next, we showed that the genomic DNA methylation status indirectly affects vitellogenin gene expression both *in vitro* and *in vivo* in young workers, and that this occurs independent of caloric restriction or JH levels, suggesting that a non-canonical circuitry may be acting in parallel with the JH/Vg module to regulate the adult life cycle of honey bee workers. Our data provide evidence that epigenetic factors play a role in regulatory networks associated with complex life history traits of a social insect.

1. Introduction

DNA methylation by chemical modification of cytosines on CpG dinucleotide sites has been suggested as an important factor in gene regulatory networks involved in aging, with strong evidence coming from methylome analyses that contrasted human newborns and centenarians (Heyn et al., 2012). Nonetheless, the full understanding on how DNA methylation impacts on aging remains far from conclusive, especially since the best studied animal models for aging, *Caenorhabditis elegans* and *Drosophila melanogaster*, lack important components of the DNA methylation machinery. In the absence of DNA methyltransferase (DNMT) enzymes, *C. elegans* completely lacks CpG methylation (Gabor Miklos and Maleszka, 2011), and in *D. melanogaster*, which has only a *dnmt2* ortholog, but no homologs of the *dnmt1* and *dnmt3* genes in its genome, DNA methylation is restricted to only few CA- and CT-rich loci in certain developmental contexts (Kunert et al., 2003; Takayama et al., 2014).

Having a complete repertoire of DNMTs encoded in its genome (Wang et al., 2006) the honey bee, *Apis mellifera*, has emerged as an interesting model for investigating the role of DNA methylation in invertebrates. Since CpG methylation is largely restricted to gene bodies, and is not widespread over promoter regions, identifying and connecting DNA methylation with specific gene functions, such as alternative splicing, could thus be a simpler task than is the case with mammals (Lyko et al., 2010; Wang et al., 2006). Furthermore, the phenotypic plasticity exhibited in the adult females, which includes drastic morphological and functional caste differences associated with reproduction and longevity, also shows great potential for studies on aging and senescence (Cash et al., 2005; Withers et al., 1993).

Adult honey bee workers typically last only 30–45 days, but can live for up to six month as winter (*diutinus*) bees in temperate climates, allowing colonies to survive through a long winter season (Page and Peng, 2001; Winston, 1987). This variability in worker lifespan has been shown to be associated with their flight activity,

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essentially with the transition from intranidal tasks, especially brood care, to the more risky tasks of foraging. This major transition in the life cycle of an adult worker involves both dietary and physiological changes, including the switch from a carbohydrate/protein to a pure carbohydrate diet, associated with a drop in hemolymph vitellogenin (Vg) levels and an increase in the hemolymph juvenile hormone (JH) titer (Hartfelder and Engels, 1998). This physiological transition, which promotes foraging behavior, is conceptually formulated as a dual repressor model (Amdam et al., 2005; Amdam and Omholt, 2002), wherein high Vg levels in young workers suppress JH synthesis, and consequently maintain a basal JH titer (Guidugli et al., 2005). With the transition to foraging, the JH levels increase and suppress *vg* gene expression (Elekonich et al., 2001). This mutually repressive Vg/JH association in honey bees, which is a novelty in insects, has conceptionally been associated with the apparent disruption of the reproduction/lifespan trade-off, a paradigm that permeates life history theory of animals (Flatt et al., 2013; Rodrigues and Flatt, 2016).

More recently, another important life history link has been evidenced, a link between JH and DNA methylation that affects honey bee caste development during the larval stages. (Foret et al., 2012; Kucharski et al., 2008) have shown by RNA interference that the knockdown of *dnmt3* gene function affected the size of the *corpora allata*, the glands that produce JH, and induced a queen-like phenotype, especially so in the ovary. Subsequently, Foret et al. (2012) demonstrated that genes of the JH signaling pathway were differentially methylated in queen and worker larvae. Hence, a question that emerged was whether DNA methylation might also play a role in the already well-established Vg/JH regulatory circuitry that underlies the behavioral transition in adult honey bee workers.

We investigated this by monitoring the expression of the DNMT genes in different age classes of honey bee workers, and we could show that *dnmt3* transcript levels in the abdomen are positively associated with age. Next, by treating adult workers with RG108 we pharmacologically inhibited DNMT activity. Such a reduction in global DNA methylation resulted in an increase in worker longevity. This lifespan increase was also found associated with an increase in *vg* expression, but without affecting JH levels in hemolymph. Based on these data we propose a non-canonical circuitry involving DNA methylation and Vg, as well as other not yet characterized components. We consider that this circuitry acts in parallel with the already well-established Vg/JH module that regulates life history plasticity in honey bees.

2. Material and methods

2.1. Bees

Workers of Africanized *A. mellifera* hybrids were obtained from the Experimental Apiary of the University of São Paulo at Ribeirão Preto, Brazil. Brood frames containing ready-to-emerge worker brood were kept in an incubator (34 °C) and checked daily for bees that had emerged within a 1–20 h interval. These bees, which were considered 0-day-old, were paint-marked and re-introduced into the colony of origin, and were sampled once they had reached the desired age (1 day, 3 days, 1 week, or 1 month).

2.2. RNA extraction and quantitative analysis of transcript levels

Upon sampling, the bees were immediately anesthetized on ice and the head, thoracic and abdominal body segments were separated. These were then directly transferred into TRIzol reagent (Invitrogen) and snap frozen for storage at −80 °C. In all experiments, the stinger and gut were removed from the abdomens. For each age class, five individual replicates were prepared. RNA extraction was done using a TRIzol (Invitrogen, Carlsbad, CA) protocol following the manufacturer's instructions, and 8 μL of each RNA sample was treated with RNase-free DNase (Invitrogen). RNA concentrations and quality were measured spectrophotometrically (NanoView Plus; GE Healthcare Life Sciences, Pittsburgh, PA). First-strand cDNA synthesis was done using 2 μg aliquots of DNase-treated RNA, Oligo(dT)$_{12\text{-}18}$ primer (Invitrogen) and SuperScript II Reverse Transcriptase (Invitrogen). The obtained cDNA was then 10x diluted in deionized (Direct-Q, Millipore) water.

Quantitative PCR (RT-qPCR) assays were carried out with 1 μL of diluted cDNA, 5 μL of SYBR Green Power Master Mix (Invitrogen), 1.25 pmol of each specific primer (Table S1 – Supplementary Material) and water to complete the 10 μL final volume. The assays were run in a StepOnePlus system (Applied Biosystems, Foster City, CA) with three technical replicates per biological replicate under the following conditions: 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. After each assay, a melting curve analysis was run to monitor product specificity. The detection threshold was adjusted manually for each primer set. Relative expression values were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The expression of the following genes was assessed: *dnmt1a*, *dnmt1b*, *dnmt2*, *dnmt3*, *vitellogenin*, *foxo*, *krüppel-homolog 1* and *tor*. The ribosomal protein encoding gene *rp49* (also known as *rpl32*) was used in all RT-qPCR reactions as endogenous control for normalization; the gene has previously been validated for use in honey bee RT-qPCR assays (Lourenço et al., 2008). Primer efficiency was calculated as E = 10[−1/slope], based on a 1 : 10 dilution series of a cDNA sample. An internal sample of each primer was used for calibration of the entire group. For further information, see Table S2 (Supplementary material).

2.3. RG108-treatment of bees: survival and food intake

In a pilot experiment, 1 μL of a 0.2 mM solution of the DNA methylation-inhibiting reagent RG108 (Cayman Chemicals, Ann Arbor MI) diluted in DMSO (Merck, Darmstadt, Germany) was topically applied on the thorax of 50 newly emerged workers. Fifty control bees were treated with DMSO only. RG108 was chosen for this experiment due to its slow decay rate and proven effectiveness with honey bees (Biergans et al., 2015; Brueckner et al., 2005), including topical treatment experiments on the thorax (Lockett et al., 2010). Following treatment, the bees were kept in 8 × 11 × 13 cm cages in queenless condition in an incubator (34 °C, 70% RH). They received sucrose (50%) and water *ad libitum*. Subsequently, the experiment was repeated four times with an increased number of bees per cage (n = 80). In these experiments, we also quantified food intake by weighing the sucrose solution remaining in the feeding tube after each 24 h interval and dividing total consumption by the number of alive bees.

2.4. In vitro and in vivo effects of RG108 on aging-related gene expression, vitellogenin and JH levels

To test whether DNA methylation affects the expression of aging-related candidate genes, we quantified the expression of vitellogenin (*vg*), forkhead box O (*foxo*) and target-of-rapamycin (*tor*), as well as the JH-response gene krüppel-homolog 1 (*kr-h1*). For the *in vitro* assays, five abdomens from 3-day-old workers were each cut in half and incubated for 6 h in Grace's insect medium (Sigma-Aldrich, St. Louis, MS) supplemented with 0.2 mM of RG108 in DMSO (experimental group) or DMSO only (control group), in a pairwise test design. RNA extraction, cDNA synthesis and RT-qPCR assays were done as described above.

Since *vg* expression was significantly affected by RCG108

treatment *in vitro*, we also analyzed its expression *in vivo*. For this we topically applied RG108 on the thorax of newly emerged bees. The bees were fed with beebread (30% of pollen collected from brood combs mixed with 50% of sucrose and water) to stimulate fat body protein synthesis activity. Bees were sampled at 1 (n = 5) and 3 days of age (n = 13–14), respectively, and RNA was extracted for RT-qPCR analysis. Since *vg* expression was higher on day 3, we also analyzed the Vg protein concentration in hemolymph of RG108-treated and control bees at the same day (n = 4). Proteins of 0.5 μL hemolymph samples were separated by SDS-PAGE (7,5%), followed by electrophoretic transfer onto nitrocellulose membrane (Hybond ECL 0.2 μM, GE Healthcare Life Sciences). The membranes were blocked with 5% skim-milk/PBS (w/v) for 1 h, and washed once before and three times after antibody incubations. A primary antibody against honey bee vitellogenin (gift by Dr. Zilá L.P. Simões) was diluted 1:20,000, the secondary antibody (ECL anti-rabbit IgG-HRP; GE Healthcare Life Sciences) was diluted 1:10,000 in 25% of skim-milk/PBS (w/v). Immunodetection of Vg was done with ECL reagents (GE Healthcare Life Sciences) and Vg band densities of four individual bees were quantified by ImageJ software 1.47v. A second 0.5 μL hemolymph aliquot of the same samples was separated by SDS-PAGE under the same conditions and the 220 kDa apolipoprotein 1 band revealed after staining with Coomassie Blue (R250) was quantified. Apolipoprotein 1 expression is stable and frequently used for normalization of protein loading of bee hemolymph samples (Guidugli et al., 2005).

At days 1, 3 and 7, five bees were randomly collected from each of the cages (control and RG108-treated bees). They were briefly anesthetized on ice and hemolymph was sampled from an incision in the abdomen for quantification of the JH titer. This was done using a standardized JH radioimmunoassay (Hartfelder et al., 2013).

2.5. Quantification of global DNA methylation

Genomic DNA extracts (n = 3–5) from abdomens of 3-day-old workers were prepared with a Wizard® Genomic DNA Purification Kit (Promega, Madison, USA) following the manufacturer's standard protocol. The quantification of total DNA methylation was assessed by the EIA DNA methylation Kit (Cayman Chemical, Michigan, USA) with few modifications. Briefly, 500 ng of total DNA were treated with DNase (Invitrogen) and with 1 U of alkaline phosphatase (Affymetrix USB, USA) for 30 min at 37 °C. The assay volume was adjusted to 50 μL and the samples were incubated in pre-coated wells in technical duplicates for 18 h at 4 °C with the kit reagents. The plates were read at 405 nm at 60 min after Ellman's Reagent (developer) was added. The analyses were done as recommended by the manufacturer.

2.6. Methoprene treatment

Abdomens (n = 5) dissected from 3-day-old honey bee workers had the gut and stinger removed and were cultured *in vitro* for 3 h in Grace's insect medium (Sigma) containing methoprene (Zoecon) at a final concentration of 10^{-5} mM. RT-qPCR assays were done to quantify *dnmt1a* and *dnmt3* transcript levels. The efficiency of the methoprene treatment was validated by monitoring the expression of the JH-response gene *kr-h1* (Belles and Santos, 2014; Minakuchi et al., 2009).

2.7. Statistical analysis

Normality of the results of all assays was checked using the Kolmogorov-Smirnov test with a 95% confidence limit on log10 transformed data. Data that passed this test were analyzed with parametric tests (Student's *t*-tests or ANOVA). Data that were not normally distributed were analyzed by appropriate nonparametric tests. Detailed information on each test and sample sizes is given in the results section and/or in the respective figure legends. For all analyses, a $p < 0.05$ was considered statistically significant. All analyses were done using GraphPad Prism 5.0 software.

3. Results

3.1. Transcript levels of DNMT genes vary with age and differ among body compartments

Transcript levels of all four DNMTs encoded in the *A. mellifera* genome were quantified by RT-qPCR in the head, thorax and abdominal compartments of workers of three different age classes: 1-day-old workers (WD), 1-week-old workers (WW) and 1-month-old workers (WM) (Fig. 1). We found that *dnmt1a* transcript levels were lower in heads and abdomens of 1-week-old workers (WW) (ANOVA - head $F_{(2, 12)} = 9.509$, $p < 0.01$, N = 5; abdomen $F_{(2, 11)} = 5.480$, $p < 0.05$, N = 4–5), whereas *dnmt1b* and *dnmt2* gene expression was diminished in heads of 1-week and 1-month-old workers (*dnmt1b* one-way ANOVA, $F_{(2,11)} = 17.51$, $p < 0.001$, N = 4–5; *dnmt2* one-way ANOVA, $F_{(2,11)} = 9.087$, $p < 0.01$, N = 4–5). For *dnmt3*, the expression was markedly increased in abdomens of 1-month-old workers (one-way ANOVA, $F_{(2,10)} = 8.130$, $p < 0.01$, N = 3–5). Thus, the results revealed clearly distinct expression patterns for the four DNMTs encoding genes in relation to worker age and with respect to body compartment.

3.2. Genomic demethylation extends the lifespan of honey bee workers

Based on these results we then asked whether and how a change in global genome methylation status would affect honey bee lifespan. We tested this by topically treating newly emerged honey bee workers with the methylation inhibitor RG108 and recording their survival rate. We found that bees treated with RG108 had a significantly increased lifespan compared to solvent (DMSO) controls (Fig. 2A, Log-rank test, $p < 0.05$). We repeated this experiment four times, and in two of these experiments (Supplementary Material Figs. S1A and C), RG108-mediated genome demethylation had the same effect, resulting in increased worker bee lifespan (Log-rank test, $p < 0.05$).

The RG108-induced reduction in global levels of DNA methylation was confirmed by an ELISA-based methylation assay done on abdomens of caged workers (Fig. 2B, Mann-Whitney test, U = 1.0, $p = 0.0357$). Interestingly, we could also show that the transcript levels of all four *dnmt* genes were upregulated after *in vitro* RG108 treatment (Supplemental Material Fig. S2, *dnmt1a* - unpaired Student's *t*-test, $t = 3.497$ $df = 10$, $p < 0.01$; N = 6; *dnmt1b* - unpaired Student's *t*-test, $t = 3.294$ $df = 10$, $p < 0.01$, N = 6; *dnmt2* - unpaired Student's *t*-test, $t = 2.592$ $df = 10$, $p < 0.05$, N = 6; *dnmt3* - unpaired Student's *t*-test, $t = 3.197$ $df = 10$, $p < 0.01$, N = 6), indicating a possible feedback loop triggered by RG108, similar to previous data reported for *dnmt3* expression in honey bee brains (Biergans et al., 2015).

3.3. RG108 treatment targets the expression of aging-related genes, but JH or vitellogenin down-regulation did not affect the expression of DNMT genes

The effect of RG108 on lifespan extension in workers made us ask: (i) whether this could have been due to an indirect effect on known lifespan-affecting factors in bees, such as caloric intake or JH levels; (ii) whether RG108 treatment may have affected the expression of aging-related genes; and (iii) whether there was an

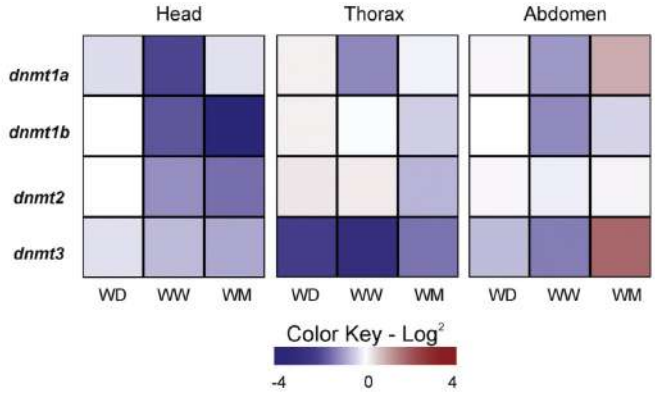

Fig. 1. DNMT gene expression levels in honey bee workers in relation to age. The transcript levels of *dnmt1a*, *dnmt1b*, *dnmt2* and *dnmt3* genes were quantified by RT-qPCR in head, thorax and abdominal tissue of workers of different age classes (WD, 1-day-old worker; WW, 1-week-old worker; WM, 1-month-old worker). The color key shows the $\log^2$ transformed data. See results section for statistical analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

effect of JH and/or Vg on the expression levels of *dnmt1a* or *dnmt3* in abdomens. Expression of the *dnmt1b* and *dnmt2* genes was not assessed because their transcript levels in abdomens did not change significantly with age (Fig. 1).

Caloric restriction is known to be an important factor for lifespan extension in *C. elegans*, *D. melanogaster* and mammals (revised in (Mair and Dillin, 2008; Speakman and Mitchell, 2011)), and a study on honey bees suggests that it can be potentially associated with lifespan in a DNA methylation-dependent mechanism (Rascon et al., 2012). Hence, we hypothesized that the RG108 treatment of newly emerged bees might have affected their feeding behavior. Upon measuring food consumption of the caged bee we found that this was not altered in the treated compared to the control group (Fig. 2C, Two-Way ANOVA, treatment – $F_{(1, 116)} = 0.08474$, $p > 0.05$), and thus we infer that the RG108-effect on lifespan was not related to a caloric restriction phenotype.

The next hypothesis was that the RG108 treatment could have had an inhibitory effect on JH biosynthesis. Since JH and Vg are configured as a mutually inhibitory module in honey bee lifespan regulation (Amdam et al., 2005; Amdam and Omholt, 2002), we used an established radioimmunoassay to measure the JH hemolymph titer in 1, 3 and 7-day-old RG108-treated and control bees (Fig. 2D–F). As we did not find any evidence for differences in JH levels these bees (day 1 - unpaired Student's *t*-test, $t = 0.4195$ $df = 8$, $p = 0.3429$; day 3 – unpaired Student's *t*-test, $t = 0.7668$ $df = 8$, $p = 0.2326$; day 7 = Mann Whitney, $U = 9.0$, $p = 0.4346$), the endocrine system function, at least as far as JH goes, was apparently not affected by the genome demethylation reagent RG108.

To further investigate this apparent lack of connection between JH function and DNA methylation at the beginning of a bee's adult life, we asked JH whether may act as an upstream element in regulating DNMT gene expression, and not as a downstream element as tested in the previous assay (Fig. 2D–F). To test this, we performed the reverse experiment where we incubated abdomens of 3-day-old workers in the presence of a 10^{-5} M final concentration of the JH analog methoprene and then analyzed the *dnmt1a* and *dnmt3* expression levels (Supplementary Material Fig. S3; *dnmt1a* - unpaired Student's *t*-test, $t = 1.159$ $df = 8$, $p = 0.14$; *dnmt3* - unpaired Student's *t*-test, $t = 0,6663$ $df = 8$, $p = 0.2620$). These results provided additional evidence for the non-existence of a direct link between JH and DNA methylation, at least not at the beginning of a worker's adult life cycle.

Finally, to obtain insights into the regulatory mechanisms triggered by DNA demethylation, we performed an *in vitro* experiment where we incubated abdomens of 3-day-old workers with RG108 or solvent (control) for six hours and analyzed the transcript levels of four candidate genes: vitellogenin (*vg*), forkhead box O (*foxo*) and target-of-rapamycin (*tor*), knowingly related to the aging process in bees (Ament et al., 2008; Corona et al., 2007; Hsu et al., 2014; Marco Antonio et al., 2008; Mazucanti et al., 2015; Munch and Amdam, 2010), as well as the JH-response gene krüppel-homolog 1 (*kr-h1*). We found that only the expression of *vg* (Fig. 3A, Wilcoxon matched-pairs signed rank test, $W = -15$, $p = 0.0313$) and *foxo* (Supplementary Material Fig. S4A, paired Student's *t*-test, $t = 2.515$, $df = 4$, $p = 0.0328$) were significantly affected by this treatment. Furthermore, and in accordance with our previous results, the expression of *kr-h1*, a primary target of JH, was not affected by the RG108-treatment (Supplementary Material Fig. S4B, paired Student's *t*-test, $t = 1449$ $df = 4$, $p = 0.1105$). This result reinforces our hypothesis that DNA methylation is not related with JH function in young adult workers.

Since *vg* expression is the best-studied gene in the context of aging in honey bees (Corona et al., 2007; Marco Antonio et al., 2008), we decided to focus our further efforts on the possible regulatory relationship of this gene with DNA methylation. First, to test whether the effect on *vg* expression seen *in vitro* would also occur *in vivo*, we quantified the levels of *vg* mRNA in abdomens of 1- and 3-day-old bees that had been treated with RG108. Since the mean *vg* expression levels were higher at both time points (Fig. 3B), but were only statistically significant in 3-day-old workers (day 1: unpaired Student's *t*-test, $t = 0.9303$, $df = 8$, $p = 0.1897$; day 3: unpaired Student's *t*-test, $t = 2.29$, $df = 25$, $p = 0.0154$), we performed a western blot analysis for vitellogenin protein levels in hemolymph of 3-day-old bees (Fig. 3C). This showed that the Vg band was indeed more prominent in RG108-treated bees.

Finally, we asked whether the Vg protein itself may be part of the regulatory circuitry that modulates DNMT activity, similar to the JH/Vg dual repressor module. To answer this, we assayed *dnmt1a* and *dnmt3* gene expression in fat body samples of 1-week-old bees that had previously been silenced for *vg* gene function by

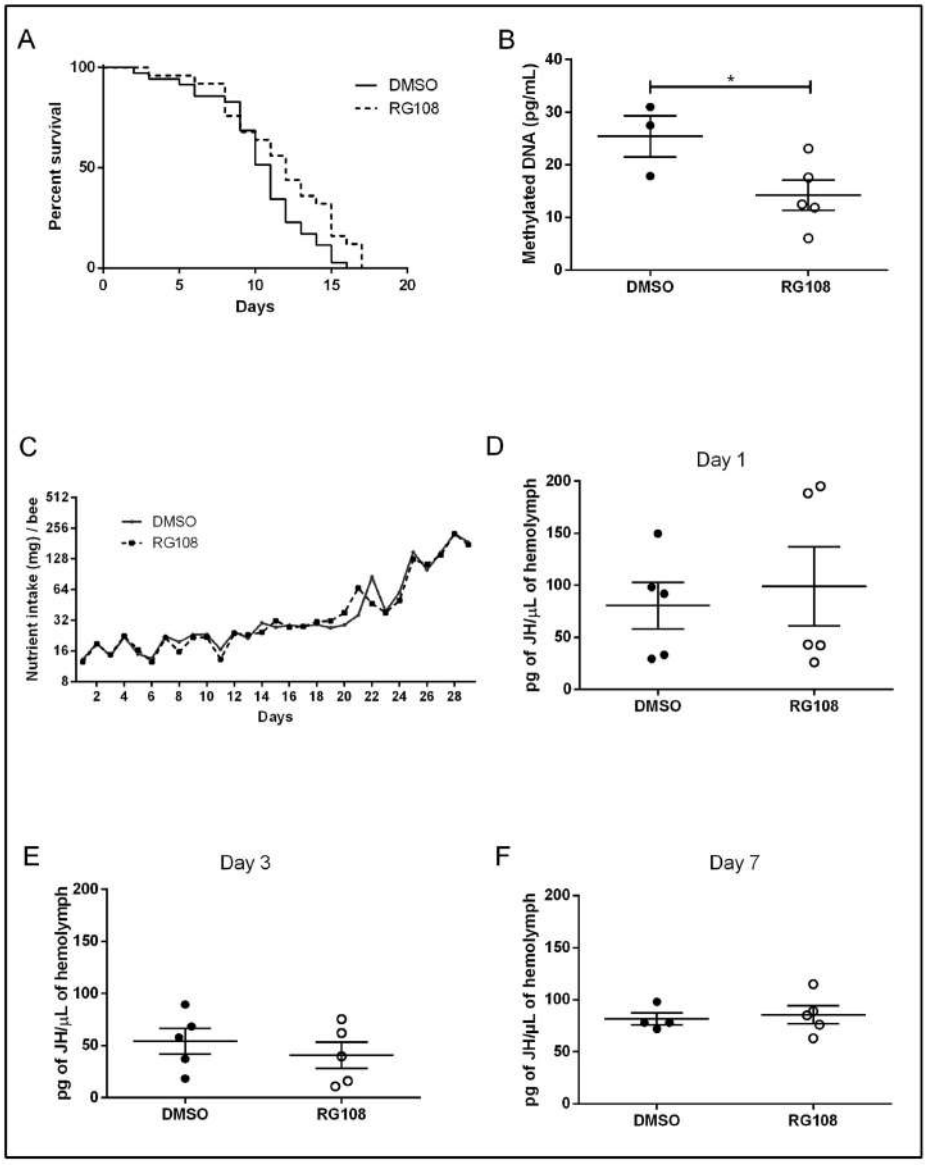

Fig. 2. Effects of the pharmacological (RG108) inhibition of DNA methylation on survival rate, feeding behavior and juvenile hormone titer. (A) Survival rate of workers treated with RG108 (dashed line) or DMSO (continuous line) (Log-rank test, $p < 0.05$, N = 50). (B) ELISA-based quantification of genomic methylation in abdominal DNA extracts of 3-day-old caged workers (*$p < 0.05$). (C) Nutrient intake of bees confined in three different experiments. The y-axis shows sucrose consumption normalized by the bees alive at each day, showing no significant change. (D–F) Juvenile hormone titers quantified by radioimmunoassay in 1-, 3- and 7-day-old caged workers. For details on statistical analyses see text.

an *in vivo* RNAi protocol (Nunes and Simoes, 2009). While, as expected, *vg* transcript levels were significantly reduced by the targeted dsRNA treatment, the expression of the *dnmt1a* and *dnmt3* genes was not significantly affected (Fig. 3D–F, *vg* – one-way ANOVA, $F_{(2,12)} = 23.52$, $p < 0.0001$; *dnmt1a* – one-way ANOVA, $F_{(2,12)} = 1.629$, $p > 0.05$; *dnmt3* – one-way ANOVA, $F_{(2,12)} = 0.8844$, $p > 0.05$), leading to infer that Vg is not an upstream but rather a downstream component in this regulatory circuitry for lifespan

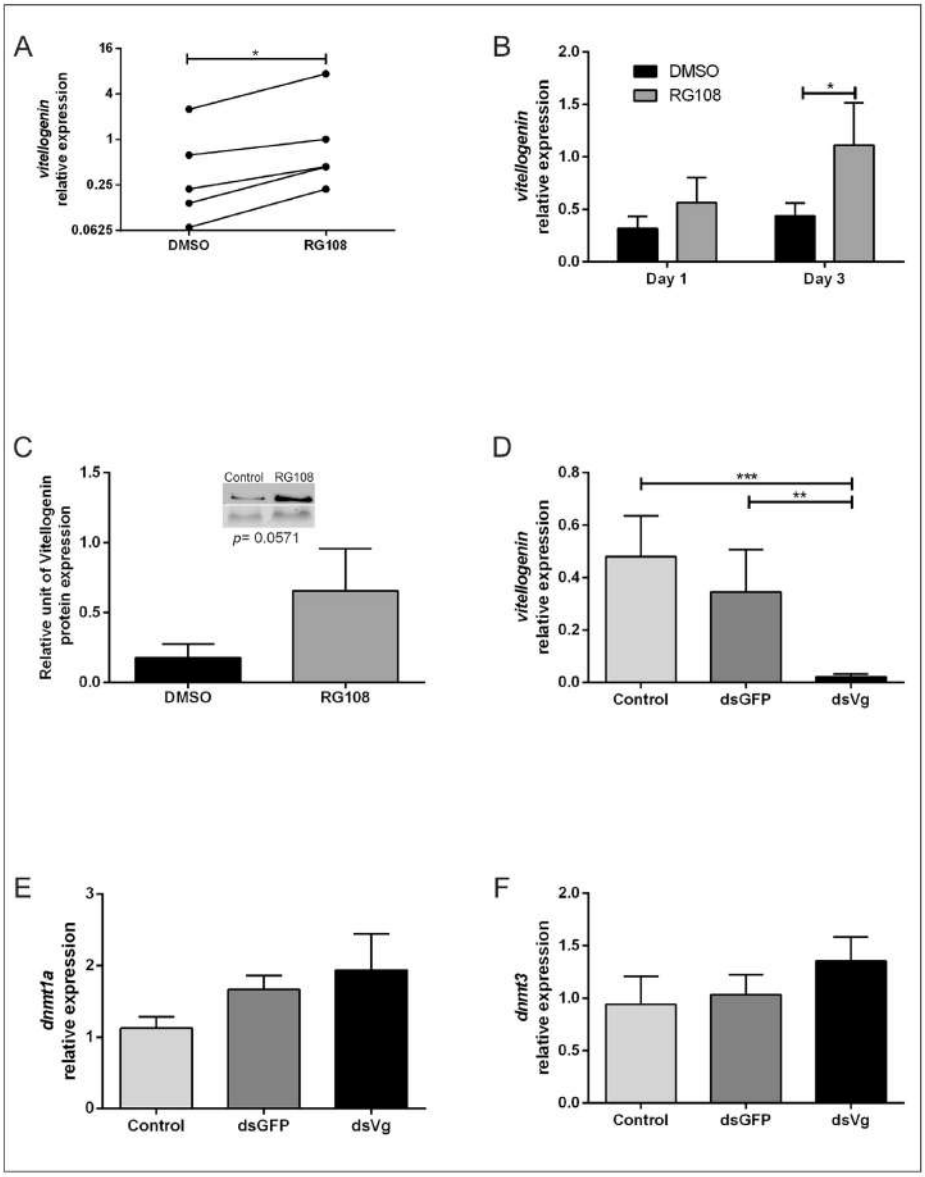

Fig. 3. RG108 effect on vitellogenin expression. (A) Relative *vg* gene expression levels assessed by RT-qPCR. Individual abdominal carcasses were cut in half and incubated *in vitro* in the presence of RG108 or DMSO solvent (control) (*$p < 0.05$). (B) Quantification of *vg* expression in abdomens 1 and 3-day-old workers treated *in vivo* with RG108 or DMSO (solvent control) (*$p < 0.05$). (C) Relative quantification of vitellogenin protein in hemolymph of 3-day-old workers revealed by western blot analysis using a honey bee-specific vitellogenin antibody. (D) Relative expression of *vg*, (E) *dnmt1a* and (F) *dnmt3* in abdomens of workers injected with dsRNA targeting vitellogenin (dsVg), a non-target dsRNA (dsGFP), or water (control) (**$p < 0.01$, ***$p < 0.001$). For details on statistical analyses see text.

regulation in honey bee workers. Furthermore, the effect caused by the RG108-mediated genomic demethylation on *vg* expression is probably not a direct one, based on prior evidence that *vg* is a hypomethylated gene (Elango et al., 2009; Herb et al., 2012; Li-Byarlay et al., 2013; Lyko et al., 2010). Hence, the link between the genomic methylation status and *vg* expression likely involves a yet unknown common regulatory factor, which is not JH.

4. Discussion

Here we found that in honey bee workers the expression of DNA methylation genes is modulated in a tissue-specific manner and varies in relation to age. Such variation in the expression of DNMTs encoding genes and in 5-methyl-cytosine marks in the genome is, of course, neither new nor exclusive to honey bees, but has long been associated with aging in mammals (Casillas et al., 2003; Gonzalo, 2010; Johnson et al., 2012). Nonetheless, such an association has not yet been demonstrated as clearly for invertebrates. Thus, our results support the idea that the CpG methylation status and aging represent a phylogenetically ancient and apparently conserved feature across the animal kingdom. With respect to insects, this view is further supported by current phylogenetic tree reconstruction, which places Hymenoptera as the basal branch in Holometabola (Misof et al., 2014).

While the relationship between DNA methylation and aging, thus, appears to hold in general terms, the respective regulatory circuitries across taxa and even across tissues of the same species appear to vary in their configurations. For instance, the transcription levels of *dnmt1* and *dnmt3* are considerably modulated in the gonads but not in the heads of castes and sexes of the fire ant *Solenopsis invicta* (Kay et al., 2016), similar to our findings in honeybees. Furthermore, we found that expression of the *dnmt3* gene, which encodes the enzyme responsible for *de novo* methylation (Bestor, 2000; Wang et al., 2006), is up-regulated in abdomens of old worker bees. This stands in contrast with findings reported for different tissues of humans, rat, and other vertebrates, where global hypomethylation during aging was seen in all compartments of the genome (gene bodies, promoters and intergenic regions). Nonetheless, certain loci are known as being hypermethylated during aging (Heyn et al., 2012; Romanov and Vanyushin, 1981; Vanyushin et al., 1973). Methylome analyses for the honey bee comparing nurses and foragers (Herb, 2014; Herb et al., 2012) identified 155 differentially methylated regions in the DNA extracted from the heads of these workers, indicating that specific changes in methylation patterns are crucial to honey bee behavioral maturation. Furthermore, the knockdown of *dnmt3* expression in abdomens of honey bee workers affected alternative RNA splicing (Li-Byarlay et al., 2013), suggesting that *dnmt3* up-regulation may also be relevant for RNA processing in the age-related nurse-to-forager transition.

When treating newly emerged honey bee workers with the DNMT inhibitor RG108 we found that lifespan was increased. Although non-epigenetic pharmacological effects of RG108 cannot be excluded *a priori*, to our knowledge, such effects have not been reported in the literature, which makes us believe that the genomic methylation status plays a role in lifespan regulation, especially since we found no caloric restriction phenotype associated with this treatment in honey bee workers. These results stand in contrast with a study that analyzed the role of DNA methylation in the fruit fly *D. melanogaster*, where overexpression of *dnmt2* was seen to extended the flies' lifespan (Lin et al., 2005). However, the functional role of DNA methylation in *D melanogaster* has been discussed controversially (Jeltsch et al., 2016; Raddatz et al., 2013; Takayama et al., 2014), as only few CA- and CT-rich motifs were found in the *Drosophila* genome, and these were not associated with DNMT2 activity. Since cytosine methylation seems to be dispensable in *D. melanogaster* (Raddatz et al., 2013), the effects of *dnmt2* overexpression on longevity in *Drosophila* (Lin et al., 2005) may possibly be related to other mechanisms, such as the regulation of siRNA pathways *via* tRNA methylation (Durdevic et al., 2013), rather than with DNA methylation.

To look into possible mechanisms related to the mode of action of RG108 on lifespan extension in honey bees we checked, as mentioned, whether DNMT inhibition may have caused a caloric restriction behavioral phenotype, which is a well-documented factor for lifespan variation (Mair and Dillin, 2008; Speakman and Mitchell, 2011). In honey bees, the ROS scavenger resveratrol was found to promote lifespan extension, possibly *via* DNA methylation (Rascon et al., 2012). In our study, however, we did not find any evidence for reduced caloric intake caused by RG108 treatment, suggesting that DNA methylation and caloric restriction are not necessarily coupled in adult bees. Furthermore, we found that the JH hemolymph titer was also not affected in the RG108-treated bees (Fig. 2D–F), and neither did the treatment of bees with the JH mimic methoprene affect the transcript levels of the *dnmt1a* and *dnmt3* (Supplemental Material Fig. S3).

The yolk protein precursor vitellogenin (Vg) is known as a major life history regulator in honey bees, and its expression is related not only with reproduction, especially so in queens, but also with aging, anti-oxidation capacity, immune system functions, foraging, as well as learning and memory acquisition in workers (Amdam et al., 2004; Corona et al., 2007; Lourenco et al., 2012; Marco Antonio et al., 2008; Salmela et al., 2015; Seehuus et al., 2006; Wang et al., 2013). Here we show that, Vg is also part of a JH-independent regulatory circuitry involving DNA methylating enzymes. In the experiment where we pharmacologically blocked DNA methylation, workers lived longer and had higher levels of *vg* expression, while in the analysis of RNAi-mediated *vg* knockdown bees we found that the expression of the two DNMTs encoding genes was not significantly affected. This indicates that DNA methylation effects are likely upstream element that indirectly affect *vg* gene function in a JH-independent non-canonical circuitry that affect the lifespan in honey bee workers.

Since *vg* is a hypomethylated gene (Elango et al., 2009; Foret et al., 2012; Li-Byarlay et al., 2013; Lyko et al., 2010), we have at present no conclusive information on what may be the factor that indirectly confer DNA methylation effects to *vg* expression. One possible, but so far merely speculative candidate could be the transcription factor *foxo*, which was hypothetically associated with aging and *vg* expression in honey bees (Corona et al., 2007). The expression of *foxo* was effectively up-regulated in our *in vitro* RG108 treatment experiment, and its expression has been shown to regulate *vg* expression in the cockroach *Blattella germanica* and the mosquito *Aedis aegypti* (Hansen et al., 2007; Suren-Castillo et al., 2012).

Even though the missing element is not yet identified, we believe that our results represent evidence for a JH-independent module for lifespan regulation of worker honey bees, which is likely to act in parallel with the already well-established Vg/JH mutual repressor circuitry (Amdam and Omholt, 2002; Flatt et al., 2013; Guidugli et al., 2005; Marco Antonio et al., 2008; Rodrigues and Flatt, 2016) as summarized in Fig. 4. This would explain certain difficulties of the Vg/JH framework in explaining why JH levels and lifespan can vary with season and daytime (Elekonich et al., 2001; Huang and Robinson, 1995), and why allatectomy did not block the nurse-to-forager transition, but only caused a delay of a view days only caused a minor delay in the behavioral maturation of these JH-deprived honey bee workers (Sullivan et al., 2000). Hence, our findings would add an epigenetic fine-tuning component that indirectly regulates *vg* expression, and further emphasize

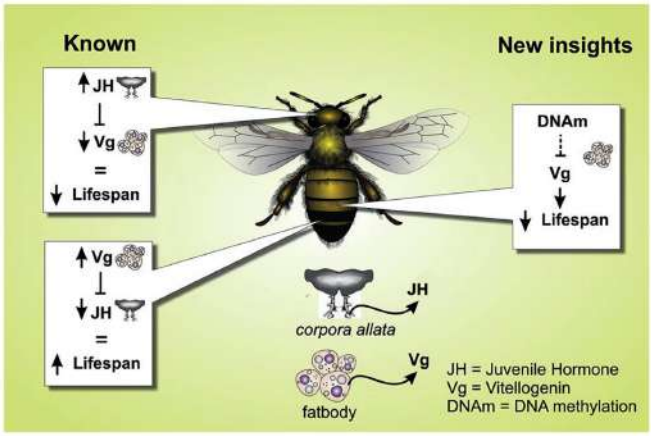

Fig. 4. Model proposed for a DNA methylation pathway regulating aging of worker honey bees in the context of the already known vitellogenin/juvenile hormone regulatory circuitry. The left boxes show the already established knowledge on honey bee lifespan regulation involving a dual-repressor regulatory circuitry between JH and Vg acting in the brain/*corpora allata* complex and the fat body. The right box shows the new insights on an epigenetic component, whereby the inhibition of DNA methylation in the fat body may extend honey bee lifespan. When DNMT activity was pharmacologically inhibited (RG108), vitellogenin expression was up-regulated, and this likely involves a not yet characterized factor. In this model honey bee lifespan would be epigenetically regulated *via* a JH-independent pathway, in addition to the parallel running Vg/JH dual-repressor module.

Vg as the crucial and shared factor in behavioral phase transition, aging and senescence in honey bee workers. Whereas the DNMT/Vg link, denoted here in honey bees for the first time in an invertebrate organism, would actually represent a phylogenetically ancient and conserved link between DNA methylation and the reproduction/lifespan trade-off syndrome, the insect-specific JH/Vg circuitry has, in honey bees, been co-opted and rewired from a gonadotropic function into a regulatory module for a complex social behavioral trait transition.

Authors contributions

CAM carried out the molecular laboratory work, participated in the design of the study and data analysis, and drafted the manuscript; KRGL participated in the design of the study and KH conceived and coordinated the study and edited the manuscript. All authors gave their final approval for publication.

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Conflicts of interest

The authors declare no conflict of interest that could be perceived as influencing the validity of the results reported herein.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ibmb.2017.11.005.

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Chapter II

3.2 Chapter II

This chapter refers to the following article:

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Social context influences the expression of DNA methyltransferase genes in the honeybee

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DNA methylation is a reversible epigenetic modification that alters gene expression without altering the nucleotide sequence. Epigenetic modifications have been suggested as crucial mediators between social interactions and gene expression in mammals. However, little is known about the role of DNA methylation in the life cycle of social invertebrates. Recently, honeybees have become an attractive model to study epigenetic processes in social contexts. Although DNA methyltransferase (DNMT) enzymes responsible for DNA methylation are known in this model system, the influence of social stimuli on this process remains largely unexplored. By quantifying the expression of DNMT genes (*dnmt1a*, *dnmt2* and *dnmt3*) under different demographical conditions characterized by the absence or presence of immatures and young adults, we tested whether the social context affected the expression of DNMT genes. The three DNMT genes had their expression altered, indicating that distinct molecular processes were affected by social interactions. These results open avenues for future investigations into regulatory epigenetic mechanisms underlying complex traits in social invertebrates.

Epigenetic modifications are non-genetic mechanisms that regulate gene expression *via cis*- and *trans*-acting factors[1]. DNA methylation occurs through the activity of DNA methyltransferases (DNMTs), which add a methyl radical to the C5 position of cytosines in CpG dinucleotide sequences[2].

Functional effects of DNA methylation have been investigated intensively over the past years. These research efforts revealed that the methylation process affects multiple functions in animals, such as genomic stability, X chromosome inactivation, embryonic development, cell division, genomic imprinting and responses to environmental changes[1,3,4]. Among others, levels of social interactions affected DNA methylation patterns in mice[5]. Conversely, social behavior of mice after maternal separation-induced stress was also affected by DNA methylation[6], showing a reciprocal interaction between this molecular process and social traits.

While studied in depth in vertebrates, the interactions between DNA methylation and complex social organization are still poorly studied in invertebrates. Several genes are differentially methylated during caste development in honeybees and in ants[7,8]. For example, the methylation of *egfr* was associated with variation of body size in workers, an important trait affecting social organization in the carpenter ant *Camponotus floridanus*[9]. Functional studies in mammalian and non-mammalian models, also demonstrated that social programming can be modulated after epigenetic impairment[5,10] and, conversely, that alterations in social stimulation can affect epigenetic modifications[6,11,12]. Hence, studying the molecular regulation of complex social traits in insects might shed light on the evolution of this molecular machinery, which could have been coopted in widely separated taxa with social environments[13,14].

Honeybee, *Apis mellifera*, colonies contain thousands of individuals interacting as a superorganism[15,16] and therefore represent an attractive model to study the impact of social context on epigenetic processes. In this species, social context varies according to demographic and seasonal changes[17,18]. In addition, honeybees possess a

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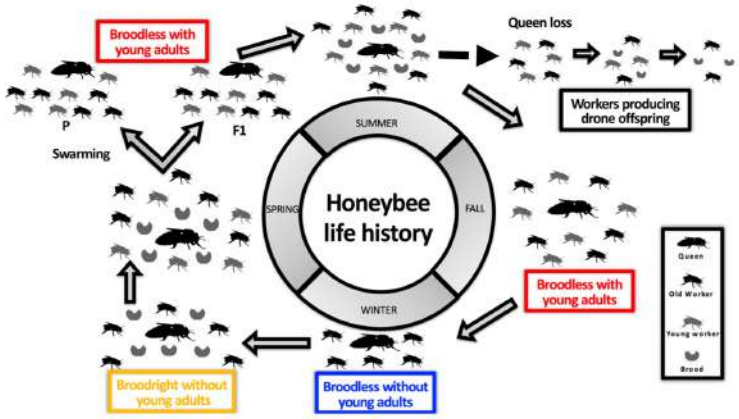

Figure 1. Honey bee life history. In fall, colonies of honeybees contain young adults, old workers and the queen. In winter, brood production is interrupted and colonies only contain the queen and aging workers. When spring comes, queens have started laying eggs, and brood and old workers are both present in the colony, soon joined by young adults emerging from the first brood. Once the colonies have grown sufficiently, reproduction by swarming can occur, and the new colonies (parental and filial – represented as P or F1, respectively) do not contain brood. Brood production starts again after a few weeks and lasts until fall. At any point, colonies can lose their queen, allowing some workers to activate their ovaries and lay unfertilized eggs that develop into drones. If no replacement queen is produced, the colony eventually dies. Large black individuals = queens; small black individuals = old workers; small grey individuals = young adults; small grey comma-shaped individuals = brood. Differently colored boxes represent the social contexts tested in this study.

functional DNA methylation system comprising a complete set of DNMT enzymes[19]. Because DNA methylation is restricted to gene bodies[20], the honeybee's methylome size is reduced compared to that of mammals. This likely facilitates the acquisition of direct evidence of its regulatory role on the transcriptome, further supporting the use of honeybees as an excellent model in epigenetics studies. Previous studies linked epigenetic modifications to social aspects of honeybee life history, such as caste differentiation and adult division of labor[8,12,21], suggesting that DNA methylation functions as mediator between environmental changes and gene expression. However, whether seasonal demographic changes are also reflected in changes in the epigenetic machinery is not yet known.

Recently, alterations in demography that reflect naturally occurring stages of the honeybee colony yearly life-cycle were shown to significantly affect the expression of genes related with aging, endocrine system functions, vitellogenesis and nutritional physiology[22]. Manipulating the presence of young adults and immatures in colonies as in Eyer *et al.*[22] we here tested the hypothesis that enzymes related with epigenetic processes are involved in the regulatory network underlying the response of honeybee workers to changing social conditions. We monitored the expression of three genes coding for enzymes involved in distinct DNA and RNA methylation mechanisms (*dnmt1a*, *dnmt2* and *dnmt3*), which are known to respond to environmental changes in vertebrates[1]. We expected their expression patterns to vary under the experimentally generated different social contexts. Experimental colonies were kept on the same site and were monitored simultaneously in order to minimize the effect of seasonal and nutritional differences on gene expression. Gene expression was measured over time to take into account age-related physiological and behavioral changes of individual workers[23]. This also allowed gaining insights on the DNMTs expression dynamics. Our results show that the expression of DNMTs encoding genes changes according to alterations in colony demography, and that this differential expression occurs primarily during the first two weeks of a worker's adult life.

Material and Methods

Honeybee source and social manipulations. The honeybees and social manipulations used in this study correspond to those of a previous study[22], in which we used free flying honeybee (predominantly *A. mellifera carnica*) colonies headed by unrelated queens (N = 9, ~16,000 workers each) that were kept in Dadant hives. The experiment was performed from June to August 2013 in Liebefeld, Switzerland. The experimental design consisted of three groups (N = 3 colonies each) displaying different social contexts that naturally occur during the annual life cycle of a honeybee colony (Fig. 1). These social contexts were created by manipulating the presence of immatures (brood) and young adult workers as follow: 1) To generate broodless colonies without young adults, the queens were caged to prevent brood production and young adult emergence during the entire experimental period. 2) The broodless group with young adult workers was generated as for group 1 and by constantly adding newly emerged individuals (N = 450 ± 213) originating from unrelated donor colonies. Additions were performed once daily on 30 occasions during the 35 days-experiment. 3) The broodright group without young adults was created by placing the queens on an empty comb kept in a frame cage, thereby allowing the queens

Name	Sequence 5′ 3′	Reference	Beebase access code
DNMT1a F	CGAGTAGTAAGCGTGCGTGAA	Cardoso-Junior, *et al.* 2017	GB47348
DNMT1a R	CAAGTGGTGGAGGAACTGC		
DNMT2 F	TGAGTCCTCCATGTCAACCTT	Biergans, *et al.* 2015	GB54141
DNMT2 R	GCCAAATTGACAAGGGCTTA		
DNMT3 F	CAGCGATGACCTGCGATCGGCGATA	Lockett, *et al.* 2010	GB55485
DNMT3 R	TACAGGG TTTATATCGTTCCGAAC		
RP49 F	CGTCATATGTTGCCAACTGGT	Lourenço, *et al.* 2008	GB47227
RP49 R	TTGAGCACGTTCAACAATGG		

Table 1. Primer sequences used in the RT-qPCR assays. Shown are the name of the genes, the respective F (Forward) and R (Reverse) primer sequences, the primer references, and the beebase database access codes.

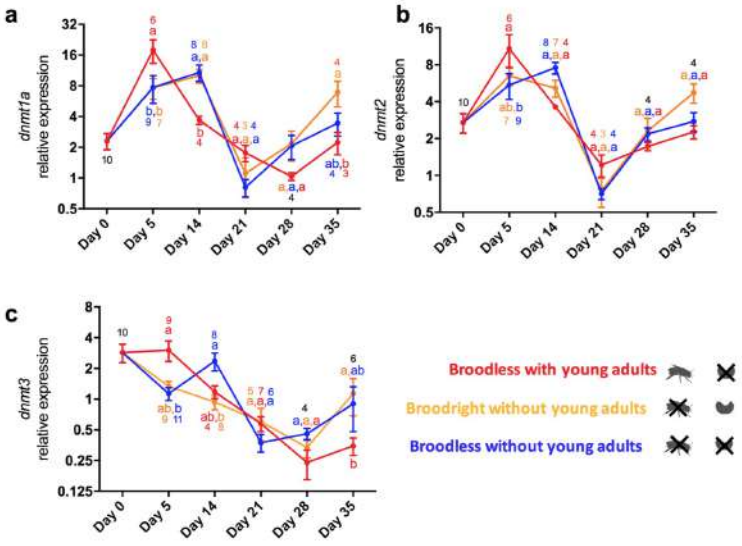

Figure 2. Relative expression of DNA methyltransferase encoding genes in adult honeybee workers of different ages and subjected to different social contexts. Shown are the expression of (**a**) *dnmt1a*, (**b**) *dnmt2* and (**c**) *dnmt3*. Numbers represent the biological sample size and different letters represent statistically significant differences ($p < 0.05$).

to oviposit on this frame for four days. Subsequently, the combs were removed from the cages and were placed into the same colonies for another nine days, after which they were removed. The repetition of this cycle ensured that open and capped brood was constantly present in the colonies during the experiment but that young adult workers did not emerge.

Prior to experimental manipulation, combs with pupae in late stages of development were incubated at 35 °C and 65% humidity. One day later, 200 newly emerged workers per colony (nine colonies; N = 1,800 individuals) were collected and marked with colored paint dots before being reintroduced into their mother colonies. Five of these marked workers were recaptured from each colony on days 0, 5, 14, 21, 28 and 35 in order to perform gene expression analysis.

RNA extraction, cDNA synthesis and RT-qPCR assays. The cDNA libraries used in this work were collected during our previous study[22]. Total RNA was extracted from whole bodies of individual workers using a NucleoSpin 96 RNA kit (Macherey-Nagel). For each sample, cDNA was prepared using random hexamer primers, M-MLV Reverse Transcriptase enzyme (Invitrogen®) and 10 µL of RNA following the manufacturers' instructions. Each sample was analyzed twice with a quantitative PCR assay (Kapa SYBR Fast) evaluating the transcript levels of three DNTM genes; *dnmt1a*, *dnmt2* and *dnmt3* using specific primers (see Table 1). The following cycling conditions were used in the RT-qPCR assays: 95 °C for 2 min, 40 cycles of 95 °C for 3 s and 60 °C for 20 s. Simultaneously, the *rp49 (rpl32)* reference gene[24] was analyzed in the same way. Product specificity was validated

Test	Day	Source of variation or Multiple comparisons	Genes		
			dnmt1a	*dnmt2*	*dnmt3*
Two-Way ANOVA	—	Social context	$F_{(2,88)} = 1.02$; $p = 0.3647$	$F_{(2,88)} = 0.07346$; $p = 0.9292$	$F_{(2,109)} = 0.2761$; $p = 0.7593$
	—	Time	$\mathbf{F_{(5,88)} = 27.9; p < 0.0001}$	$\mathbf{F_{(5,88)} = 29.65; p < 0.0001}$	$\mathbf{F_{(5,109)} = 30.75; p < 0.0001}$
	—	Interaction between 'social context' and 'time'	$\mathbf{F_{(10,88)} = 3.216; p = 0.0014}$	$F_{(10,88)} = 1.755$; $p = 0.081$	$F_{(10,109)} = 2.574$; $p = 0.0078$
Tukey-Kramer post-hoc test	5	Broodless with young adults vs broodless without young adults	$\mathbf{p = 0.0084}$	$\mathbf{p = 0.0481}$	$\mathbf{p = 0.0093}$
		Broodless with young adults vs broodright without young adults	$\mathbf{p = 0.0469}$	$p = 0.3979$	$p = 0.0853$
		Broodless without young adults vs broodright without young adults	$p = 0.8576$	$p = 0.5334$	$p = 0.7353$
	14	Broodless with young adults vs broodless without young adults	$p = 0.0238$	$p = 0.077$	$p = 0.3829$
		Broodless with young adults vs broodright without young adults	$p = 0.0381$	$p = 0.6805$	$p = 0.7266$
		Broodless without young adults vs broodright without young adults	$p = 0.9729$	$p = 0.2569$	$\mathbf{p = 0.0315}$
	21	Broodless with young adults vs broodless without young adults	$p = 0.1558$	$p = 0.3666$	$p = 0.3852$
		Broodless with young adults vs broodright without young adults	$p = 0.4044$	$p = 0.3946$	$p = 0.9949$
		Broodless without young adults vs broodright without young adults	$p = 0.9017$	$p = 0.9987$	$p = 0.502$
	28	Broodless with young adults vs broodless without young adults	$p = 0.3495$	$p = 0.817$	$p = 0.1895$
		Broodless with young adults vs broodright without young adults	$p = 0.4291$	$p = 0.9286$	$p = 0.638$
		Broodless without young adults vs broodright without young adults	$p = 0.9885$	$p = 0.9689$	$p = 0.6728$
	35	Broodless with young adults vs broodless without young adults	$p = 0.3437$	$p = 0.8725$	$p = 0.1729$
		Broodless with young adults vs broodright without young adults	$\mathbf{p = 0.019}$	$p = 0.1253$	$\mathbf{p = 0.0215}$
		Broodless without young adults vs broodright without young adults	$p = 0.3113$	$p = 0.3085$	$p = 0.6417$

Table 2. Statistical analysis details for the expression of the DNMTs encoding genes. Shown are the results for the Two-Way ANOVA and Tukey-Kramer *post-hoc* tests, including the *F* value of the ANOVA test and adjusted *p*-values for source of variation and multiple comparison analysis. Significant differences ($p < 0.05$) are represented in bold.

for all samples by running a melting curve analysis after the last amplification step. The detection threshold was adjusted manually for each primer set. Relative expression values were calculated using the $2^{-\Delta\Delta Ct}$ equation[25]. The numbers of biological replicates are indicated in Fig. 2.

Statistical analysis. Log^{10} transformed data was shown to be normally distributed with the Kolmogorov-Smirnov test. We thus used a two-way ANOVA and a Tukey-Kramer *post-hoc* test with correction for multiple comparisons to compare the gene expression of workers under the three social conditions at each time-point (Table 2). A p-value < 0.05 was considered significant. All analyses were performed with the software GraphPad Prism version 5.0.

Results

Expression of the three DNMT genes did not differ significantly between social contexts over the whole experimental period, but varried significantly over time (Table 2). In addition, the interaction between social context and time was statistically significant for *dnmt1a* and *dnmt3* expression but not for *dnmt2* (Table 2). The RT-qPCR analyses performed over 35 days, which cover most of the lifespan of an adult worker in summer, revealed that the expression levels of *dnmt1a* were significantly higher at day 5 in workers from colonies with young adults, when compared with the two groups lacking young adults (Fig. 2a). At day 14, the expression of *dnmt1a* was significantly lower in the group with young adults compared with the two groups lacking young adults (Fig. 2a). As for day 5, no statistical difference was found between the broodright and broodless groups lacking young adults (Table 2). For 21 and 28 days-old individuals, gene expression was not significantly different between any of the groups (Table 2). At day 35, a statistically significant difference was found between the broodless group with young adults and the broodright group lacking young adults, but not between the broodless groups either with, or without young adults (Table 2). Moreover, no statistical difference was found between the broodright and broodless groups lacking young adults.

The expression pattern of the RNA methyltransferase *dnmt2* gene over time was similar to that of *dnmt1a* (Fig. 2b) but showed fewer time points with significant differences between the groups. The only difference was observed at day 5, when gene expression was statistically higher in broodless colonies containing young adults compared to broodless colonies lacking young adults (Table 2).

The expression of *dnmt3* was significantly higher in 5-day-old individuals from broodless colonies with young adults than in individuals from broodless colonies lacking young adults. In contrast, there was no significant difference in expression compared to individuals from broodright colonies without young adults (Fig. 2c). Once more, no statistical difference was found between the broodright and broodless groups without young adults (Table 2). At day 14, *dnmt3* expression was statistically different between individuals from broodright and from broodless colonies lacking young adults. No statistical differences were observed for the other combinations of groups. For individuals aged 21 and 28 days, gene expression was not significantly different between any of the groups. At day 35, individuals from broodless colonies with young adults showed a significantly lower expression of *dnmt3* than individuals from broodright colonies lacking young adults. No difference was observed for the other combinations of groups (Table 2).

Discussion

Gene expression has been shown to be modulated by reversible epigenetic marks in different animal models[26]. Here, we show for the first time an effect of social context on the epigenetic machinery in the honeybee. Hence, our results suggest a role of epigenetic mechanisms as mediators of adaptive responses to social environment.

In this study, we focused on the effect of social context on the expression of three genes belonging to distinct classes of nucleotide modifiers[1]. Our results imply that DNA methylation can be activated by brood and young adults, unlike RNA methylation that seems to be only affected by the presence of young adults. These data, together with the fact that Hymenopterans show dynamic methylome process[12,27,28] possibly involving DNMTs mRNA levels[29], suggest that activation of different epigenetic pathways appears necessary to fine-tune the levels of effector proteins required by honeybees to adapt to their social context.

Expression of all the DNMTs encoding genes tested here was variable during the adult life of workers. This finding is in accordance with previous studies in honeybees and other social insects that reported changes in DNMT expression and/or DNA methylation levels involved in the regulation of multiple processes, such as development, aging, behavior and reproduction[12,21,27,30–32]. Although, social context alone was not a significant source of variance in the expression of *dnmts*, their interaction with time affected *dnmt1a* and *dnmt3* expression. This indicates that social context affects the expression of these genes differently at specific time points. Large differences in expression were observed during the first days of adult life, suggesting that honeybee workers can quickly respond to social stimuli that are likely to regulate their behavioral maturation. We found that the presence of young adults affected the expression of all three DNMTs encoding genes early in the adult life cycle, whereas the presence of brood seemed to only affect the expression of *dnmt3* at day 14. A possible explanation for this pattern is that social manipulation accelerated the behavioral maturation dynamics of nestmates, making them age faster. This hypothesis is supported by our previous study[22], which showed that the presence of young adults and brood altered the expression of aging-related genes and survival rates in their nestmates. The *dnmt1a* and *dnmt3* transcript levels were also different between 35 days-old workers from broodless colonies that contained young adults and from broodright colonies without young adult colonies. The ability to respond to external changes is, thus, not restricted to the beginning of adult life, but can also be expressed at later stages, when the workers have typically started foraging[33].

DNA methylation was previously associated with different degrees of behavioral maturation in honeybees, whereby distinct patterns of DNA methylation were shown to be associated with workers age polyethism, *i.e.*, the transition from within-colony to foraging task performance[12]. These patterns were found to be reversible and contingent on the respective social task. Our results support the hypothesis that social environments, here represented by demographic changes related to the presence of brood and young adults, alter nucleotide methylation that may mediate behavioral maturation networks. For example, the expression of *dnmt1a*, an enzyme necessary for maintenance of global DNA methylation levels, peaked at day 5 in the group with young adults, but was delayed until day 14 in workers from colonies lacking young adults, independently of whether these colonies had brood or not. This peak may promote distinct genomic methylation patterns that drive alterations in gene activity and ultimately, worker behavior and aging. Furthermore, *dnmt2* expression, which was upregulated at day 5 in the group with young adults, has been associated with tRNA protection against ribonuclease cleavage and microRNA biogenesis following environmental stress in *Drosophila melanogaster*[34,35]. Thus, DNMT2 seems to act downstream of gene expression by regulating microRNA decay. On the same day (5), *dnmt3* expression also exhibited a peak in this group (with young adults). Interestingly, variation in *dnmt3* expression, a *de novo* DNA methyltransferase, was shown to affect aging and alternative splicing in honeybee workers[29,31], thus suggesting that fluctuations in *dnmt3* transcript levels may represent a molecular signature of behavioral maturation through mRNA regulation. Therefore, it is possible that the expression of all three DNMTs encoding genes studied here is sensitive to external stimuli, especially early in a worker's adult life. Consequently, the resulting epigenetic modifications might influence gene regulatory networks associated with the workers behavioral ontogeny in colonies containing young adult workers.

We found that the presence of brood affected *dnmt3* expression. This effect is possibly mediated through a brood pheromone[36]. A recent study showed that *dnmt3* gene expression is also affected by a pheromone produced in the mandibular gland of *A. mellifera* queens[37]. This pheromone spreads within the colony and signals the presence of the queen and influences worker behavior[38]. Through their pheromonal signals, queens and brood, thus, appear to affect the same epigenetic component in the regulatory circuitry underlying behavioral plasticity of workers.

Finally, our results provide empirical evidence for an epigenetic response to seasonal demographic variation in honeybee colonies that is in line with previous studies[10,12,21]. The results of these studies, together with ours, highlight the important role of DNA methylation in insect societies, acting in processes that are analogs to those described in mammals[5,6,11]. This indicates that social insects may make use of an evolutionarily conserved epigenetic machinery to modulate behavioral responses according to social context.

Although we herein focused on the social context resulting from alterations in colony demography, our results shed light on social interactions at the molecular level in general, thus expanding the available knowledge on epigenetic mechanisms of complex traits seen in social insects. Future investigations will be necessary to elucidate the possible roles of DNMTs and DNA methylation in other socially regulated processes, such as hygienic behavior, worker policing, aggressiveness, and regulation of reproduction.

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Author Contributions

M.E. conduced the field and molecular work. C.A.M.C.J. carried out the RT-qPCR, analyzed the results. Conception and experimental design of present work was done by C.A.M.C.J., V.D. and M.E. with support of B.D. and K.H. The manuscript draft was written by C.A.M.C.J. and V.D. with critical revisions of K.H., B.D. and M.E. B.D. supervised the lab work and V.D. supervised the project.

Additional Information

Competing Interests: The authors declare no competing interests.

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Attachments to the Chapter II

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Publisher Correction: Social context influences the expression of DNA methyltransferase genes in the honeybee

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This Article contains formatting errors in Table 2. The correct Table 2 appears below, with p values < 0.05 highlighted in bold.

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Test	Day	Source of variation or Multiple comparisons	Genes		
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Two-Way ANOVA	—	Social context	$F_{(2,88)} = 1.02$; $p = 0.3647$	$F_{(2,88)} = 0.07346$; $p = 0.9292$	$F_{(2,109)} = 0.2761$; $p = 0.7593$
	—	Time	$\mathbf{F_{(5,88)} = 27.9}$; $\mathbf{p < 0.0001}$	$\mathbf{F_{(5,88)} = 29.65}$; $\mathbf{p < 0.0001}$	$\mathbf{F_{(5,109)} = 30.75}$; $\mathbf{p < 0.0001}$
	—	Interaction between 'social context' and 'time'	$\mathbf{F_{(10,88)} = 3.216}$; $\mathbf{p = 0.0014}$	$F_{(10,88)} = 1.755$; $p = 0.081$	$\mathbf{F_{(10,109)} = 2.574}$; $\mathbf{p = 0.0078}$
Tukey-Kramer post-hoc test	Day 5	Broodless with young adults vs broodless without young adults	$\mathbf{p = 0.0084}$	$\mathbf{p = 0.0481}$	$\mathbf{p = 0.0093}$
		Broodless with young adults vs broodright without young adults	$\mathbf{p = 0.0469}$	$p = 0.3979$	$p = 0.0853$
		Broodless without young adults vs broodright without young adults	$p = 0.8576$	$p = 0.5334$	$p = 0.7353$
	Day 14	Broodless with young adults vs broodless without young adults	$\mathbf{p = 0.0238}$	$p = 0.077$	$p = 0.3829$
		Broodless with young adults vs broodright without young adults	$\mathbf{p = 0.0381}$	$p = 0.6805$	$p = 0.7266$
		Broodless without young adults vs broodright without young adults	$p = 0.9729$	$p = 0.2569$	$\mathbf{p = 0.0315}$
	Day 21	Broodless with young adults vs broodless without young adults	$p = 0.1558$	$p = 0.3666$	$p = 0.3852$
		Broodless with young adults vs broodright without young adults	$p = 0.4044$	$p = 0.3946$	$p = 0.9949$
		Broodless without young adults vs broodright without young adults	$p = 0.9017$	$p = 0.9987$	$p = 0.502$
	Day 28	Broodless with young adults vs broodless without young adults	$p = 0.3495$	$p = 0.817$	$p = 0.1895$
		Broodless with young adults vs broodright without young adults	$p = 0.4291$	$p = 0.9286$	$p = 0.638$
		Broodless without young adults vs broodright without young adults	$p = 0.9885$	$p = 0.9689$	$p = 0.6728$
	Day 35	Broodless with young adults vs broodless without young adults	$p = 0.3437$	$p = 0.8725$	$p = 0.1729$
		Broodless with young adults vs broodright without young adults	$\mathbf{p = 0.019}$	$p = 0.1253$	$\mathbf{p = 0.0215}$
		Broodless without young adults vs broodright without young adults	$p = 0.3113$	$p = 0.3085$	$p = 0.6417$

Table 2. Statistical analysis details for the expression of the DNMTs encoding genes. Shown are the results for the Two-Way ANOVA and Tukey-Kramer *post-hoc* tests, including the *F* value of the ANOVA test and adjusted *p*-values for source of variation and multiple comparison analysis. Significant differences ($p < 0.05$) are represented in bold.

Chapter III

3.3 Chapter III

This chapter refers to the following article:

Title: Queen pheromone modulates the expression of epigenetic coding genes in the brain of honeybee workers

Authors: Carlos A. M. Cardoso-Júnior, Isobel Ronai, Klaus H. Hartfelder, Benjamin P. Oldroyd

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Queen pheromone modulates the expression of epigenetic coding genes in the brain of honeybee workers

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Running title: Epigenetic communication in honeybees

Abstract

Pheromones are used by many insects to mediate social interactions. In the highly eusocial honeybee (*Apis mellifera*) queen mandibular pheromone is involved in the regulation of reproduction and the rate of ageing of workers. The molecular mechanisms by which queen mandibular pheromone acts remain largely unknown. Here we investigate how genes associated with epigenetic modifications in the DNA, RNA and histones respond to the presence of queen mandibular pheromone in the brain of honeybee workers. Several of these genes are upregulated in workers exposed to queen mandibular pheromone. This suggests that epigenetic mechanisms are mediated by pheromonal communication between queens and workers in honeybees, and that the interaction of pheromone signalling and epigenetics may mediate the neurological plasticity that underlies ageing-associated traits in social insects. Moreover, we suggest that sophisticated communication systems, such as those used by social insects, were built making use of efficient epigenomic machineries able to respond to environmental clues.

Keywords: DNMTs, HATs, HDACs, SIRT, honey bees, aging

53 **Introduction**

54

55 The transition to social living in the eusocial insects required that the reproductive
56 interests of individual workers be subsumed by the collective interests of the colony [1–3]. In
57 particular, workers should normally be sterile, though they should switch to laying eggs when
58 their queen dies [4]. Furthermore, because insect societies are self-organized, it was necessary
59 for systems to evolve so that tasks are distributed among workers in ways that enhance colony-
60 level productivity [5–8]. Both the regulation of worker fertility and the efficient allocation of tasks
61 among workers required the evolution of effective inter- and intra-caste communication
62 systems that can respond rapidly to changes in colony needs. Communication between
63 nestmates mainly occurs via pheromones [9,10], chemical signals that are produced by one
64 individual, and cause changes in the behaviour or physiology of another [11,12].

65 In the western honeybee (*Apis mellifera*) a key pheromone is queen mandibular
66 pheromone (QMP). This pheromone is a blend of fatty acids secreted by the mouthparts of the
67 queen that regulates several important traits of workers, including their reproductive capacity
68 [13], foraging behaviour [14], learning capacity [15], nestmate recognition [16] and age at onset of
69 foraging [14]. Furthermore, phenotypic variation in these traits is associated with differential gene
70 expression in the brains of workers [17,18]. Nonetheless, little is known about the intermediate
71 steps between QMP production by the queen, the regulation of gene expression in workers, and
72 changes in their behaviour [19–22]. Epigenetic mechanisms are a likely mediator between the
73 social environment and global gene expression responses [23]. Several classes of epigenetic
74 mechanism have been described in honeybees that are potentially associated with
75 environmental change [24–27]. For example, DNA methylation, a reversible chemical
76 modification on carbon 5 of cytosines in a CpG context, is associated with behavioural
77 maturation in the brains of honeybee nurses and foragers [28]. DNA methylation is catalysed and
78 maintained by the DNA methyltransferase (DNMT) family of enzymes [29,30]. Interestingly, the
79 expression of genes associated with the maintenance of DNA methylation levels after DNA
80 replication (*Dnmt1a* and *Dnmt1b*), as well *de novo* DNA methylation patterns (*Dnmt3*), are
81 modulated by different social stimuli in honeybees [23,31–33]. In addition, the expression of *Dnmt2*
82 (also called Trdmt1), a gene that codes for an enzyme that methylates RNA substrates [34], is
83 affected by different social contexts in honeybees [23,28], suggesting that epigenetic alterations of
84 DNA and RNA are affected by environmental stimuli.

85 Another epigenetic mechanism, histone post-translational modifications (HPTMs)
86 change chromatin structure by altering the physiochemical affinity between DNA and histones.

HPTMs are associated with honeybee queen-worker differentiation [35] and are likely triggered by their different larval diets [36]. This demonstrates that, like DNA methylation, differential amount of HPTMs is sensitive to environmental signals (e.g. food, pheromones) in honeybees and other social insects [37–39].

HPTMs are produced by histone modifier proteins [30], which can be divided into three functional classes. First, "writer" enzymes, such as lysine acetyltransferases (KAT), promote acetylation of lysine residues [30]. Acetylation reduces the affinity between DNA and nucleosomes, which in turn induces chromatin relaxation and is often associated with increased gene expression [40]. Second, "eraser" enzymes, such as histone deacetylases (HDACs), remove acetyl groups from lysine residues, resulting in chromatin compaction (heterochromatin) and, consequently, inhibition of gene expression [30,41]. Notably, there is a histone deacetylase protein class that comprises NAD^+-dependent enzymes, the Sirtuins (SIRT). SIRT enzymes are key regulators of ageing in several species [42]. Thus, it is possible that SIRTs genes may play a role in QMP modulation of ageing for honeybee worker. Third, "reader" enzymes recognise epigenetic modifications and induce chromatin remodelling through the recruitment of protein complexes [30]. A proteome study [25] has shown that the histone tails of honeybees are extensively modified by epigenetic marks, indicating that writer, eraser and reader enzymes are all coded by the honeybee genome.

As QMP promotes behavioural maturation in honeybee workers [14] we hypothesised that the proteins associated with DNA methylation, RNA methylation and HPTMs (particularly acetylation/deacetylation processes) respond to QMP exposure. These epigenetic mechanisms can serve as a proxy to understand the regulation of global changes in gene expression in a complex social environment. Specifically, we investigated the expression of epigenetics genes in the brains of honeybee workers exposed or not exposed to QMP.

Material and Methods

Biological material

To obtain age-matched honeybee workers we collected brood frames from four queenright *A. m. ligustica* source colonies and kept them in an incubator overnight at 34.5 °C. From each source colony, newly-emerged workers were randomly allocated to two cages (n = 150 bees per cage, eight cages in total). Four cages (QMP^+) contained a 0.5 queen equivalent per day QMP strip (Phero Tech Inc. Canada), which is an effective queen mimic in cage

experiments with young workers [19,43]. The other four cages (QMP^-) contained no QMP strip. Pollen, honey and water were provided *ad libitum*. Food was replenished when necessary, and the number of dead workers was recorded each day, which was nearly the same in both control and QMP-treated cages. Cages were kept in an incubator at 34.5 °C for four days. Workers were collected on dry ice at Day 0 (directly from the brood comb), Day 1 and Day 4. Day 1 was chosen to investigate genes with a quick response to the QMP treatment, and Day 4 was chosen to identify the genes that are still influenced by the QMP exposure after the majority of QMP-sensitive genes had returned to their basal expression line [17]. We then dissected the brains of the workers on dry ice [44].

Identification of the honeybee DNA methyltransferases and histone modifiers

We identified the DNA methyltransferases and histone modifiers in the honey bee genome (Amel_4.5) [45] searching manually for the names of each epigenetic gene in GeneBank [46] (Table S1) based on a large list of histone-modifier genes present in eukaryotes [47,48]. We filtered this list by selecting those associated with acetylation and deacetylation processes. From this short list we identified the proteins that are predicted to reside in the nucleus using their amino acid sequence and Protcomp software version 9.0 (Softberry). The genes were characterised following a previously described workflow (see [13]), briefly: gene information was obtained from GenBank [46]; protein information from UniProt [49]; insect orthologues (genomes of 116 species) from OrthoDB hierarchical catalogue v9.1 [50]; and TBLASTN to the *Drosophila melanogaster* genome using GenBank [46].

Gene expression quantification and bioinformatics analysis

Each sample consisted of a single brain. We extracted total RNA from the brains through maceration in TRIzol (Invitrogen) and a Direct-zol™ RNA Miniprep kit (Zymo Research). The RNA was treated with Turbo DNase Kit (Thermo Fisher Scientific) and quantified with a Qubit 2.0 Fluorometer (Invitrogen). The cDNA was synthesized from 600 ng of RNA using a SuperScript™ III Reverse Transcriptase Kit (Invitrogen) with oligo(dT) primer. Then, the cDNA was diluted to 5 ng/µL in ultrapure water.

The expression of the three DNA methyltransferases (*Dnmt1a*, *Dnmt1b* and *Dnmt3*), one RNA methyltransferase (*Dnmt2*) and eleven histone modifiers genes (*Kat2a*, *Kat3b*, *Kat6b*, *Kat7*, *Kat8*, *Hdac1*, *Hdac3*, *Sirt1*, *Sirt6*, *Sirt7* and *Rcs1*) was quantified by reverse transcription quantitative real-time PCR (RT-qPCR) according to [43,51]. RT-qPCR assays were set up with

153 2.5 µL SsoAdvanced™ Universal SYBR® Green Supermix (Bio Rad), 1.25 pmol of each
154 primer, 1 µL diluted cDNA (5 ng) in a total volume of 5 µL using a CFX384 Real-Time System
155 (Bio-Rad). For each biological replicate (n = 8 per source colony and treatment) three technical
156 replicates were conducted. RT-qPCR cycle conditions were as follows: 95 °C for 10 min; 40
157 cycles of 95 °C for 10 s, 60 °C for 10 s and 72 °C for 15 s. At the end of the RT-qPCR protocol
158 a melting curve analysis was run to confirm a single amplification peak. Primer efficiencies
159 (Table S2) were calculated based on an amplification curve of 10 points obtained through serial
160 dilution of mixed cDNA samples. The expression of the genes of interest was normalised using
161 the expression of two reference genes (*Rpl32* and *Ef1α*), whose expression was found stable
162 according to *BestKeeper* software [52]. Relative expression levels were calculated as in [51] using
163 a relative gene expression formula that takes into account the efficiency of each primer. The
164 list of genes, primer sequences and efficiencies can be found in Table S2.
165
166 **Statistical analysis**
167 To compare the expression of the QMP^+ and QMP^- treatments at Day 1 and Day 4 we
168 used a generalised linear mixed model (GLMM) with 'source colony' as random effect and
169 treatment and time (e.g. age) as fixed effects. To model the gene expression data, we used link
170 = identity, family = Gaussian. Where necessary a transformation function was applied (see
171 Table S3 for details). We used Day 0 data as a baseline of gene expression data. GLMM
172 analyses were performed in R [53] loading the packages lme4, car and lsmeans. An adjusted *p*-
173 value (Bonferroni correction for each gene) lower than 0.05 was considered significant for all
174 statistical tests.
175
176 **Results**
177 Using the protein sequences of the fifteen genes studied here, we first acquired *in-silico*
178 evidence that each gene was an authentic epigenetic modifier of DNA, RNA or histone tails
179 (Table S1). Next, RT-qPCR assays show that in 1-day-old honeybee workers the expression of
180 eight genes associated with epigenetic processes (*Dnmt3*, *Kat2a*, *Kat6b*, *Kat8*, *Hdac1*, *Hdac3*,
181 *Sirt 1* and *Rcs1*) was affected by exposure to QMP (GLMM, $p < 0.05$, Figure 1 and Table S3).
182 However, only four genes (*Dnmt1b*, *Dnmt2*, *Kat3b* and *Sirt7*) continued to be differentially
183 expressed at the age of four days (GLMM, $p < 0.05$, Figure 1 and Table S3). Time was
184 statistically significant for 14 of the 15 epigenetics genes (GLMM, $p < 0.05$, Figure 1 and Table

S3), the only exception being *Kat 7*. An interaction between treatment and time was found for *Hdac1*, *Sirt1* and *Kat6b* ($p < 0.05$, Table S3).

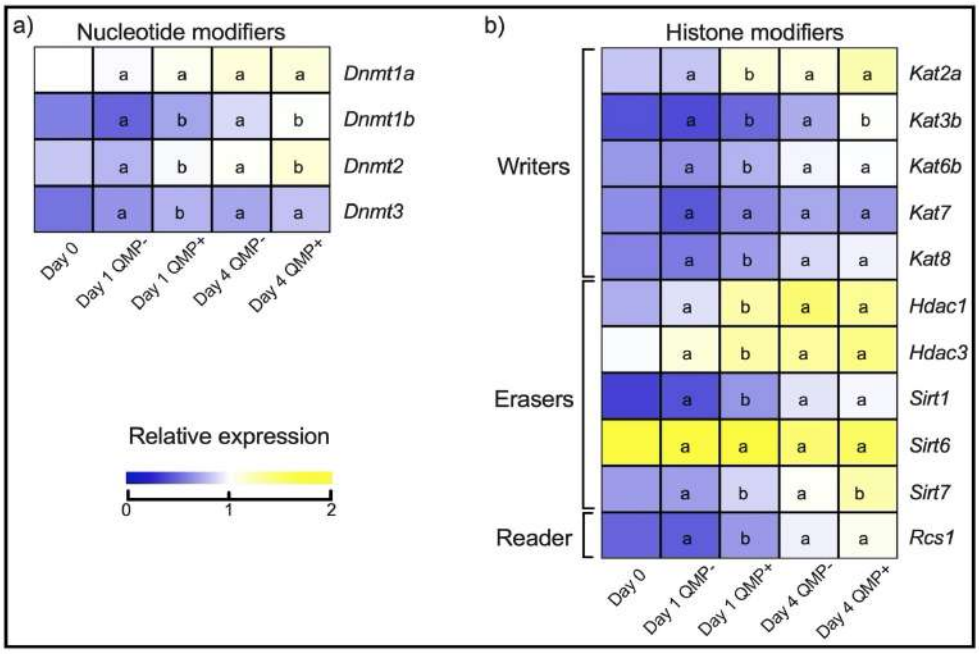

Figure 1. Heat maps representing the relative expression of epigenetic mechanisms-associated genes in the brains of 0-, 1- and 4-days old honeybee workers, exposed or not to QMP (QMP^+/QMP^-). a) Transcript levels of DNA methyltransferases. b) Expression of histone modifiers genes. The colour grading for each box represents the mean gene expression level of the biological replicates (N = 32; N=8 from each of the four colonies) for each of the three time points. Different letters represent statistical significance after Bonferroni correction for each time point, 1- or 4-days after QMP exposure (GLMM test, $p < 0.05$). Day 0 was used as baseline for gene expression.

Discussion

Our study shows that QMP affects the expression of 12 genes that are associated with epigenetic processes in the brain of honeybee workers across a short time scale. Rapid activation of these genes within 24 hours of exposure to QMP suggests that epigenetic mechanisms are a likely mediator between queen pheromone signalling and the regulation of gene expression. In particular, we show that genes associated with epigenetic mechanisms and involved in ageing are strong candidates for mediating gene expression changes in response to QMP in honeybees. QMP alters foraging onset in honeybee workers, a key marker of

206 behavioural maturation in *A. mellifera* [14,54,55]. Thus, our results suggest that genes associated
207 with epigenetic processes underlie the effects of QMP on behavioural maturation in honeybees.
208 We detected differential expression of three *DNMTs*. The expression of *DNMT*s
209 changes with chronological ageing and is sensitive to diverse social stimuli that affect ageing
210 in honeybee [23,32] and ant [32,56,57] workers. Interestingly, the expression of the RNA
211 methyltransferase *Dnmt2* is upregulated at both day 1 and day 4 of QMP treatment. While the
212 role of DNMT2 is currently unknown in the honeybee, it is the evolutionarily most conserved
213 and supposedly most ancient of the DNA methylating enzymes [34]. DNMT2 is present in insects
214 that have lost several or all of the other DNMTs, as is the case in Drosophilids [58]. Hence, our
215 results now suggest a potential role of *Dnmt2* mediating pheromonal communication in a
216 eusocial insect. In the fruit fly *D. melanogaster*, DNMT2 plays a critical role regulating the
217 biogenesis of microRNAs and tRNA stability upon environmental stress [24,59,60]. Moreover,
218 DNMT2 is associated with aging in *Drosophila* [61], suggesting that QMP may drive alterations
219 in the non-coding RNA pathways in honeybee workers.
220 Another two ageing-associated genes [42,62] regulated by the QMP exposure were *Sirt1*
221 and *Sirt7*. SIRT1 activity is known to modulated by resveratrol, a plant polyphenol that mimics
222 caloric restriction effects [63,64] and alters lifespan in honeybees [65] and other species [66–68].
223 Therefore, these results show that queens modulate the expression of ageing-associated genes
224 of honeybee workers, a process that possibly facilitates the behavioural maturation changes
225 associated with QMP exposure [14]. The precise epigenetic mechanisms by which each of these
226 genes act reprograming neurological gene networks to modulate aging process upon QMP
227 signalling remains to be elucidated.
228 We have also determined that two HDACs encoding genes (*Hdac1* and *Hdac3*) are
229 differently expressed in response to QMP treatment. However, unlike *Sirt1* and *Sirt7*, *Hdac1*
230 and *Hdac3* are only indirectly related to aging in honeybees. The two genes were first reported
231 [69] to be differentially expressed in honeybee workers treated with a diet supplemented with 10-
232 Hydroxy-2-decenoic acid (10-HDA), an abundant component of royal jelly that inhibits HDAC
233 activity in mammalian cells and is considered to play a role in caste determination [36]. While
234 the effects of 10-HDA on honeybee ageing have been not yet investigated, 10-HDA has been
235 shown to affect lifespan in other organisms used to study the effects of aging including *D.*
236 *melanogaster* and the worm *Caenorhabditis elegans* [70,71], suggesting that QMP makes use of
237 environmental-sensitive pathways, such as histone (de)acetylation, to promote altered
238 behavioural maturation changes in honeybee workers' brain.

239 Another gene promoting HPTM, *Kat8,* is also differentially expressed in honeybees
240 exposed to QMP versus those not exposed. *Kat8* was previously identified as being
241 differentially spliced in the transcriptome of *Lasius flavus* ants treated with their own queen
242 pheromones [56] suggesting that (de)acetylation processes may be regulated by queen
243 pheromones not only in honeybee but also in other social species. In line with this hypothesis,
244 acetylation was reported as an important regulator of foraging activities in the ant *Camponotus*
245 *floridanus* [38]. Together, these results highlight the role of epigenetic mechanisms in chromatin
246 reprogramming upon the exposure to new environmental clues.
247 Our discovery of QMP-responsive genes that were previously unidentified [17,18,56] can
248 be attributed to the methodological strategies we adopted in this study, such as QMP dosage,
249 age of workers at the time of sampling and tissue analysed. For example, our data shows that
250 the *Dnmt1a* expression is not affected by the QMP treatment, contrasting with data from a
251 previous study [32]. While we have analysed the expression of *Dnmt1a* in brains of workers
252 exposed to QMP for one day, the previous study focused on *Dnmt1a* expression after 3 days
253 of QMP treatment in whole-body RNA preparations. Such contrasting results indicate a tissue
254 and temporal specificity in response to QMP, highlighting the dynamics and complexity of
255 social interaction between queens and workers.
256 Pheromonal modulation of gene expression in honeybee workers changes over time
257 [17,18]. Gene expression in QMP^- workers was relatively stable from Day 0 to Day 1 when
258 compared to QMP^+, suggesting QMP *actively* promotes gene expression within 24 hours. Also,
259 we have found only a small number of epigenetic related genes (4 out of 15) are persistently
260 regulated by QMP-treatment over different time-points, indicating that dynamic regulatory
261 waves switch their expression on and off [17].
262 In conclusion, our study provides evidence that genes associated with epigenetic
263 mechanisms are differentially expressed in the brain of honeybee workers in response to queen
264 pheromone. We propose that epigenetic mechanisms are key responders to the honeybee social
265 environment. These critical environmental-sensitive mediators are likely candidates to drive
266 the transcriptomic changes triggered by queen pheromones, both as a short-term response
267 indicating queen presence (a "releaser" pheromone attribute of QMP) and a long-term response
268 related to behavioural maturation and ageing (a "primer pheromone characteristic of QMP).
269 We propose that the evolutionary success of complex communication systems found in highly
270 eusocial insects made use of efficient epigenomic machineries capable of responding quickly,
271 as well as persistently to social clues.
272

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Attachments to the chapter III

Table S1 Characterisation of the four honey bee DNA methyltransferases and eleven honeybee histone modifiers.

Gene name/ Accession number	Dnmt1a/ GB47348	Dnmt1b/ GB48403	Dnmt2/ GB54141	Dnmt3/ GB55485	Kat2a/ GB54338	Kat3b/ GB55583	Kat6b/ GB41680	Kat7/ GB44187	Kat8/ GB41293	Hdac1/ GB53438	Hdac3/ GB43894	Sirt1/ GB53035	Sirt6/ GB51490	Sirt7/ GB51465	Res1/ GB42921
Location in genome	Group 10	Group 10	Group 14	Group 2	Group 16	Group 2	Group 14	Group 4	Group 5	Group 9	Group 8	Group 4	Group 3	Group 11	Group 6
Sequence length (nts)	5,872	6,044	1,603	1,1377	3,521	16,043	18,815	23,297	2,529	4,840	1,797	7,704	3,826	2,250	12,796
Exons	15	15	3	18	7	20	8	13	7	9	1	10	7	4	29
Amino acid sequence residues	1,366	1,431	359	1,160	811	2,614	1,444	937	455	492	433	892	407	523	1,651
Protein molecular mass (Da)	157,077	163,009	41,516	132,692	93,622	284,372	159,433	103,370	53,420	56,124	49,437	101,393	45,952	59,670	188,137
Protein domains	CXXC-type, BAH (x2)	CXXC-type, BAH (x2)	No data	PWWP, PHD-type	N-acetyltransferase, Bromo	TAZ-type, KIX, Bromo, CBP/p300-type HAT, ZZ-type, TAZ-type	PHD-type, MYST-type HAT	MYST-type HAT	MYST-type HAT	Hist_deacetyl	Hist_deacetyl	Deacetylase sirtuin-type	Deacetylase sirtuin-type	Deacetylase sirtuin-type	Bromo (x5), BAH (x2), HMG box
Phyletic profile of protein in insects	106 genes in 59 species (25 single & 34 multi-copy)	106 genes in 59 species (25 single & 34 multi-copy)	114 genes in 109 species (104 single & 5 multi-copy)	70 genes in 39 species (35 single & 4 multi-copy)	145 genes in 114 species (89 single & 25 multi-copy)	147 genes in 113 species (89 single & 24 multi-copy)	133 genes in 110 species (89 single & 21 multi-copy)	117 genes in 112 species (107 single & 54 multi-copy)	125 genes in 112 species (101 single & 11 multi-copy)	123 genes in 106 species (92 single & 14 multi-copy)	120 genes in 114 species (108 single & 6 multi-copy)	85 genes in 83 species (81 single & 2 multi-copy)	225 genes in 116 species (15 single & 101 multi-copy)	94 genes in 92 species (90 single & 2 multi-copy)	127 genes in 115 species (105 single & 9 multi-copy)
Consensus gene median protein length (±sd)	1282 (±505)	1282 (±505)	341 (±99)	350 (±469)	799 (±242)	2313 (±1116)	2302 (±846)	795 (±231)	457 (±195)	489 (±84)	433 (±74)	821 (±182)	285 (±133)	578 (±234)	1647 (±553)
Consensus gene median exon count (±sd)	5 (±7.7)	5 (±7.7)	2 (±0.9)	13 (±5.5)	4 (±4.9)	13 (±6.7)	6 (±4.4)	7 (±2.6)	2 (±2.9)	5 (±3)	3 (±4.9)	3 (±3.8)	4 (±3.8)	2 (±1.8)	4 (±11.7)
BLAST best match *Drosophila melanogaster* BLAST [% identity]	Lysine (K)-specific demethylase 2 [56%]	Lysine (K)-specific demethylase 2 [56%]	Methyltransferase 2 [45%]	ADD domain-containing protein 1(ADD1) [27%]	Histone acetyltransferase GCN5 (Gcn5) [53%]	Nejire (nej) [65%]	Enoki mushroom (enok) [59%]	Chameau (chm) [57%]	Males absent on the first (mof) [54%]	Histone deacetylase 1 (HDAC1) [82%]	Histone deacetylase 3 (HDAC3) [76%]	Sirtuin 1 (Sirt1) [62%]	Sirtuin 6 (Sirt6) [56%]	Sirtuin 7 (Sirt7) [57%]	Polybromo [42%]

Table S2. Primer sequences used in the RT-qPCR assays. Shown are the name of the genes, the respective forward and reverse primer sequences (5'→ 3'), the database access codes, primer efficiency and references. The values marked with asterisks represent the second round of amplification.

Gene	Forward	Reverse	BeeBase access code	Primer efficiency (%)	Reference study
Dnmt1a	CGAGTAGTAAGCGTGCGTGAA	CAAGTGGTGGAGGAACTGC	GB47348	90.38 / 106.14*	[1]
Dnmt1b	GAAATTACATGGGTGGGAGAA	GTCACTGCCTCTTCGAAACC	GB48403	96.69 / 92.84*	[2]
Dnmt2	TGAGTCCTCCATGTCAACCTT	GCCAAATTGACAAGGGCTTA	GB54141	93.87 / 91.54*	[2]
Dnmt3	CAGCGATGACCTGCGATCGGCGATA	TACAGGGTTTATATCGTTCCGAAC	GB55485	93.93 / 95.86*	[3]
Hdac1	TCCTGGCACTGGTGACCTCCG	GCAACCTAACCGATCCCCTGTCA	GB53438	91.64 / 80.99*	[4]
Hdac3	TTGGAGCAGAAAGTGGGAGA	CTATTGCTGTCGGTTGGAAAA	GB43894	101.88 / 84.96*	[4]
Sirt1	ATGGTGGAGATAGTGGATTCAA	ATTAGGCAACCAGTGTCATCA	GB53035	98.45 / 82.68*	This study
Sirt6	ACCAAAAATCTTCCATACGGC	TCAAACAACACCAACGCTCATA	GB51490	101.26 / 83.74*	This study
Sirt7	GGCGTTCTGCTGCTCTTAAA	TAAGTATTCCTGTTTCCTCTGC	GB51465	98.05 / 85.17*	This study
Kat2a	ACTCGTTGGCTTGTGTTCTGT	GTGCCAATTTATCTCGCTCAC	GB54338	84.85 / 84.41*	This study
Kat3b	GGAATGGCAGGAGGAAGTTT	TGATGTGGACTTTGACCTCG	GB55583	88.75 / 84.3*	This study
Kat6b	CGGGAGAGGGAAATTCTAAAGT	AGGACTCGGAACTGGTATCAC	GB41680	85.47 / 78.55*	This study
Kat7	TCTCCTCTGTCCTTTTCCTC	GATTCGTTCTCGGTGATTTCTC	GB44187	86.27 / 80.14*	This study
Kat8	ATCAGCAAGTGCAATGTTCATC	GCCATTTGTCAACGTATTCTCA	GB41293	84.71 / 75.51*	This study
Rsc1	TAAGCAGCACAGTTCCAAAAAT	GCAGGTTCTTCTTCTTCATCA	GB42921	87.93 / 75.5*	This study
Rp49	CGTCATATGTTGCCAACTGGT	TTGAGCACGTTCAACAATGG	GB47227	82.25 / 69.68*	[5]
Ef1α	TGCAACCTACTAAGCCGATG	GACCTTGCCCTGGGTATCTT	GB52028	88.64 / 74.54*	[5]

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Table S3. Statistical details of GLMM test used to model the expression of epigenetic coding genes.

	Main effect			Bonferroni Post-hoc test		
Gene	**QMP treatment**	**Time**	**Interaction**	**Day 1**	**Day 4**	**Type of data transformation**
Dnmt1a	0.212429	**0.003911**	0.289477	0.2148	0.8021	none
Dnmt1b	**5,60E-02**	**1,47E-09**	0.2181	**0.0001**	**0.0239**	none
Dnmt2	**2,72E-02**	**6,36E-05**	0.1681	**0.0001**	**0.0265**	sin
Dnmt3	**0.04042**	0.17324	0.73346	**0.0426**	0.1195	none
Kat2a	**0,0210687**	**5,07E-05**	0,7099964	**0,0228**	0,0774	none
Kat3b	**0,04428**	**2,19E-08**	0,50982	**0,0465**	**0,0039**	sin
Kat6b	**0,02177**	**1,91E-09**	**0,09817**	**0,0235**	0,9647	log
Kat7	0,3798	0,1259	0,2746	0,3829	0,5091	log
Kat8	**0,02577**	**8,68E-12**	0,81189	**0,0276**	0,0607	exp
Hdac1	**4,88E-05**	**3,55E-10**	**0,0001046**	**2,28E-06**	**0,0001**	none
Hdac3	**0,03735**	**0,01095**	0,51968	**0,00306**	**0,0395**	none
Sirt1	**0,001395**	**2,00E-11**	**0,094228**	**8,94E-15**	**0,0018**	none
Sirt6	0,341144	**0,006409**	0,842772	**4,71E-05**	0,343	none
Sirt7	**0,0182**	**1,51E-05**	0,9352	**1,16E-10**	**0,0198**	none
Rcs1	**0,0003643**	**2,20E-16**	0,1826989	**0,0005**	0,0955	none

Chapter IV

3.4 Chapter IV

This chapter refers to the following article:

Title: Social regulation of DNA methyltransferase 3 and its association with aging in the honeybee, *Apis mellifera*

Authors: Carlos A. M. Cardoso-Júnior, Mário Cervoni, Cibele Cardoso, Lucas Tavares, Elaine E. da Silva, Klaus H. Hartfelder

Journal: Manuscript in preparation

Social regulation of DNA methyltransferase 3 and its association with aging in the honeybee, *Apis mellifera*

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Running title: Social regulation of honeybee DNMT3

Keywords: honeybee, ageing, epigenetics, microRNA 29b, DNA methylation

Abstract

DNA methylation, a reversible epigenetic mark, has emerged as an important regulatory mechanism to explain phenotypic and behavioral plasticity in social insects. The *de novo* DNA methyltransferase 3 (DNMT3), a key diver of epigenetic (re)programming, is considered a critical player in caste development and the adult life cycle in honeybees. Here we investigated the regulation of the honeybee DNMT3 gene (*Dnmt3*) at the transcriptional, post-transcriptional and post-translational level during the major transition in the adult life of a honeybee worker, the behavioural maturation from brood care to forager tasks. First, we show that the *Dnmt3* mRNA levels are higher in foragers compared to nurses. Next, we show that the *ame-miR-29b*, a microRNA that regulates the expression of *Dnmt* genes in mammals, is also a *bona-fide* regulator of the honeybee *Dnmt3*. Pharmacological genomic demethylation increased the expression of *ame-miR-29b*, indicating a regulatory cross-talk between these epigenetic mechanisms. Unexpectedly and contrary to our hypothesis, the DNMT3 protein was found primarily inside lipid vesicles of the cytoplasm of fat body cells, not in the nucleus as we first hypothesized. Yet, *in silico* prediction of the DNMT3 protein three-dimensional structure is consistent with such a subcellular location, suggesting that DNMT3 might play other functions not directly linked to DNA methylation. Our results provide new insights into the regulation of the epigenetic machinery in honeybees.

69 **Introduction**

70

71 Social insects are notable for their capacity to produce different phenotypes from the
72 same genome. For example, in the highly eusocial honeybee insects, differential feeding of
73 the female larvae produces two morphologically and functionally distinct castes, workers and
74 queens[1]. While the primary function of long-lived queens is reproduction, the short-lived
75 workers display a more diverse repertory of age-related behaviours, a process called
76 behavioural maturation [2].

77 After emergence, young workers first spent most of their time cleaning themselves,
78 and then start to perform different tasks within the colony primarily tending the brood as so-
79 called nurses, as well as cleaning the hive and feeding the queen through trophallaxis. Then,
80 as they age, they perform more risky tasks, such as foraging for nectar, pollen and water and,
81 as guards, they defend the colony against intruders [2,3]. This so-called nurse-to-forage
82 transition (NFT) represents a major change in the life cycle of a honeybee worker, regulated
83 by social and environmental clues. The endogenous regulatory circuitries underlying this
84 transition involve distinct molecular and physiological processes in several tissues, such as
85 the brain, flight muscle, fat body and the ovaries [2]. The time point at which the NFT occurs
86 actually defines a worker's life span [4], since after this transition, a worker typically lives for
87 another two weeks. However, since colony needs and colony demography vary throughout
88 the year, the timing of the NFT can be delayed, accelerated or even reverted with respect to a
89 worker's chronological age [2,5]. In temperate climates, for example, workers that emerge in
90 late autumn have extraordinarily long life spans, as their NFT is delayed until the beginning
91 of the next spring [6]. In this sense, foraging onset is a key marker of ageing for honeybee
92 workers, as it is associated with the deleterious effects of immunosenescence [7]. Thus,
93 efficient molecular systems are needed that adjust the perception and response to
94 environmental, social cues (e.g., queen pheromones, colony demographic composition) to the
95 NFT timing in workers, allowing the plasticity needed in the workers' life history.

96 Epigenetic mechanisms are superb candidates for regulating the extraordinary
97 behavioral plasticity seen in social insects [8,9], including aging-associated traits [10–12]. For
98 example, the DNA methylation, a chemical modification of CpG dinucleotides, was shown to
99 be a molecular signature of behavioral maturation in the brain of honeybee workers [10].
100 Nonetheless, since the discovery of the presence active and functional epigenetic systems in
101 the honeybee genome [13], little progress has been made concerning the transcriptional,
102 translational and post-translational regulation of honeybee DNA methyltransferases

103 (DNMTs), the enzymes that promote DNA methylation. Four *Dnmt* genes were annotated in
104 the honeybee genome: two copies of a *Dnmt1* ortholog (*Dnmt1a* and *Dnmt1b)* and a single
105 copy each of a *Dnmt2* and *Dnmt3* [13]. DNMT1a and DNMT1b enzymes are typically predicted
106 to maintain the methylation levels after somatic cell divisions, due to their higher specificity
107 to hemi-methylated DNA, while DNMT3 is hypothesized to promote *de novo* DNA
108 methylation due to its higher affinity to unmethylated DNA strands [13]. In contrast, the
109 metazoan *Dnmt2* genes, also called *Trdmt1*, code for a highly conserved RNA
110 methyltransferase [14]. While the role of DNMT2 has so far not been studied in honeybees, it
111 has been proposed in *Drosophila melanogaster* that DNMT2 methylates tRNAs and regulates
112 microRNA (miRNA) biogenesis under stress conditions [15,16].

113 Here, we investigated the social regulation of *Dnmt3*, at the transcriptional and post-
114 transcriptional levels, as well as the subcellular location of DNMT3 protein and its potential
115 post-translational regulatory mechanisms in the abdominal fat body of adult honeybee
116 workers. We focused on this tissue, as it is the major site of the intermediary metabolism in
117 insects, equivalent to the liver and adipose tissue of vertebrates [17]. Previous studies [10–12]
118 reported that the expression of *Dnmt3* is modulated by various social stimuli, indicating that
119 the NFT is an ideal condition to investigate the regulation of *Dnmt3* expression and function.
120 We found that a microRNA, *ame-miR-29b*, likely plays an important role in the regulation of
121 *Dnmt3* expression. Another novel finding was that the honeybee DNMT3 protein is primarily
122 localized in lipid vesicles of fat body cells, and not in the nucleus, thus suggesting that
123 DNMT3 may play a role in other contexts than DNA methylation. We expect that these
124 findings will provide novel insights into the functional role of the DNA methylation system
125 in honeybee workers, as well as in other social insects that exhibit active epigenetic systems.
126
127 **Results**
128 **Social regulation of *Dnmt3* mRNA expression**
129 First, we compared the *Dnmt3* expression levels in the fat body of honeybee nurses
130 and foragers. The transcript levels of *Dnmt3* were significantly higher in the fat body of
131 foragers compared to nurses in all the four assessed colonies (Figure 1 and Table S1).
132

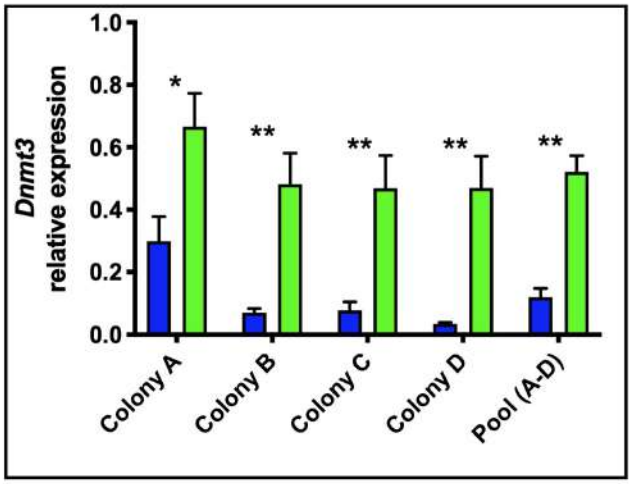

Figure 1. Real Time-PCR quantification of *Dnmt3* transcripts in the fat bodies of nurse and forager workers from four different colonies. Statistical analysis: Colonies A-D - Student's *t*-test; * $p < 0.05$; ** $p < 0.01$, N=7; Pool – GLMM, ** $p < 0.01$; N=28.

Dnmt3 expression is regulated by *ame-miR29b*

The honeybee genome codes for a single member of the miRNA29 family, *ame-miR29b*, and members of this microRNA family are known microRNAs involved in the regulation of *Dnmt* genes in mammals [18]. Hence, we hypothesized that the *miR29b* may regulate *Dnmt3* expression in honeybees. To test this, we first performed an *in silico* search for putative *ame-miR-29b* binding sites in the *Dnmt3* 3' untranslated region (3' UTR), and we found on such site (Figure 2A). We next determined whether *ame-miR29b* is expressed in the same tissues as *Dnmt3,* and how their temporal relate to one another (Supplementary figure 1). For this, we analysed the expression of these two genes in the same RNA extracts. Furthermore, to confirm whether *ame-miR29b* can actually regulate *Dnmt3* expression, we performed luciferase reporter assays, where the *Dnmt3* 3'UTR was cloned downstream of the luciferase coding sequence. Hek cells were used to express either *ame-miR-29b* or *hsa-miR-210*, a human miRNA that does not binding to the *Dnmt3* 3' UTR. Relative luciferase light units were significant lower in *ame-miR29b*-expressing cells compared to the controls (Figure 2B).

Another experimental approach taken to investigate the relationship between *Dnmt3* and *miR29b*, was the analysis of *ame-miR29b* expression in fat body samples of bees that had been treated with a biologically active dose of the genomic demethylation reagent RG108 [11].

The *ame-miR29b* expression levels in the RG108-treated samples were higher than in the controls (Figure 2C, Two-tailed *t*-Student test, t=4.038, df = 4, p<0.05, N=5), further confirming the postulated negative feedback loop involving this microRNA in DNA methylation of honeybee workers.

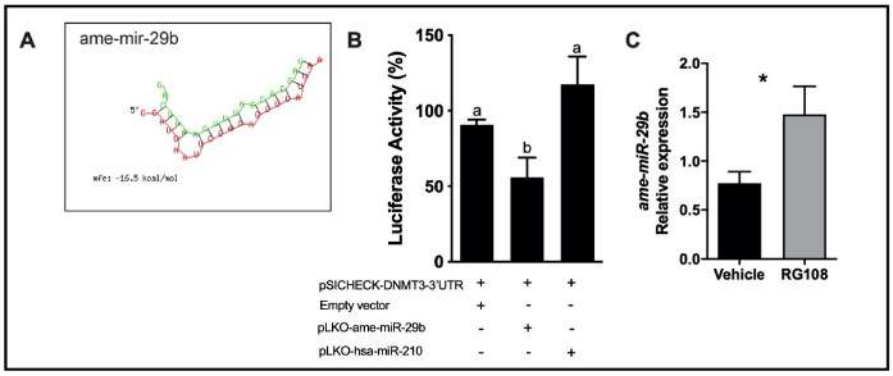

Figure 2. Functional analysis of *ame-miR-29b* and its target *Dnmt3*. (A) *In silico* prediction of the interaction of *ame-miR-29b* (green) and its potential binding site in the *Dnmt3* 3'UTR (red). The minimal free energy (mfe) for this interaction is shown. (B) Luciferase assay results validating the interaction between *ame-miR-29b* and *Dnmt3* 3'UTR. The *ame-miR-29b* and *hsa-miR-210* were both expressed in Hek 239-T cells, transfected with a vector that expresses luciferase mRNA downstream of the *Dnmt3* 3'UTR. The human microRNA 210 (*hsa-miR-210*) was used as a positive control, as does not have a binding site in the *Dnmt3* 3'UTR. (C) Real-time quantitative PCR results for fat body samples of worker bees that had been treated with the genomic demethylation agent RG108 in comparison to control (vehicle treated). Statistics: Two-tailed paired Student's *t*-test, * p<0.05, N=5.

DNMT3 protein is predominantly localized in the cytoplasm of fat body cells

For the immunolocalization of honeybee DNMT3 protein we used a heterologous (human) DNMT3 antibody, which binds to an epitope that is 44.4% identical and 62.8% similar to its honeybee ortholog. To confirm the cross-reactivity of this antibody with the honeybee DNMT3 protein we performed western blot assays, which identified a single band with a molecular weight slightly below that predicted from the *Dnmt3* gene sequence (Supplementary figure 2). The immunofluorescence staining of fat body tissue of honeybee nurses and foragers (Figure 3) was generally weak, indicating a relatively low abundance of this protein, consistent with the relatively low levels of *Dnmt3* mRNA (Figure 1).

Surprisingly, we found that the DNMT3 protein was predominantly localized in the cytoplasm of oenocytes, and not in the nucleus, both in nurses and foragers. Nonetheless, the levels of DNMT3 in fat body cells, measured by total DNMT3-immunofluorescence, were higher in foragers in comparison to nurse bees (Supplementary figure 3).

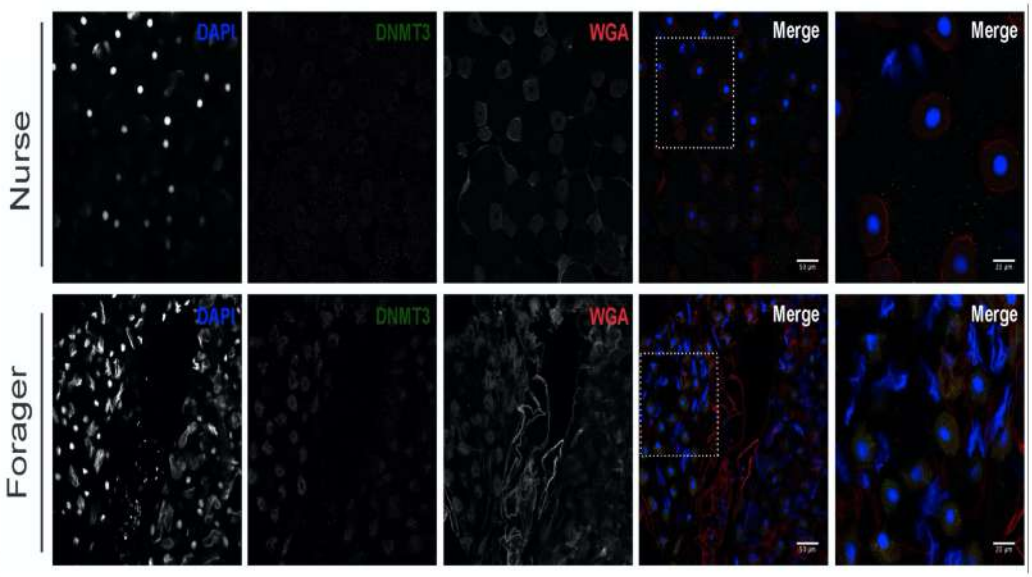

Figure 3. Laser confocal microscopy images of DNMT3 localization in honeybee fat body cells. Two cell types can be distinguished: oenocytes are the smaller cells with rounded nuclei, while trophocytes are bigger and have irregular nuclei. The two columns at the right are merged images, with the last one showing an optical amplification (2.5x) of the dashed box inserts. DAPI was used to identify nuclei and WGA to stain lipid membranes.

DNMT3 protein is localized inside lipid vesicles of fat body cells

To further investigate the cytoplasmic localization of DNMT3, we performed immunogold labelling on ultrathin sections of forager fat body preparations, followed by transmission electronic microscopy. Forager fat body was chosen because of the higher DNMT3 levels. The images revealed that in trophocytes, DNMT3 was predominantly localized inside lipid vesicles of the cytoplasm (Figure 4B). Also, DNMT3 was frequently observed on mitochondria and endoplasmic reticulum close to these lipid vesicles (Figure 4B). In oenocytes DNMT3 labelling was also found inside the nucleus, though its nuclear location is rare (Figure 4D).

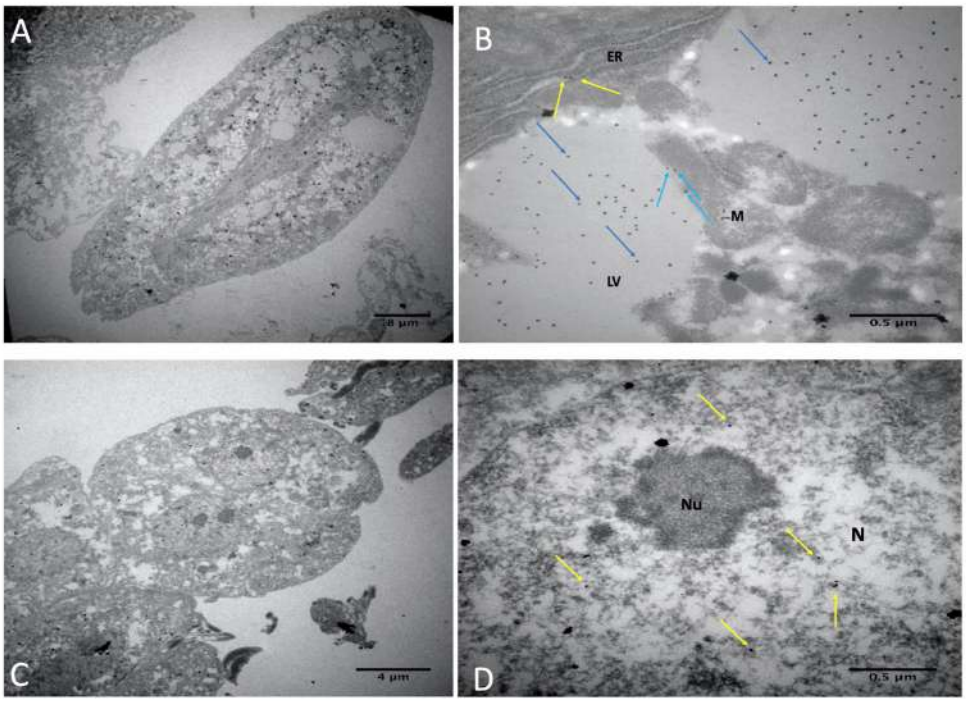

Figure 4. DNMT3 subcellular localization in trophocytes and oenocytes of fat body of foragers. DNMT3 was detected by immunogold staining in transmission electronic microscopy sections. (A and B) Trophocyte images. (A) General view, (B) DNMT3 protein labelling inside lipid vesicles (LV) is evidenced by dark blue arrows. Light blue and yellow arrows show DNMT3 labeling close to mitochondrial membranes (M) and endoplasmic reticulum (ER), respectively. (C and D) Oenocyte images. (C) General view, (D) Nucleus image with yellow arrows showing DNMT3 in the nucleoplasm (N); Nu represents the nucleolus.

***In silico* analysis of DNMT3 protein**

To gain further insights into the regulation and function of DNMT3, we used a bioinformatic approaches to *in silico* predict its biochemical properties and three-dimensional structure (Figure 5A). In line with the immunofluorescence results (Figure 3), DNMT3 was predicted to be a cytoplasmic protein by the Protcomp software. NLS mapper software then explained this cytoplasmic location by the lack of a nuclear localization peptide, addressing this protein to the nucleus. Further structure predictions identified a small hydrophobic region in the DNMT3 sequence that is possibly a transmembrane domain (see methods). Due to its

hydrophobic properties, this domain may be responsible for the interaction with lipids. The predicted three-dimensional structure of DNMT3 shows that this hydrophobic region is composed by an α-helix and a β-sheet structure, which are exposed on the surface of the DNMT3 three-dimensional structure (Figure 5B).

Finally, we investigated whether DNMT3 possesses protein-binding sites in its structure, indicating that association with other proteins can shuttle this protein to the nucleus. Seven regions were predicted to be protein-binding sites (amino acid numbers – 4, 390-391, 417, 469, 647, 683, 693-695 on NP_001177350.1 GenBank access number). It is worthy of note that one of these amino acids is close to the hydrophobic region (see the orange region in Figure 5C), suggesting that the DNMT3 protein may interact with other proteins to regulate its subcellular location or functions.

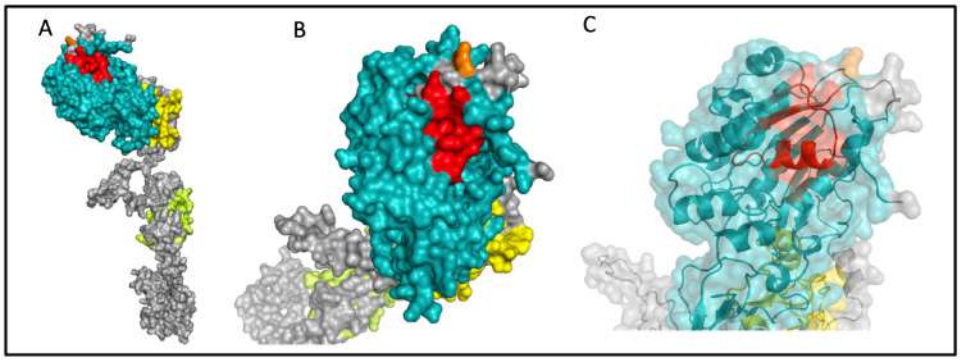

Figure 5. Predicted model for the DNMT3 three-dimensional structure. (A) Overall view. Colour schemes: blue – AdoMet domain; yellow – ADDz domain; green – PWWZ domain; red – hydrophobic region and orange – protein-binding site (B) Surface structure of DNMT3 highlighting the AdoMet domain (blue), hydrophobic region (red) and protein-binding site (orange). (C) Secondary structures of the AdoMet domain (blue) and the hydrophobic region (red).

Discussion

DNA methylation has been implicated in behavioral maturation of honeybee workers [10]. However, little is known about the mechanistic aspects underlying the regulation of this process. Here we show that the *de novo* DNMT3 is regulated at the transcriptional, post-transcriptional, and post-translational levels during a major transition in the life cycle of honeybee workers, the NFT. The multiple regulatory layers controlling the expression of

DNMT3 highlights the participation of this protein in social behavior, namely, the nurse and forager phenotypes.

Our data show that *Dnmt3* expression is higher in foragers compared to nurses the adjustment of worker behaviors, indicating a relationship with age-related behaviors. Here we show that the higher expression of *Dnmt3* in foragers involves a post-transcriptional regulatory step by *ame-miR-29b.* The microRNA 29b was the first epi-microRNAs reported [19]. Epi-microRNAs target genes encoding epigenetic factors, such as *Dnmt*, histone modifier, or *Tet* genes, the latter involved in active demethylation processes [20]. The cross talk between two epigenetic mechanisms, DNMT3 and an epi-microRNA is a novel finding for honeybees, and is little addressed in invertebrates in general. Similar to humans, the honeybee microRNA 29b is regulated by DNA methylation, and in turn, displays a repressive function in *Dnmt3* expression [19,21]. Such similarity in the regulatory network for *de novo* DNA methylation suggests an evolutionary conservation in this epigenetic mechanism that deserves closer attention.

Another relevant finding was the association of honeybee DNMT3 protein with lipid vesicles in the fat body of honeybee workers, and only marginally so with the nucleus. This suggests that DNMT3 may play a role in lipid storage in fat body cells, both in trophocytes, as well as in oenocytes, and it is especially in the latter where DNMT3 protein abundance seemed be higher in foragers. This cell type is known to store lipids in honeybees [22] and to have important metabolic functions in fruit flies [17]. Furthermore, it has been hypothesized that oenocytes play a role in the NFT through insulin-like pathway signaling, which in turn mobilizes stored nutrients [23,24]. Such a link between signaling through a major nutrient sensing pathway and epigenetic regulation has clearly been shown by the knockdown of *Dnmt3* gene function in worker larvae, mimicking the effect of royal jelly in caste determination [25]. Furthermore, dietary manipulation in adult workers resulted in altered levels of *Dnmt3* expression [26]. Therefore, it is possible that DNMT3 participates in a cascade of events that results in nutrient (lipid) mobilization in oenocytes during NFT [27].

But why a protein that methylates DNA would be so prominently located in the cytoplasm of honeybee fat body cells, especially so in lipid vesicles, and only marginally in the nucleus? In mechanistic terms, our *in-silico* analyses suggest the presence of a highly hydrophobic α-helix in DNMT3 and a lack of a nuclear localization signal in its sequence. Thus, this hydrophobic region exposed on the surface of the honeybee DNMT3 might be responsible for retaining the protein in the cytoplasm, especially so with lipid vesicles. But

278 then, how does DNMT3 get into the nucleus, as shown for example for oenocytes (Fig. 4D)?
279 A possibility is that other proteins function as carriers, as we found a protein-protein
280 interaction site close to the hydrophobic region. Thus, we propose that the DNMT3
281 subcellular location is regulated by the interaction with other proteins. This could explain that
282 honeybee DNMT3 has a diversity of functions beyond DNA methylation, including ones
283 associated with metabolic regulation. Actually, such a function has already been shown for
284 the DNMT2 enzyme of *D. melanogaster,* which regulates lipid homeostasis in an aging-
285 dependent manner [28].
286 In conclusion, our biochemical, computational, and functional characterization of
287 DNMT3 regulation in honeybee workers sheds new light on its role in the context of
288 honeybee aging-related behavioral change. Our results open new roads to explore the
289 functional roles of DNMT3 in the behavioral maturation of workers, as well as the epi-
290 microRNAs in the epigenetic reprograming and fine-tuning of worker behaviors in this social
291 insect model organism.
292
293 **Material and Methods**
294
295 **Bees**
296 Adult honeybee workers were obtained from managed colonies (Africanized *A.*
297 *mellifera* hybrids) kept in the Experimental Apiary of the University of Sao Paulo at Ribeirao
298 Preto. Nurses were collected when observed attending larvae, with their heads inside brood
299 cells. Foragers were collected as they returned from foraging flights harbouring pollen in
300 their pollen baskets on the hindlegs. Further confirmation of the workers' status was
301 obtained by the dissection of their hypopharyngeal glands. Nurses typically present
302 developed hypopharyngeal glands, whereas in foragers these are atrophied. Bees were
303 immediately anesthetized on ice, dissected, and kept in a -80 °C ultra-freezer until analysis.
304 The gut was removed from abdominal carcases to avoid bacterial contamination prior to
305 RNA and protein extraction. Abdominal carcases (dorsal and ventral) were used as a source
306 of fat body tissue. One-week-old workers were sampled as indicated in [11].
307 **RNA extraction, cDNA synthesis and RT-qPCR assays**
308 RNA samples were extracted with TRIzol reagent (Invitrogen) following the
309 manufacture's recommendations. RNA extracts (8 μL) were treated with RNase-free DNase
310 (Invitrogen, Carlsbad, CA, USA) to remove residual DNA. RNA quantity and purity were

311 assessed spectrophotometrically (Nanoview, GE Healthcare Life Science, USA). To measure
312 the expression of *Dnmt3*, cDNA was synthetized with Oligo(dT)$_{12-18}$ primer, 1 μg of DNA-
313 free RNA, and Super Script II (Invitrogen) following the manufacturer's protocol. The
314 obtained cDNA was 10x diluted in deionized water (Direct-Q, Millipore, Burlington, MA,
315 USA). Quantitative PCR (RT-qPCR) assays were carried out with 5 μL of Sybr Green Mix
316 (Applied Biosystem, Foster City, CA, USA), 1.25 pmol of each primer, 1 μL of diluted
317 cDNA, and water for completing the final reaction volume to 10 μL. The following
318 amplification parameters were applied: 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60
319 °C for 1 min. Each biological sample (N=10 for each colony) was analyzed as the average of
320 three technical replicates for each sample. The *Dnmt3* expression was normalized against
321 *Rpl32* Ct levels[29], a stable housekeeping gene used in honeybee studies [11,26,29].
322 The expression of *ame-miR-29b* was quantified in cDNA samples known to express
323 *Dnmt3* [11]. To amplify the *ame-miR-29b*, the first strand cDNA was prepared using the NCode
324 kit (Invitrogen), following the manufacturer's protocol. The RT-qPCR assays were ran with
325 the same parameters as those described for *Dnmt3*, only altering the cDNA volume to 2 μL
326 and the annealing/extension time to 30 s. These modifications were necessary to gain higher
327 specificity for *ame-miR-29b*. As primers we used a forward primer corresponding to the
328 actual miR sequence (substituting U for T) and as reverse primer the universal primer
329 provided by the NCode kit. The expression of *ame-miR-29b* was normalized by the U6
330 miRNA, which has been previously validated for microRNA RT-qPCR in the honeybee and
331 other insects [30,31]. Each biological sample (N=3-5) was analyzed as the mean of three
332 technical replicates.
333 A melting curve was run for all RT-qPCR assays (*Dnmt3* and miRNA expression
334 analysis), and only the samples with a single peak were used in further analyses. Relative
335 expression was calculated with the formula $2^{\wedge-\Delta\Delta Ct}$ [32]. Primer sequences and their sources are
336 listed in Table S2.
337 To investigate whether DNA methylation regulates the expression of *ame-miR-29b,*
338 we induced genomic demethylation by incubating freshly dissected fat body for 6 h in
339 Grace's insect medium (Sigma-Aldrich) in the presence of 0.2 mM RG108 (Cayman
340 Chemicals, Ann Arbor, MI, USA) diluted in DMSO (Merck, Darmstadt, Germany) in a
341 pairwise test design with DMSO serving as control. The RG108 dosage was validated by us
342 in a previous study elsewhere [11].
343

344 ***In silico* analysis of miRNAs**

345 We *in silico* predicted putative *ame-miR-29b* binding sites in the *Dnmt3*
346 3'untranslated region (3'UTR) using RNA hybrid software [33].
347
348 **Luciferase assay**
349 For vector construction, the genomic *Dnmt3* 3'UTR was amplified and cloned into the
350 *Not*I and *Xho*I sites of the psiCHECK2 vector (Promega, Madison, WI, USA). The sequences
351 of *ame-miR-29b* and *hsa-mir-210* were cloned into the pLKO expressing vector (Addgene,
352 Watertown, MA, USA). *hsa-mir-210* was used as negative control because it does not have
353 predicted binding sites in the 3'UTR of *Dnmt3* mRNAs. All constructs were verified by
354 Sanger sequencing. Primers are listed in the Table S2.
355 We used human Hek 239-T cells gently provided by Dr. David E Fisher, Harvard
356 Medical School. Hek 239-T cells were maintained in Dulbecco's modified Eagle's medium
357 (DMEM) supplemented with 10% fetal bovine serum, 50 U/mL penicillin, and 50 mg/ml
358 streptomycin at 37 °C under a 5% CO_2 humidified atmosphere. Cells were seeded in 24-well
359 dishes at a density of 30,000 cells/well. The next day, cells were transfected with either the
360 pLKO- ame-miR-29b, pLKO-hsa-210, or pLKO-empty vector, together with the
361 psiCHECK2-DNMT3-3'UTR using PEI MAX (Polysciences, Inc., Warrington, PA, USA)
362 according to the manufacturers' protocol. After 48 hours post transfection, cells were
363 harvested and assayed using the Dual-Luciferase Reporter Assay Kit (Promega) according to
364 the manufacturer's instructions. The luciferase signal was detected with SpectraMax L
365 microplate reader (Molecular Devices, San Jose, CA, USA). Renilla/Firefly normalization
366 was performed according to recommendations at the Promega website:
367 https://www.promega.com.br/resources/pubhub/cellnotes/normalizing-genetic-reporter-
368 assays/.
369
370 **Western Blot**
371 Protein extracts from fat bodies were obtained from pools of either 10 nurses or 10
372 foragers. Tissue was lysed on ice for 20 min with lysis buffer (1 mM EDTA, 50 mM TRIS-
373 HCl pH 7.5, 1% Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 10 mM
374 DTT and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride) supplemented with protease
375 inhibitor mixture (cat. P8340, Sigma-Aldrich, St. Louis, MI, USA) and centrifuged for 20
376 min/16.000 g/ 4° C. The supernatants were collected and mixed with sample buffer (4% SDS,
377 160 mM Tris-HCl (pH 6.8), 20% glycerol (v/v), 100 mM DTT, and 0.1% bromophenol blue)

and boiled for 5 min. Equal sample amounts of the protein extracts were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane (cat. 10600002, GE Healthcare). The membrane was then incubated in blocking solution (PBS-T supplemented with 5% nonfat dry milk and 1% BSA) for 1 h, incubated with primaries antibodies [anti-DNMT3, cat. 64B1446, Novusbio (Littleton, CO, USA) at a 1:1000 dilution; anti β-actin, cat. sc-47778, Santa Cruz Biotechnology (Dallas, TX, USA) at a 1:1000 dilution; anti GAPDH, Sigma-Aldrich, cat. G9545, dilution 1:500] overnight at 4 °C in a solution containing PBS-T and 1% BSA. After 5 washes with PBS-T, the membranes were incubated for 1 h in blocking solution supplemented with the respective secondary antibody [Horseradish-peroxidase-conjugated donkey anti-mouse immunoglobulin G (IgG) (NA931V, GE Healthcare, 1:10,000) or donkey anti-rabbit IgG (NA934V, GE Healthcare, 1:10,000)]. After 5 washes with PBS-T, the proteins were detected by using enhanced chemiluminescence (ECL) solutions (solution 1: 1 M Tris-HCl pH 8.5, 250 mM luminol, 90 mM p-coumaric acid; and solution 2: 30% H_2O_2, 1 M Tris-HCl pH 8.5) and visualized in the ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA).

Immunofluorescence

Immunofluorescence analyses were done on whole-mount preparations of fat body tissue. Immunostaining was done as described in [34] with some modifications. Briefly, tissues were dissected and fixed in 3.6% paraformaldehyde diluted in PBS (v/v) for 25 min. After washing three times with PBS, tissues were blocked with 5% of BSA diluted in PBS for 1 h, followed by incubation for 1 h with a human anti-DNMT3 antibody (Novusbio, # 64B1446) diluted 1:1000, and then washed 5 times with PBS. After incubation with anti-mouse alexa-flour 488 antibody (GE Life Sciences, Chicago, IL, USA) diluted 1:5000 for 1 h in the dark. Fat bodies were washed 5 times and then incubated for 5 minutes with a Fluoroshield mounting medium with DAPI (ABCAM, Cambridge, MA, USA) and WGA, two fluorophores to stain the nucleus and lipid membranes, respectively. Finally, tissues were mounted between two cover slips and images were captured with a TCS SP5 confocal microscope system (Leica, Wetzlar, Germany). Image quantification was performed with ImageJ Fiji version 10.

Immunogold labeling and transmission electronic microscopy (TEM)

Immunogold labelling and TEM were performed as in [35] with minor changes. For pre-embedding immunoelectron microscopy, tissue was dissected and incubated for 20 min in 4%

412 paraformaldehyde in PBS and subsequently rinsed with 50 mM glycine in PBS for 15 min.
413 Subsequently, the samples were permeabilized with 0.1% Triton in PBS for 30 min and
414 blocked for 45 min with 1% BSA in PBS. Next, they were incubated overnight at 4 °C with
415 anti-DMTT3 (1:100 dilution), rinsed, and subsequently incubated with goat anti-mouse IgG
416 conjugated to nanogold particles (Nanoprobes, Yaphank, NY, USA) at 1:1000 dilution) for 1
417 h at room temperature. After rinsing in 3 times with 1% BSA in PBS, the samples were post-
418 fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 1 h, rinsed twice with 0.1 M
419 cacodylate buffer, rinsed 3 times with 50 mM glycine in PBS, 3 times with 1% BSA in PBS,
420 and 3 times with deionized water (Milli-Q, Milllipore). The nanogold staining was enhanced
421 using GoldEnhance™ Electron Microscopy Plus (Nanoprobes) for 10 min according to the
422 manufacturer's directions. The samples were then post-fixed in 1% OsO4 in 0.1 M
423 cacodylate buffer (pH 7.4) for 2 h, rinsed in Milli-Q water, and dehydrated in a graded
424 ethanol series and embedded in EMBED 812 (Electron Microscopy Sciences, Hatfield, PA,
425 USA). Thin sections were cut with a diamond knife, mounted on copper grids, and stained for
426 10 min each in Reynolds's lead citrate and 0.5% aqueous uranyl acetate, and examined with a
427 JEOL JEM-100CXII (JEOL Ltd., Tokyo, Japan) transmission electron microscope.
428
429 ***In silico* analyses of DNMT3 protein structure**
430 For all *in silico* analyses we used the DNMT3 sequence available under the access
431 number NP_001177350.1 in GenBank. The NCBI server was used to identify the AdoMet,
432 ADDz and PWWZ domains. Subcellular location was predicted with Softberry ProtComp
433 software version 9.0 (available at
434 http://www.softberry.com/berry.phtml?topic=protcompan&group=programs&subgroup=prol
435 oc). The DAS transmembrane prediction server [36], TMpred from ExPASy [37], Split 4 server [38],
436 and Phobius prediction [39] were used to identify hydrophobic regions of DNMT3. NLS
437 mapper [40] was used to find nuclear localization signals in DNMT3. The three-dimensional
438 structure of DNMT3 was predicted with RaptorX [41] and visualization was done in PyMol
439 version 2.3.
440
441 **Statistical analysis**
442 Gene expression was analyzed separately for each colony with a one-tailed Student's
443 *t*-test, based on the *a priori* prediction that *Dnmt3* expression would be higher in foragers
444 compared to nurses [11]. A Generalized Linear Mixed Model (GLMM) was used to analyze
445 *Dnmt3* expression from multiple colonies. We used "colony" as random factor, "social

446 function" as a fixed factor, link = identity, family = Gaussian, and a $\log^{10}$ transformation.
447 GLMM analyses were performed in R [42] with the packages lme4, car, and lsmeans.
448 Luciferase data were analyzed using a two-tailed One-Way ANOVA test after checking
449 normality assumptions (Kolmogorov-Smirnov test). The effect of RG108 treatment on *ame-*
450 *miR-29b* expression was analyzed with a two-tailed paired Student's *t*-test after checking
451 normality assumptions (Kolmogorov-Smirnov test). Protein levels were compared by a Two-
452 Way ANOVA test, as the data followed a Gaussian distribution. An adjusted p-value < 0.05
453 was considered significant for all statistical tests.
454
455 **Competing interests**
456 The authors declare no competing interests
457
458 **Author contributions**
459 CAMCJ and KH designed and coordinated the study; CAMCJ performed the *in silico*
460 analyses, RT-qPCR experiments, and immunofluorescence assays, statistical analyses; MC
461 helped with immunogold and western blots; CC performed the luciferase assays; LT helped
462 with the western blots; EEdaS performed the immunogold TEM analysis; CAMCJ and KH
463 drafted the manuscript, and all authors approved the final version.
464
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468 TEM.
469
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476
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478
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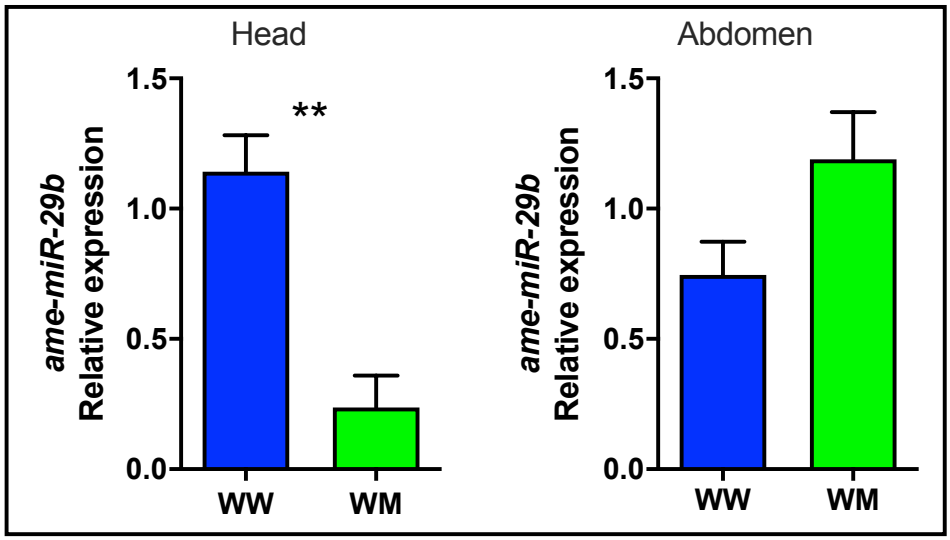
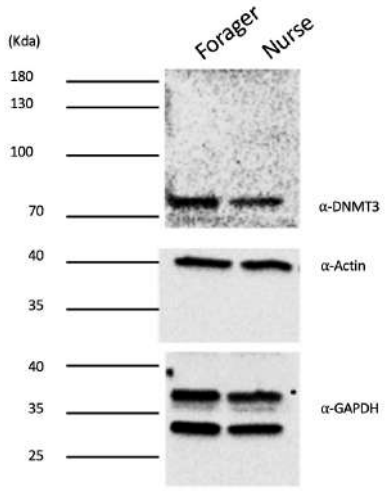
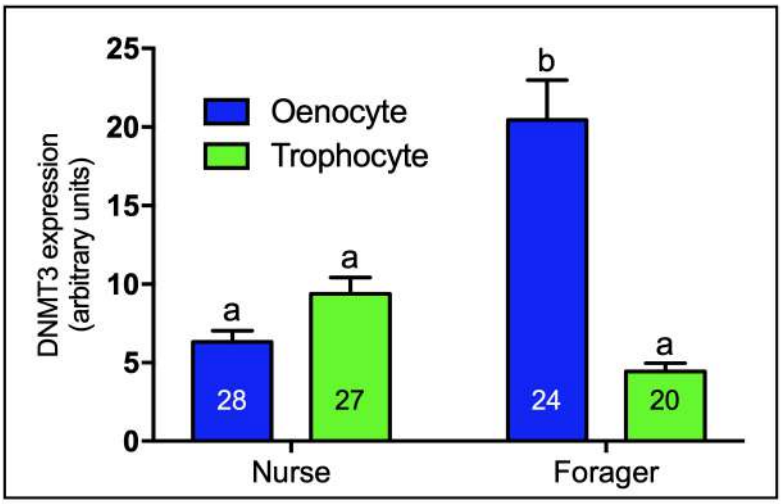
Attachments to the chapter IV



Supplementary Figure 1. Real Time-PCR quantification of *ame-miR-29b* transcripts in the head and abdomen of one-week-old workers (WW) and one-month-old workers (WM). Statistical analysis: unpaired Student's *t*-test, ** $p < 0.01$; N=3-5.



Supplementary Figure 2. Western blot validation of antibody α-DNMT3 in honeybee fat body tissue. Actin and GAPDH were used as loading control. * represents unspecific bands informed by manufacturer.



Supplementary Figure 3. Quantification of DNMT3 fluorescence from immunofluorescence images in both oenocyte and trophocyte cells of nurse and foragers. Different letters represent statistically significant differences (Two-Way ANOVA, $p < 0.05$, N=20-28 cells per group)

Table S1. Statistical details of *Dnmt3* gene expression.

Test	Comparison	Function applied	*Dnmt3* expression
Two-tailed Student's *t*-test	Colony A - Nurse *vs.* Forager	log	$t = 2.981$, $df = 12$, $p = 0.0115$
	Colony B - Nurse *vs.* Forager	none	$t = 4.073$, $df = 12$, $p = 0.0015$
	Colony C - Nurse *vs.* Forager	none	$t = 3.592$, $df = 12$, $p = 0.0037$
	Colony D - Nurse *vs.* Forager	none	$t = 4.265$, $df = 12$, $p = 0.0011$
Generalized Linear Mixed Model	Pool - Nurse *vs.* Forager	log	$X^2 = 8.3867$, $p = 0.00378$

Table S2. List of primers used in RT-qPCR and luciferase assays.

	Name	Sequence (5' → 3')	GenBank or miRBase access code	Reference
Gene expression	Dnmt3 - F	CAGCGATGACCTGCGATCGGCGATA	GB55485	DOI: 10.1097/WNR.0b013e32833ce5be
	Dnmt3 - R	TACAGGGTTTATATCGTTCCGAAC		
	Rpl32 - F	CGTCATATGTTGCCAACTGGT	AF441189.1	DOI: 10.1051/apido:2008015
	Rpl32 - R	TTGAGCACGTTCAACAATGG		
	ame-miR-29b - F	TAGCACCATTTGAAATCAGT	MI0005736	This study
	U6 - F	CGATACAGAGAAGATTAGCATGG	GB50324	DOI: 10.1016/j.ibmb.2013.03.001
	U6 - R	GTGGAACGCTTCACGATTTT		
Luciferase assay	Dnmt3 3' UTR - F	CCGCTCGAGTGCGTTGAAACCAATTT	GB55485	This study
	Dnmt3 3' UTR - R	AAGGAAAAAAGCGGCCGCTTTTTGAAGAGCATTATTCGTG		
	ame-miR-29b - F	CCGGACTGATTTCAAATGGTGCTACTCGAGTAGCACCATTTGAAATCAGTTTTTTG	MI0005736	This study
	ame-miR-29b - R	AATTCAAAAAACTGATTTCAAATGGTGCTACTCGAGTAGCACCATTTGAAATCAGT		
	hsa-miR-210 - F	CCGGTCAGCCGCTGTCACACGCACAGCTCGAGCTGTGCGTGTGACAGCGGCTGATTTTTG	MI0000286	This study
	hsa-miR-210 - R	AATTCAAAAATCAGCCGCTGTCACACGCACAGCTCGAGCTGTGCGTGTGACAGCGGCTGA		

Chapter V

3.5 Chapter V

This chapter refers to the following article:

Title: DNA methylation is not a driver of behavioral reprogramming in young honeybee workers

Authors: Carlos A. M. Cardoso-Júnior, Boris Yagound, Isobel Ronai, Emily Remnant, Klaus H. Hartfelder, Benjamin P. Oldroyd

Journal: Manuscript in preparation

DNA methylation is not a driver of behavioral reprogramming in young honeybee workers

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Running title: Social environment does not regulate DNA methylation

Keywords: honey bees, whole genome bisulfite sequencing, amplicon sequencing, DNMT, epigenetics

Abstract

Intragenic DNA methylation, also called gene body methylation, is an evolutionarily conserved epigenetic modification in animals and plants. Its functional role is, however, still unclear. In social insects, gene body methylation has been suggested as a mechanism underlying behavioral plasticity, which is a hallmark of social organization. However, recent studies indicate that gene body methylation is sequence-specific, rather than a mediator of social interactions. To address this apparent contradiction, we examined the whole methylomes of brains and ovaries of young honeybee workers (*Apis mellifera*) subjected to two different social contexts, the presence or absence of the queen in a colony. Despite the remarkable and well-known effect of the queen on gene expression in workers, we found no significant differences in the methylomes of workers from queenright or queenless colonies. Interestingly, thousands of differentially methylated regions (DMRs) were associated with colony genotype, but these differences were not associated with differential gene expression. Actually, we found only nine DMRs comparing the methylomes of both tissues regardless their social condition, indicating that DNA methylation is not a driver of gene expression in these tissues. Furthermore, the finding that the queen presence or absence affects the expression of DNA methyltransferase genes suggest that these genes might have additional roles beyond DNA methylation. Hence, the functional role of the DNA methylation in social insect genomes is once again an open question.

Introduction

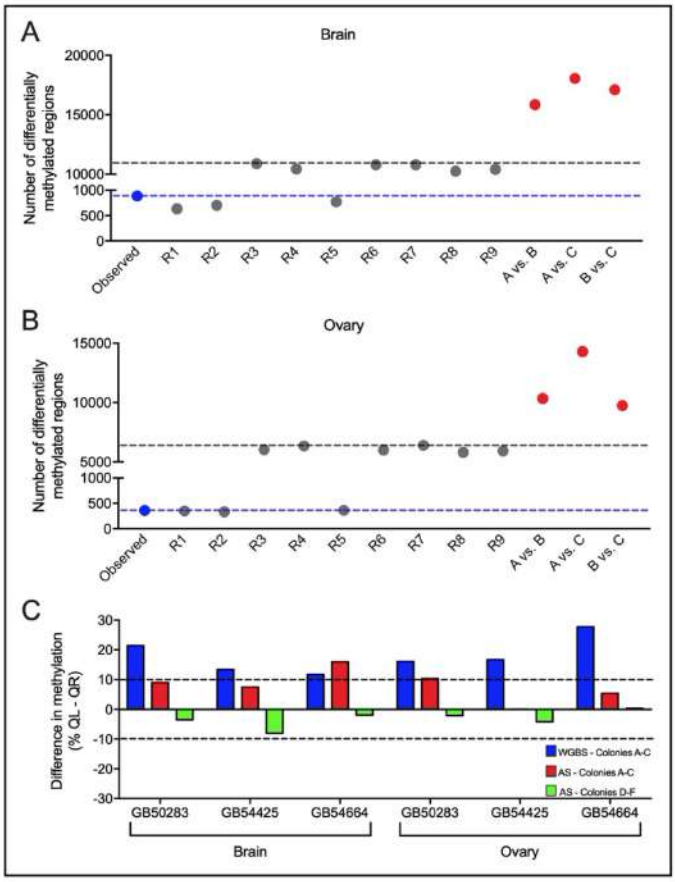
DNA methylation is a reversible chemical modification whereby methyl groups are added to CpG dinucleotides by enzymes of the DNA methyltransferase family (DNMTs). DNA methylation is known to play an important role in regulating gene expression in mammals, such as the maintenance of chromosome X inactivation and genomic imprinting [1]. Its role in invertebrate genomic regulation, however, remains obscure, particularly because the main invertebrate model organisms, the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans,* do not have a methylated genome (*C. elegans*) or show only sporadic levels of methylation at specific developmental time-points (*D. melanogaster*) [2,3]. As the genome sequencing project for the honeybee (*Apis mellifera*) revealed an active and functional epigenetic system comparable to vertebrates [4], this highly eusocial insect has emerged as a model to study epigenetics in invertebrates, also because of its fascinating behavioural repertoire In contrast to mammals, however, the methylation marks in social insect genomes, including honeybees, are sparse and restricted to the gene bodies (intragenic DNA methylation) [5,6], providing thus an excellent opportunity the uncover the role(s) of gene body methylation, in distinction to methylation on promoters or transposable elements.

Gene body methylation has been associated with behavioural and phenotypic plasticity in honeybees and other social insects (reviewed in Yan et al. 2015, 2014). For example, methylome differences were found between larval and adult honeybee queens and workers [5,9] and also in ants [6]. Gene body methylation was also found associated with division of labour among workers of ants [6] and bees [10]. Next, the RNA interference-mediated functional knockdown of *DNA methyltransferase 3* (*Dnmt3*), the gene that codes for the enzyme responsible for *de novo* DNA methylation, was shown to affect 14% of the honeybee worker transcriptome [11]. Even more spectacularly, the knockdown of *Dnmt3* in larvae resulted in the development of a queen-like phenotype, mimicking the effects of royal jelly [12].

74 Despite this apparent support for the hypothesis that gene body methylation drives gene
75 expression alterations in social insects, it is important to note that most of the evidence is
76 correlational and not causal, and most of the analyses were not done in a genetically controlled
77 or homogeneous background. Hence, recent studies taking this into account now provide
78 evidence that argues against this hypothesis, suggesting that DNA methylation is in fact colony
79 genotype-specific rather than playing a major role in the regulation of gene expression.
80 Particularly clear evidence for such colony genotype associated DNA methylation comes from
81 a study that took advantage of the clonal reproductive system of the raider ant (*Ooceraea biroi*),
82 which reproduces asexually, and therefore allows to design experiments without genotypic
83 variants. This study clearly showed that DNA methylation is not associated with phenotypic
84 plasticity in the female castes and suggest that this also may not be the case in other ants or
85 honeybees [13], as a critical re-analysis of the variation reported in previous studies [5,6,9] could be
86 attributed to random effects or as a result of combinations of colony-specific methylation
87 patterns. In line with this, several DNA methylation patterns in honeybees were since seen
88 associated with genotypic DNA signatures [14–16]. Furthermore, a reanalysis of the *Dnmt3*
89 knockdown data published by Li-Byarlay et al. (2013) indicated an overestimate in the number
90 of regulated genes, and that the *Dnmt3* knockdown has only a minor effect on the honeybee
91 gene body methylation pattern [17]. Due to the lack of functional studies (e.g. activity of DNMTs
92 proteins) and sequencing of multiple tissues and time points of social insects, the role of gene
93 body methylation in honeybees and more broadly in other social insects is not yet clear.
94 In this study, our aim was to determine whether gene body methylation may play a role
95 in the fine-tuning gene expression in adult honey bee workers as they are exposed and adapt to
96 different social environmental conditions. To do so, we generated data on gene expression and
97 the methylomes of brains and ovaries of young honeybee workers kept in the presence or
98 absence of the queen. Honey bee queens, through their mandibular pheromones, regulate the

99 behavioural maturation and reproductive capacity of workers [18,19], and this involves changes
100 in the expression of hundreds of genes in the two tissues [20–22]. As the presence or absence of
101 the queen is equivalent to social stability in the colony or of social instability, respectively, this
102 contrast provides a great opportunity to test whether honeybee gene body methylation is
103 associated with or mediates the effect of environmental changes on gene expression, so as to
104 facilitate adaptation to changes in social context.
105
106 **Results**
107 **The queen presence/absence does not cause changes in the brain and ovary methylomes**
108 **of young honeybee workers**
109 We sequenced and analysed the whole methylomes at single base-pair resolution for
110 brains and ovaries of honeybee workers reared in queenright and queenless colonies,
111 respectively. After removing adaptors and reads of low quality and aligning the methylomes to
112 the reference genome, we obtained high coverage (an average of ~35 times) in all sequenced
113 samples. The conversion rate of bisulfite treatment was above 99.5% resulting in an only minor
114 amount of false-positive methylated CpGs. The level of methylated CpGs (~1%) was
115 consistent with previous observations in honeybees and other holometabolic insects [5,6,10,23].
116 To investigate whether the queen's presence promotes alterations in the worker
117 methylomes, we compared those of workers reared in queenright or queenless colonies in two
118 tissues known to respond to the presence of the queen, the brain and the ovaries. We found 885
119 differently methylated regions (DMRs) for the brain (Figure 1A – blue dot, Table S1) and 358
120 DMRs for the ovary methylomes (Figure 1B – blue dot, Table S2). To assess whether these
121 differences were indicative of an effect of the social environment or more simply arose due to
122 between-samples variability, we repeated the analysis by randomly shuffling the samples being
123 compared irrespective of their social condition. The number of DMRs is much higher in the

124 random comparisons (Figure 1A-B – R1 to R-9, grey dots), thus suggesting that finding DMRs
125 in queenless *vs.* queenright comparisons is no more likely than finding DMRs in random
126 pairwise comparisons, and therefore may be false-positives. In contrast, we found 10,000-
127 15,000 significant DMRs in pairwise comparisons between the different colonies, regardless
128 of the social context (Figure 1A-B, red dots). This data indicates that the effect of colony on
129 the worker methylomes is of much greater magnitude than the effect of social environment',
130 which could be masking the effect of social environment on the worker methylomes, if there
131 is any at all.



132

133 **Figure 1.** Differentially methylated regions (DMRs) comparing brains and ovaries of honeybee
134 workers kept in queenright and queenless colonies, respectively. The comparison of the brain
135 (A) and ovary (B) methylomes of workers from queenright and queenless colonies shows fewer

136 DMRs than expected by chance (grey dots). In contrast, when comparing the different colonies
137 (red dots) regardless of the social environment, this returned more DMRs than expected by
138 chance in both tissues. Grey dots represent the number of DMRs obtained by shuffling the
139 methylomes, resulting in nine possible random comparison (R1 to R9) that do not have any
140 biologically relevant basis. Blue dots (observed comparison) represent the number of DMRs
141 retrieved when comparing the methylomes of three queenright *vs.* three queenless colonies.
142 The blue and the grey dashed lines represent the highest number of DMRs found in the
143 observed or random comparisons, respectively. (**C**) Methylation level of three DMRs found in
144 the brain and ovaries for the six colonies (A-F). Blue bars represent the whole-genome bisulfite
145 sequencing (WGBS) for colonies A-C; red bars representing the amplicon sequencing (AS)
146 results for the same samples (A-C) shown in the blue bars. The green bars show the amplicon
147 sequencing data for samples from three additional colonies, described as colonies D-F. Dashed
148 line represents the 10% threshold for considering a region as differentially methylated.
149

150 To further investigate whether and how the social context may impact on the workers'
151 methylomes, we selected 6 DMRs (3 for each tissue) from the queenright *vs.* queenless
152 comparison for amplicon sequencing (AS). Bisulfited-treated DNA extracts from the same
153 colonies used for WGBS (colonies A-C) were PCR-amplified and sequenced in a high-
154 throughput platform. In addition, we added samples from three new pairs of colonies (colonies
155 D-F) to this analysis to check for data reproducibility. We found that the methylation
156 differences (queenless *vs.* queenright) observed in the WGBS data did not reach the differential
157 methylation threshold set at 10%, even after increasing the coverage of same samples by
158 amplicon sequencing (Figure 1C – red bars), or by the addition of samples from three new
159 independent colonies (Figure 1C – green bars). Importantly, the differential methylation pattern
160 of some regions (5 out of 6) from colonies D-F was in an opposite direction to that of colonies
161 A-C. This indicates that the differences found in WGBS comparisons of queenright *vs.*
162 queenless samples are indeed a result of random combinations of colonies A-C, and are not
163 associated with social context neither in the original colonies used in the experiment, not in the
164 three new colonies included to increase coverage.
165

Differential methylation is not associated with differential gene expression when comparing different colonies and social contexts

To investigate whether methylation level differences are associated with alterations in gene expression we first determined the expression levels of genes contained in DMRs of the WGBS data. When comparing workers from queenright *vs.* queenless colonies we found no overall correlation (Figure 2, Two-tailed Pearson correlation, $p = 0.2302$, N = 12, Table S3) between differential methylation and differential expression.

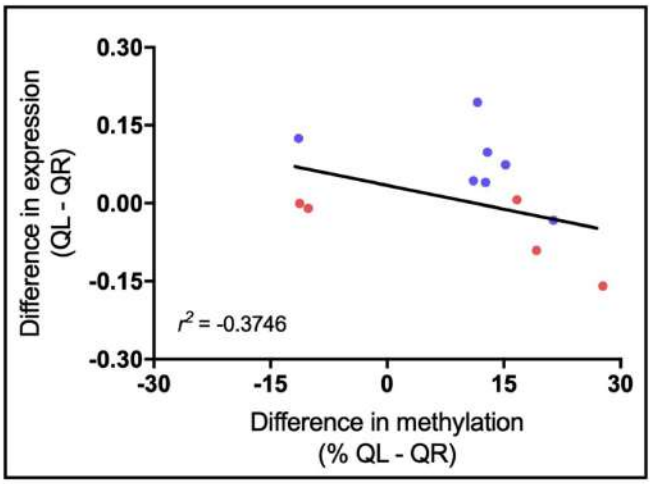


Figure 2. Relative gene expression analysis of differentially methylated genes between queenright (QR) and queenless (QL) colonies. Overall there is no correlation between the level of expression and the degree of methylation (Two-tailed Pearson correlation, $p = 0.2302$, N = 12). Gene expression data were obtained by real-time PCR quantification of eight samples (N=8) per colony (N=3) in each social condition (N=2), resulting in a quantification of 64 samples per gene (N=9). The black line represents the trend line, blue dots represent the expression of differentially methylated genes in the brains and red dots represent the expression of differentially methylated genes in the ovaries.

Given that the genome methylation differences appeared more related to the colony genotype differences than to differences in the social context (Figure 1A-B, red dots), we decided to test whether colony-specific methylation patterns may be associated with differential gene expression between colonies. To do so, we first examined the methylation

187 differences between colonies. These differences between colonies are statistically significant,
188 as they returned a much higher number of DMRs (10,000-15,000) than expected by chance
189 (Figure 1A-B – red dots).

190 We next investigated the colony effect by ascertaining how many regions had altered
191 methylation levels in the brain and ovary of workers, regardless of the social context
192 comparison (Figure 1A-B, red dots). We found large numbers of DMRs in the brain (N= 3,603,
193 Figure 3A) and ovary (N= 1,997, Figure 3B) that differed in their methylation level by at least
194 10% when comparing the same genomic region in the all three colony pairwise comparisons.
195 This indicates that there is a core set of genomic regions hypervariable regarding their
196 methylation status.

197 Next, we determined whether changes in DNA methylation were associated with
198 differential gene expression between colonies (Figure 3C). For these analyses we selected 12
199 genes that were either hypermethylated or hypomethylated in one colony, but differentially
200 methylated in the opposite direction in the other two colonies (e.g., hypermethylated in colony
201 A but hypomethylated in colonies B and C). We first validated the methylation pattern of these
202 regions by AS for colonies A-C (Figure 3C). The results revealed remarkably colony-specific
203 methylation patterns. Thus, if DNA methylation would play a role in regulating gene
204 expression, these genes would be favourite candidates to assess differences in gene expression,
205 as their differential methylation across the different colonies was much more relevant than the
206 differences in social context.

207 For most of the genes we found that even huge alterations on DNA methylation profiles
208 (Figure 3C) did not affect gene expression (Figure 3D). The only exception was the gene
209 GB50283, which was seen differentially methylated in colony C compared to the other two
210 colonies, and which was also differentially expressed (Figure 3C, $p < 0.01$, Table S4). Hence,

after comparing different colonies and strongly opposed social contexts, we conclude that overall, differences in DNA methylation do not drive alterations in gene expression.

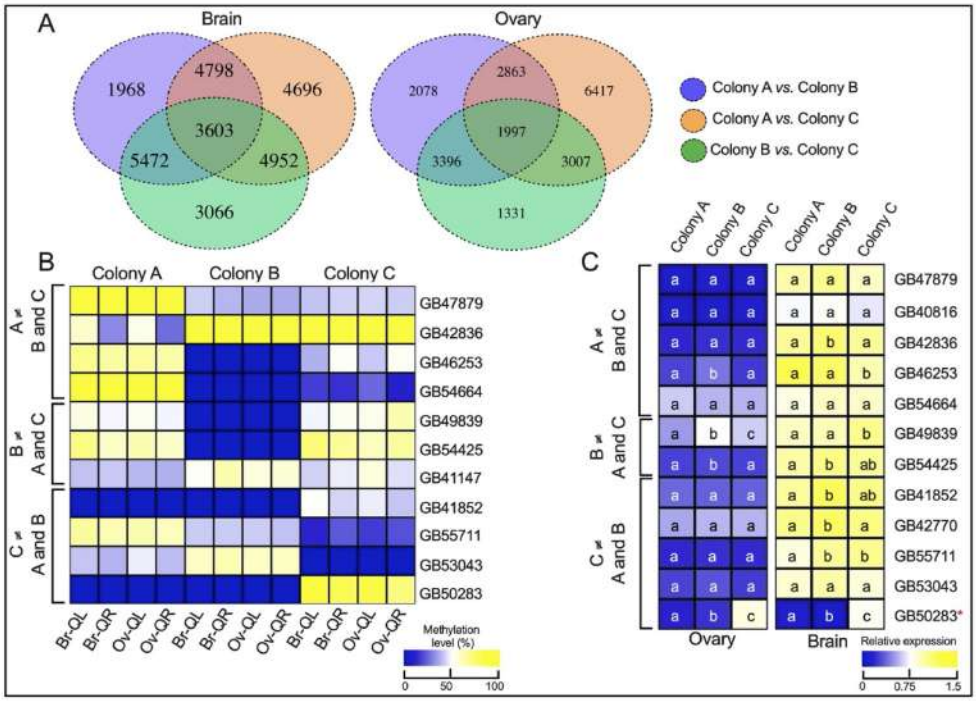


Figure 3. Differences in the methylomes and gene expression levels between colonies. Venn diagrams for the number of DMRs in brains and ovaries **(A)** comparing three different colonies. Numbers inside the intersections represent the regions that present a differential methylation of at least 10% in one colony compared to the other two colonies. For example, the red section represents the regions from colony A that differ in methylation level by at least 10% from the same regions in colony B and C. The intersection of all three comparisons represents the regions with three degrees of DNA methylation levels (for example, 0% methylation level for colony A, 20% for colony B and 70% for colony C). **(B)** Heatmap showing colony-specific methylation obtained by amplicon sequencing of 11 DMRs. For this analysis, we selected genes that have a specific pattern for one colony, but differ from the patterns observed in the other two colonies. Methylation level for the colonies A-C is shown as follow: Br-QL – brain queenless; Br-QR – brain queenright; Ov-QL – ovary queenless; Ov-QR – ovary queenright. (**C**) Colony-specific gene expression analysis of differentially methylated genes. Note that the gene GB50283 (red asterisk) had a compatible methylation pattern (Figure 3B) with its gene expression level. Different letters inside the heatmap boxes indicate statistical differences between colonies (GLMM, N=16 per colony and tissue).

231 Differential expression between tissues was observed for all of the tested genes (Table S4),
232 with the exception of the gene GB54664.
233

234 **The brain and ovary methylomes are highly similar**

235 After showing that DNA methylation is colony-specific rather than influenced by the
236 social context (presence/absence of the queen), we investigated whether there are tissue-
237 specific differences between brain and ovary methylomes. We found only 9 DMRs across all
238 three colonies when comparing the methylomes of the two tissues, regardless of social context
239 (Figure 4A). These regions are allocated within 4 genes. An AS analysis done for two of these
240 regions confirmed the differences in brain and ovary methylation patterns, in line with the
241 WGBS data (Figure 4B). Finally, we tested whether alterations in gene expression between
242 tissues are associated with alterations in the DNA methylation levels. Although 3 out of 4 genes
243 tested were found differentially expressed (Figure 4C, Table S3), the observed pattern (e.g.,
244 higher expression in brain compared to ovaries) was the same for most of the samples analysed
245 in this study (see Figure 3C, Table S4). Thus, while it is not yet clear whether the alterations
246 in methylation content may have driven alterations in gene expression in the comparison of the
247 two tissues, overall, the methylomes of brains and ovaries are very similar, with significant
248 differential methylation seen for only 4 genes.
249

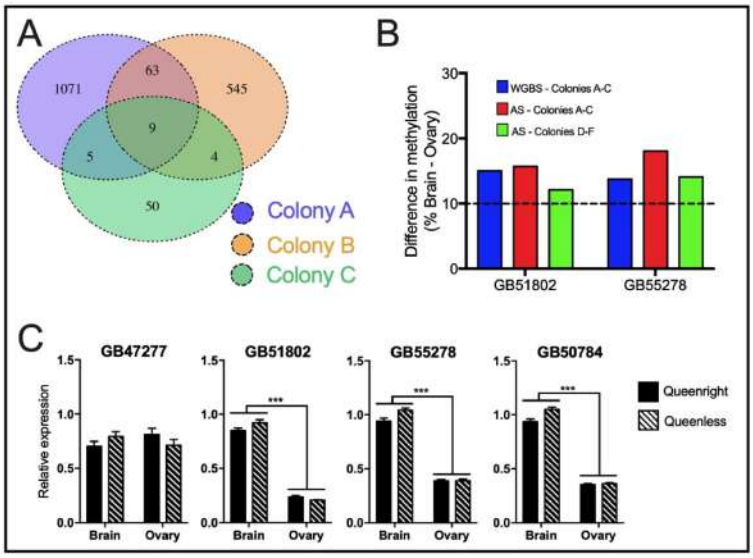


Figure 4. Differentially methylated regions (DMRs) and expression of the respective genes comparing brain and ovaries. **(A)** Venn diagram showing the number of DMRs consistently shared by all three colonies (A-C) comparing the methylomes of brains and ovaries. These nine regions are associated with four genes. **(B)** Validation by amplicon sequencing of two DMRs in colonies A-C (red bars) and D-F (green bars). Blue bars represent the whole-genome bisulfite sequencing (WGBS) data. The Dashed line represents the 10% threshold applied to consider a region as differentially methylated. (**C**) Relative gene expression of the four differentially methylated genes quantified by real-time PCR. Bars represent the mean ± SEM, *** p <0.001. Sample size and statistical analysis are the same as in Figure 3D.

The expression of *Dnmt* genes is regulated by social context

Our data indicate that the presence/absence of a queen does not drive significant alterations in the gene body methylation patterns in the brain and ovaries of young honeybee workers (Figure 1A-C). Overall, there is also no relationship between alterations in either DNA methylation content and gene expression (Figure 2). Nonetheless, previous studies show that the expression of *Dnmt* genes can been used as a *proxy* for global DNA methylation in honeybees [12,24–30]. Therefore, determining whether the expression *Dnmt* genes is affected by the presence/absence of the queen provides a unique opportunity to understand the relationship between *Dnmt* gene expression and gene body methylation. When quantifying *Dnmt* gene

expression in our samples we found that all the 4 *Dnmt* genes predicted in the *A. mellifera* genome are affected by the presence/absence of a queen (Figure 5A and B), both in the brain and the ovary. This suggests that expression of the *Dnmt* genes strongly respond to social clues, regardless of whether this expression results in alterations in the methylomes of honeybee workers.

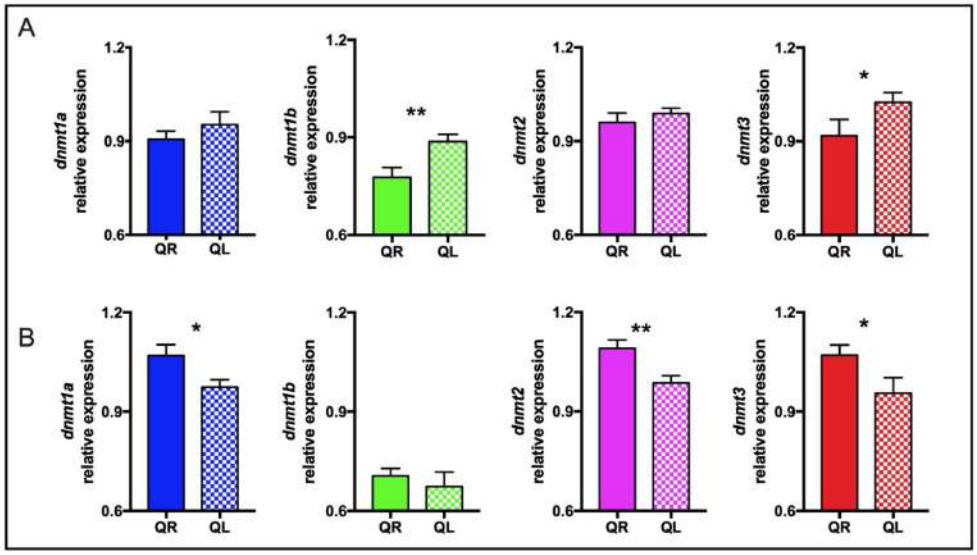


Figure 5. Relative expression of *DNMTs* encoding genes in honeybee workers kept in distinct two social conditions, queen presence (QR) or queen absence (QL). Transcript levels were assessed by quantitative RT-PCR in the brain (A) and ovaries (B) of young honeybee workers. Bars represent the means ± SEM, * $p < 0.05$, ** $p < 0.01$. Sample size and statistical analysis are the same as in Figure 1.

Discussion

Gene body methylation is an evolutionarily conserved epigenetic modification that is phylogenetically widespread across animals and plants [31,32]. Nonetheless its functional role remains obscure for many taxa [33–35]. Taking advantage of the gene body-restricted methylomes of social insects, it was hypothesized that gene body methylation plays a major role in the

287 regulation of gene expression, acting as a mediator between environmental changes and gene
288 activity (revised in [8]). The data that we now obtained in our study clearly contradict this
289 hypothesis. We found that the presence *vs.* absence of a queen, is not associated with significant
290 alterations in the methylomes of young honeybee workers. This is surprising because the
291 presence of a queen is known to strongly affect the behaviour and reproductive traits of
292 workers, associated with major differences in gene expression in the brain and ovaries [18–22].

293 In contrast to our findings, earlier studies showed that honeybee workers kept under
294 different social contexts exhibit altered rates of behavioural maturation, together with discrete,
295 but still significant alterations in their methylomes that were found associated with differential
296 gene expression patterns [10]. A plausible explanation for these contrasting results [15] is that
297 previously observed differences in the methylomes of honeybee workers in response to
298 different social stimuli were in fact a by-product of genotype-associated methylation variants,
299 as these studies used workers from different colonies, i.e, individuals of different genetic
300 backgrounds. Indeed, when the genetic background was controlled by Herb and co-authors
301 (2012), no differences were found comparing the methylomes of newly-emerged queens and
302 newly-emerged workers. The possibility of such genotype-specific methylation patterns, or in
303 other terms colony effects, have been neglected in previous studies and might be the source of
304 epigenetic alterations found in previous studies [13]. Thus, we conclude that the methylomes of
305 honeybee workers do not respond dynamically to drastic periods of social instability (e.g., loss
306 of the queen), whereas gene expression levels do so, thereby indicating that the gene body
307 methylation pattern is not a cause underlying changes seen in gene expression.

308 In line with the hypothesis that gene body methylation is not a driver for gene
309 expression in social insects, we found thousands of DMRs when comparing the methylomes
310 of genotypically-different honeybee workers that are not associated with gene expression. Even
311 when differences in methylation were greater than 70%, as seen when analysing individuals

312 from different colonies (see Figure 3C for details), there was no apparent association between
313 DNA methylation and gene expression, except for one gene (GB50283, Figure 3D).

314 Interestingly, making use of the unique biology of the clonally reproducing raider ant
315 *O. biroi*, DNA methylation patterns were found not to differ between ants in the reproductive
316 and brood-care phases [13], despite major differences in transcription levels [36]. The same authors
317 showed that previous studies that reported epigenetic alterations associated with castes in
318 honeybees and ants failed to control for genotype-specific methylation as a major source of
319 epigenetic variation [13]. This lack of association between epigenetic variation and gene
320 expression has now also been confirmed in bumble bees [16] and in the small carpenter bee
321 *Ceratina calcarata.* Also in this subsocial bee that provides maternal care, alterations in
322 methylomes are not associated with differential gene expression and alternative splicing [37].
323 Moreover, a reanalysis of the *Dnmt3* knockdown data obtained by Li-Byarlay et al. (2013)
324 showed that only a small fraction of the honeybee transcriptome was associated with
325 differential methylation [17]. Together with the results obtained on the clonal raider ant [13,36] and
326 our results, thus, cast doubts on the existence of a link between the gene body methylation
327 pattern and gene expression levels not only in honeybees, but possibly and more broadly also
328 in other social insects.

329 The lack of association between gene expression and gene body methylation is
330 particularly evident in our comparison of the methylomes of the brain and the ovaries.
331 Although brains and ovaries differ widely in their biological functions and gene expression,
332 the methylation state was found nearly identical between these two tissues, with only nine
333 significant DMRs across the three colonies that we studied (Figure 4). Furthermore, we only
334 detected three genes that were both differentially methylated and differentially expressed.
335 These differences may not be causal, as the expression pattern of the three genes were very

similar to most of the genes studied here (Figure 3D). Taken together, we propose that gene body is not a driver for the reprogramming of global gene expression in honeybee workers.

With this it becomes important not only to re-evaluate the role of gene body methylation in social insects [6,9,10,12,23,26,27,29,38], but also to address the role of the expression of gene encoding DNA methyltransferases in these contexts, as these were frequently seen associated with one another [12,24–26,29,39,40]. The expression of *Dnmt* genes has been used as a proxy for actual epigenetic modifications in the DNA [24,25,29,30,41]. The results of our current study show that *Dnmt* genes expression is affected by the presence of a queen, similar to what is seen in other social insects [41]. This suggests that queens influence the expression of *Dnmt* genes in workers over evolutionary time scales in social Hymenoptera, even though this is apparently not reflected in alterations in the workers' methylomes in response to queens. Hence, the expression of *Dnmt* genes should not (cannot) be used as indicating differences in DNA methylation patterns or levels.

The lack of relationship between DNA methylation and *Dnmt* genes expression is evidenced by studies that showed important roles for DNA methyltransferase 1 (DNMT1) that are not associated with alterations in methylomes or transcriptional activity. For example, the knockdown of the *Dnmt1* expression in the red flour beetle *Tribolium castaneum,* which has an unmethylated genome [31,39], results in offspring showing developmental arrest and high lethality [39]. Similar developmental defects in response to *Dnmt1* knockdown was observed in the milkweed bug *Oncopeltus fasciatus*, with remarkable deleterious effects in the reproductive system of females. These alterations are not a consequence of differential gene expression, even though the *Dnmt1* knockdown successfully reduced the global levels of DNA methylation [17]. Thus, it is suggested that the role of *Dnmt1* in milkweed bug development is independent of DNA methylation [17], similar to the red flour beetle [39]. Therefore, we suggest that the DNMTs are required to participate in several biological processes in a methylation-independent manner

361 and there might be other functions for these proteins that go beyond regulating gene expression
362 through differential methylation.

363 The apparently non-functional gene body methylation shown here for the honeybee is
364 not restricted to social insects, but extends other metazoans, as well as to plants. For example,
365 gene body methylation in plants does not regulate gene expression, and gene body methylation
366 is not correlated with the chromosomal placement of H2A.Z, a histone variant that regulates
367 gene expression by preventing genic silencing though spurious methylation of promoters [35,42].
368 It was also shown that gene body methylation is not required for viability in angiosperm plants
369 [35]. Gene body methylation is also not required for viability in social insects, as evidenced in
370 the social paper wasp *Polistes dominula*, which has an almost completely unmethylated
371 genome [43]. A possible explanation is that gene body methylation may act as a standing
372 epigenetic mark that could be useful under new environmental pressures, producing multiple
373 epialleles in a population that could be important to adaptation [44]. While this may be relevant
374 for plants, this seems not to be the applicable to social insects, because even though sequence-
375 specific methylation patterns have been observed in bees [14–16,45] and that DNA methylation is
376 stably inherited [46], gene body methylation in social insects apparently regulates expression in
377 only a very small fraction of methylated genes (this study; Bewick et al. 2019). Thus, it is
378 unlikely that the primary function of gene body methylation in social insects is a standing
379 source for adaptive responses, though this may be possible for a few genes. Another hypothesis
380 to explain gene body methylation in social insects is that this epigenetic modification reflect
381 the degree of social organization, increasing the repertoire of regulatory processes encoded in
382 the genomes of highly eusocial species [47]. Nevertheless, this hypothesis was also contested
383 because no association was seen between DNA methylation and the degree of eusociality [48].

384 With an apparent non-functional role for gene body methylation in the honeybee, we
385 speculate that gene body methylation may have structural functions, orchestrating cell

386 divisions, genomic stability, and other cellular processes that are not directly associated with
387 gene expression, similar to what is currently proposed for other insects [17,39]. In mammals,
388 alternative roles for DNA methylation have also been proposed as acting in genomic integrity
389 and regulation of the cell cycle [49,50], we suggest that dynamic global methylation waves
390 occurring over developmental phases [23,51,52] may contribute to the regulation of such basic
391 biological processes. Hence future work in social insects should consider as a priority
392 functional analyses done to enlighten the relationship between DNMTs, gene body
393 methylation, and basal cellular processes (e.g., cell division), in order to understand the
394 functional role of the enigmatic, evolutionarily conserved gene body methylation. Clearly,
395 alternative explanations going beyond the regulation of gene expression are needed to explain
396 the role of gene body methylation in the genomes of social insects

397 In conclusion, all evidence that we generated in this work went against the hypothesis
398 that gene body methylation could be an important driver for the behavioural programming in
399 adult honey bee workers. With our experimental design using several colonies of controlled
400 genetic backgrounds we had actually expected to provide evidence in favour of this hypothesis
401 and to see clearly defined gene sets with correlated methylation and expression patterns.
402 Nonetheless, as it turned out, the conclusion is that gene body methylation patterns are
403 essentially colony-specific and not related to social context. Furthermore, the results make it
404 clear that future studies need to consider the possible effect of genotype variants (colony
405 genotypes) in their experimental design. In this sense, we believe our work makes a significant
406 contribution to the understanding of the meaning of gene body methylation in a classical
407 epigenetic model, the honey bee. As it stands now, the functional role of gene body methylation
408 in social insects is still an unsolved issue.

409

410 **Methods**

411 **Bees and social environment manipulation**

412 We used workers (primarily *A. m. ligustica*) from six colonies (source colonies) hosted
413 at the University of Sydney and headed by unrelated queens (colonies A-F). The queens were
414 single-mated using a standard protocol [53] of artificial insemination with sperm of a single
415 drone. Hence, the workers used in this study were full-sisters for each colony, with a genetic
416 relatedness of 75%, allowing a maximum control over genotypic heterogeneity in this species.
417 The social manipulation experiments were during the southern hemisphere summers of
418 2017 (colonies A-C) and 2018 (colonies D-F). Queenright and queenless colonies were
419 prepared by splitting each of the six host colonies into two (N=12), thus generating colony
420 pairs of the same genetic background but differing completely in terms of social context. Six
421 of these colonies [colonies queenright A-C and queenless A-C] were initially used for
422 methylome sequencing and subsequently also for the region-specific analyses, whereas the
423 other six host colonies [colonies queenright D-F and queenless D-F] were used for the region-
424 specific analyses only, which served to confirm the reproducibility of the methylome results.
425 Brood frames were equalized among the colony pairs, as the brood pheromones are known to
426 affect the expression of several genes [54,55], including *Dnmts* [24]. To avoid the workers to return
427 to their original hives, colony splitting was done in colonies housed at more than 10 Km far
428 from the University of Sydney one day prior social manipulation. Thus, workers from splitted
429 colonies (QR and QL host colonies) do not fly back to their sister colonies and therefore cannot
430 be merged naturally. One day after splitting the host colonies, the nucs were brought to the
431 University of Sydney, where the newly-emerged workers from six source colonies were
432 introduced to the new social context (e.g. pairs of queenright and queenlees host colonies).
433 Newly-emerged workers were obtained after keeping sealed brood frames overnight in an
434 incubator at 34 °C. These newly-emerged workers were divided in two groups (N=200 bees
435 per group), painted with different colours and then introduced into the splitted pair colonies,

436 queenright and queenless. After four days in the respective social context, the marked workers
437 were collected, snap frozen on dry ice, and stored at -80 °C. Before any further analysis, these
438 workers were dissected for checking their ovary status because we wanted to make sure that
439 only workers without any morphological signs of ovary activation (as may occurred in
440 queenless colonies) would be used in the subsequent molecular analyses.
441
442 **Whole Genome Bisulfite Sequencing (WGBS) and Amplicon Sequencing (AS)**
443 We first performed Whole Genome Bisulfite Sequencing (WGBS) to identify which
444 genes were differentially methylated in the samples from queenright and queenless colonies.
445 For each sample we pooled either 8 brains or 20 pairs of inactive ovaries. Pooling was
446 necessary to obtain sufficient DNA yield for high-throughput sequencing. This resulted in a
447 total of 12 samples, represented by six brains and six ovary samples for each of the social
448 context (three queenright and three queenless). DNA was extracted with the DNeasy Blood
449 and Tissue kit™ (Qiagen) and quantified with Qubit 2.0 Fluorometer system (Invitrogen).
450 Before sequencing, 0.01% (w/w) of unmethylated Lambda DNA (Promega) was added to each
451 sample to be later used in the calculation of bisulfite conversation efficiency. For WGBS, the
452 DNA samples were sent to the Beijing Genomics Institute (China), where library construction,
453 bisulfite treatment and sequencing was performed. Bisulfite treatment was done with the EZ-
454 DNA methylation kit (Zymo Research) and paired-end WGBS was performed on an Illumina
455 NovaSeq platform. Each library was sequenced twice in two separated lanes for high coverage.
456 In the bioinformatics analysis, the data from the two lanes were then merged, as we did not
457 detect relevant differences between the two runs.
458 After processing of the WGBS data and identification of differentially methylated
459 regions (DMRs), we performed paired-end Amplicon Sequencing (AS) for 13 of these DMRs,
460 and we now used a total of six colonies pairs, the original three colony pairs (QR A-C and QL

461 A-C) plus three new colony pairs (QR D-F and QL D-F) to check the reproducibility of WBGS
462 results. DNA extractions were done as described above. Before bisulfite treatment, samples
463 were spiked with 0,1 ng of unmethylated Lambda DNA to calculate bisulfite conversion
464 efficiency, and 200 ng of DNA from each sample (12 for brains and 12 for ovaries) was treated
465 with the EZ-DNA Methylation Kit™ (Zymo Research) following the manufacturer's
466 recommendations. Columns were eluted with 20 µL of ultrapure water, and 1 µL of the eluted
467 solution was used as template in the PCR assays. Bisulfite PCR primers were designed to
468 amplify fragments between 140-300 bp of the forward strand of each differentially methylated
469 region and the Lambda spike-in control (Table S5). Bisulfite-treated DNA was amplified using
470 the primers listed in Table S5 and the KAPA HiFi Uracil+ Kit™ (Roche). PCR amplifications
471 were performed with 5 µL of Kapa Master Mix, 0.3 µL of each primer (forward and reverse),
472 1 µL of bisulfite-treated DNA and 3.4 µL of H_2O, with annealing temperatures as described in
473 Table S5. For multiplexing of the samples into two libraries, Nextera barcodes were added to
474 the 5'ends of all primers. Amplicons from different primers were pooled to generate a separate
475 library for each sample (24 samples in total: queenright and queenless brains and ovaries from
476 each of the colonies A-F). Samples were purified, and Nextera paired-end libraries were
477 constructed at the Australian Genome Research Facility (AGRF). Libraries were constructed
478 in duplicate and sequenced (150 bp paired end) in a single flow cell of an Illumina MiSeq
479 platform. Data from the two libraries were merged for downstream analyses.
480
481 **Differential methylation analyses from WGBS data**
482 Quality of the raw data was assessed by FastQC 0.11.8
483 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) and reads with quality < 20
484 were removed. Trimming was performed with TrimGalore 0.5.0
485 (www.bioinformatics.babraham.ac.uk/projects/trim_galore/) with a stringency error of 2 bp.

Overall, we removed ~ 0.5% of the total sequencing reads after checking read quality and trimming of the adaptors. The remaining reads were mapped onto the honeybee reference genome assembly Amel_4.5 [56] using Bismark 0.16.1 [57] and Bowtie 2 [58]. The coverage varied between 29-41 times across samples. Methylation calling was performed with Bismark software. As threshold for sequencing coverage of each cytosine we established a 10x coverage, same as in Herb et al. (2012), to make these data comparable. Methylation levels were assessed by the C/T ratio of converted cytosines to unconverted bases [48]. Significantly methylated sites were identified using a binomial probability model that takes into account the bisulfite conversion rate for each sample as the probability of success, followed by Bonferroni corrections at the 1% significance level using BWASP [43]. We removed methylated CpGs with >500x coverage to avoid biased analyses.

The MethylKit program [59] was used for differential methylation analysis. First, the honeybee genome was sectioned into 200 bp sliding windows (step size = 100 bp). Only windows containing at least 4 sufficiently covered CpGs (2 in each strand) were analyzed. A difference threshold of 10% in the methylation level between pairwise comparisons was applied, as in Herb et al. (2012). A threshold >10 methylated cytosines (sum for all CpGs inside given window) in at least one of the libraries was used to reduce methylome complexity. Thus, sufficiently covered but unmethylated CpGs in all samples were removed from differential analyses. The list of DMRs was then FDR-corrected and a q-value <0.01 was considered significant. A gene containing at least one DMR was defined as a differentially methylated gene (DMG). Gene annotation was performed with Homer 4.9.1 software [60]. Analyses were performed under *R* environment [61].

We computed the effect of social context on methylation profiles by comparing the three queenright samples (from colonies A-C) against the three queenless samples (from the same colonies). To calculate the number of DMRs in the random comparisons, we shuffled the

511 samples into all nine three-by-three possible combinations. These nine random comparisons
512 lack any biological meaning, as the "social context effect" is completely confounded with the
513 "colony effect" - for example by comparing two queenright colonies plus one queenless colony
514 *vs*. two queenless colonies plus one queenright colony.
515
516 **Differential methylation analyses by amplicon sequencing**
517 Reads were checked for quality using FastQC 0.11.8
518 (www.bioinformatics.babraham.ac.uk/projects/fastqc), followed by trimming of adaptors and
519 removal of low quality sequence (Phred score < 20) using Trimmomatic [62]. Between 85-90%
520 of the reads were retained for each library. BS-converted DNA sequences of the 13 amplicon
521 regions of interest and the Lambda control sequence were used as templates to generate a
522 Bowtie2 index prior to alignment. Data from each of the two duplicate libraries for each sample
523 were aligned with Bowtie2 2.3.5.1 [58] using paired-end default parameters and then converted
524 to BAM files with Samtools [63]. BAM files were imported into the Geneious 10.2.4 program [64],
525 and alignments for each amplicon were manually checked for each sample. C-to-T variant
526 frequencies were calculated using the 'Find variant' function for all CG sites, with a minimum
527 coverage of 50 and minimum variant frequency of 2%. Additional SNP variants that were still
528 visible after bisulfite conversion, such as G-to-A polymorphisms, were also recorded. The
529 overall cytosine methylation frequency was determined for each of the four treatment groups
530 (QR brain, QL brain queenless, QR ovary, QL ovary) for all of the 13 amplicons by dividing
531 the total amount of C (methylated cytosines) per the total amount of C+T (total amount of
532 methylated and unmethylated cytosines). Data was expressed in percent, and when appropriate,
533 regions were compared in relation to their colony, origin social context and tissue type.
534
535 **Gene expression analysis**

536 Each biological replicate consisted of four pairs of inactive ovaries or an individual
537 brain (N=8 per colony and treatment). Ovaries needed to be pooled to obtain a sufficient
538 amount of RNA. Brains and ovaries were macerated in TRIzol (Invitrogen) and total RNA was
539 extracted using the Direct-zol™ RNA™ Miniprep kit (Zymo Research) according to the
540 manufacturer's instructions. Samples were treated with Turbo DNase™ (Thermo-Fisher
541 Scientific) and RNA concentrations were determined using a Qubit 2.0 Fluorometer system
542 (Invitrogen). RNA samples were diluted with ultrapure water to a final concentration of 40
543 ng/µL (brain) and 15 ng/µL (ovary). We used 142.5 ng of ovary RNA and 600 ng of brain
544 RNA to synthesise cDNA using the SuperScript™ III Reverse Transcriptase Kit (Invitrogen)
545 with Oligo (dT) primer (Invitrogen). Ovary cDNAs were diluted to 2 ng/µL whilst brain
546 cDNAs were diluted to 5 ng/µL in ultrapure water. Relative gene expression analyses were
547 done as in [28,65]. We used two endogenous control genes (*Rpl32* and *Ef1α*) to normalize the gene
548 expression of target genes which were stable according to the BestKeeper software [66]. These
549 control genes are validated for honeybee RT-qPCR analyses (Lourenço et al., 2008). The list
550 of primers used is given in Table S5. Primer specificities were confirmed with Sanger
551 sequencing of the respective amplicons (Macrogen).
552
553 **Statistical analysis**
554 Gene expression levels were analysed as dependent variable using a Generalized Linear
555 Mixed Model (GLMM). To investigate the role of social environment on gene expression, the
556 data was modelled with 'colony' as random effect, and 'social context' and 'tissue' as fixed
557 effects, family was set as "Gaussian" and a log function or another type of data conversion was
558 applied whenever needed (see Table S3). As the 'social environment' (presence/absence of the
559 queen) might influence gene expression in opposite directions in different tissues we performed
560 Tukey's *post-hoc* test for all genes (Table S3). To investigate whether colony origin influenced

561 gene expression, 'colony' was treated as fixed effect and 'social environment' as a random
562 effect. Tukey's *post-hoc* test was performed for those genes where 'colony main effect' was
563 significant. The correlation between gene expression and differential methylation was tested
564 using a two-tailed Pearson's correlation analysis. Statistical testing was performed in *R* (R Core
565 Team, 2018) using the packages lme4, car and lsmeans, or in the GraphPad Prism 7 statistics
566 package . For all analyses, a p-value >0.05 was considered significant.
567
568 **Data access**
569 All raw sequencing data generated in this study will be submitted to the NCBI Gene Expression
570 Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/).
571
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579
580 **Author's contribution**
581 CAMCJ conceived the study, performed the field work, carried out the dissections, laboratory
582 molecular work and bioinformatics analyses, and wrote a first draft of the manuscript; BY
583 contributed to the design of the study, performed field work and statistical analyses. ER
584 participated in the design of the study and performed bioinformatics analyses. IR helped with
585 the dissections and participated in the gene expression analyses. KH conceived the study. BPO

participated in the design the study and contributed to the statistical analyses. All authors made critical contributions during the writing of the manuscript and gave final approval for publication

Disclosure declaration

There are no competing interests.

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Attachments to the Chapter V

Table S1. List of differentially methylated regions found in the contrast of the methylomes of workers' brains from queenright (QR) and queenless (QL) colonies (Page 1 out 20).

Nearest promoter	Scaffold Amel 4.5	start	end	pvalue	qvalue	Methylation Difference QL - QR (%)	Annotation
GB54338	16.2	340901	341100	1,1E-13	2,2E-10	31,88951751	K(lysine) acetyltransferase 2A(KAT2A)
GB45692	5.20	171801	172000	3,4E-23	1,7E-18	25,07515907	dentin sialophosphoprotein(LOC552782)
GB52195	6.14	517201	517400	2,4E-12	3,2E-09	23,16673467	dystrobrevin beta(LOC411673)
GB53011	4.16	248201	248400	7,1E-13	1,2E-09	22,96224224	polyphosphoinositide phosphatase(LOC410980)
GB47746	1.2	779801	780000	6,5E-07	8,5E-05	22,35173689	AP-3 complex subunit beta-2(LOC552064)
GB53725	3.9	1155401	1155600	2,1E-12	3,0E-09	21,68858076	splicing factor 3B subunit 1(LOC551331)
GB44977	11.18	3035401	3035600	1,3E-07	2,6E-05	21,41238338	
GB50283	15.19	3804901	3805100	7,2E-11	5,1E-08	21,37045242	uncharacterized LOC551908(LOC551908)
GB49839	11.13	109501	109700	1,4E-06	1,5E-04	21,2889018	sorting nexin-8-like(LOC412842)
GB51222	6.1	582201	582400	9,1E-18	4,5E-14	21,13359344	
GB53011	4.16	248301	248500	2,3E-09	9,9E-07	21,00774189	polyphosphoinositide phosphatase(LOC410980)
GB52192	6.14	470901	471100	1,0E-06	1,2E-04	20,47037977	ATPase family AAA domain-containing protein 2-like(LOC411496)
GB43824	8.8	732401	732600	4,7E-08	1,2E-05	20,37950539	lysoplasmalogenase-like protein TMEM86A(LOC409538)
GB47736	1.2	514001	514200	8,4E-09	2,8E-06	20,33627376	alkyldihydroxyacetonephosphate synthase(LOC725343)
GB50283	15.19	3804801	3805000	6,8E-11	5,0E-08	20,32018025	uncharacterized LOC551908(LOC551908)
GB53245	1.7	546801	547000	1,0E-05	5,8E-04	20,05117763	UBX domain-containing protein 6(LOC409584)
GB53230	1.7	448001	448200	1,9E-09	8,3E-07	19,89308026	adipokinetic hormone receptor(Akhr)
GB52195	6.14	517101	517300	4,6E-10	2,5E-07	19,85214569	dystrobrevin beta(LOC411673)
GB41348	5.18	325101	325300	5,1E-11	4,0E-08	19,21212121	Protein FAM208A
GB53197	1.7	491901	492100	4,2E-05	1,5E-03	18,98266553	
Intergenic	7.21	301	500	1,8E-08	5,4E-06	18,767507	
GB49169	3.14	62301	62500	1,7E-05	8,2E-04	18,71578277	uncharacterized LOC725122(LOC725122)
Intergenic	1.18	50501	50700	3,9E-07	5,7E-05	18,69802388	
GB44164	4.13	909301	909500	2,2E-06	2,1E-04	18,633078	
GB41847	8.9	105301	105500	1,8E-06	1,8E-04	18,57897766	vacuolar protein sorting-associated protein 13D(LOC412701)
GB51555	1.37	1486201	1486400	6,3E-05	1,9E-03	18,57142857	
GB46735	5.9	141601	141800	1,6E-05	7,9E-04	18,48965479	eukaryotic translation initiation factor 2A(LOC552764)
GB44881	11.18	4498101	4498300	2,5E-09	1,0E-06	18,45874416	nuclear pore complex protein Nup133(LOC411735)
GB54541	8.7	927101	927300	8,1E-05	2,2E-03	18,367713	leukocyte elastase inhibitor-like(LOC100577408)
GB52985	4.16	711901	712100	4,7E-06	3,4E-04	18,36458996	ankyrin repeat domain-containing protein
Intergenic	7.21	201	400	2,9E-07	4,5E-05	18,24779267	
GB54119	14.13	904201	904400	1,6E-07	3,0E-05	18,226748	vacuolar protein sorting-associated protein 11 homolog(LOC410493)
GB41448	6.20	798101	798300	5,4E-07	7,3E-05	18,18572089	rho GTPase-activating protein 26(LOC727108)
NA	17.94	51201	51400	6,1E-06	4,1E-04	18,17903192	
NA	17.308	5401	5600	2,4E-05	1,0E-03	18,07858957	
GB50730	2.11	1408001	1408200	3,3E-09	1,3E-06	17,96592844	heat shock protein 70Cb ortholog(LOC408706)
GB42014	5.11	714501	714700	6,5E-05	1,9E-03	17,80689299	E3 ubiquitin-protein ligase TRIP12(LOC409382)
GB51222	6.1	582301	582500	9,4E-13	1,4E-09	17,72844273	
GB52997	4.16	532201	532400	3,6E-06	2,9E-04	17,71576227	lysosomal-trafficking regulator(LOC408774)

Table S2. List of differentially methylated regions found in the contrast of the methylomes of workers' ovaries from queenright (QR) and queenless (QL) colonies (Page 1 out 10).

Nearest promoter	Scaffold Amel 4.5	start	end	pvalue	qvalue	Methylation Difference QL - QR (%)	Annotation
GB54664	15,1	79801	80000	3,5E-15	4,5E-11	27,71505447	multiple epidermal growth factor-like domains protein 10(LOC552479)
GB45135	11,18	1772101	1772300	1,4E-15	2,2E-11	22,6557712	transcription elongation factor SPT6-like(LOC410331)
GB44164	4,13	909801	910000	5,3E-12	1,9E-08	22,63988522	
GB49909	13,12	629801	630000	3,5E-09	3,5E-06	20,29411765	protein argonaute-3(LOC725111)
NA	17,18	34301	34500	1,9E-06	3,6E-04	20,20453173	
GB44056	4,13	910201	910400	1,4E-07	6,2E-05	20,19446522	Stress-induced-phosphoprotein 1
GB43824	8,8	733701	733900	8,9E-09	7,6E-06	19,69207537	lysoplasmalogenase-like protein TMEM86A(LOC409538)
GB45135	11,18	1772201	1772400	2,9E-12	1,2E-08	19,66544458	transcription elongation factor SPT6-like(LOC410331)
GB48371	10,23	630501	630700	9,4E-05	3,5E-03	19,61462451	lebercilin(LOC726219)
GB44164	4,13	909901	910100	1,6E-12	8,0E-09	19,37525944	
GB49839	11,13	110201	110400	1,1E-09	1,5E-06	19,17087542	sorting nexin-8-like(LOC412842)
GB53788	7,1	307301	307500	7,2E-08	3,8E-05	18,56306112	tyrosine-protein phosphatase non-receptor type 9(LOC411227)
GB50274	15,19	3555801	3556000	4,3E-07	1,3E-04	18,52687587	transitional endoplasmic reticulum ATPase TER94(LOC409377)
GB54939	1,41	479001	479200	3,2E-07	1,1E-04	18,35512552	WD repeat-containing protein 7(LOC412288)
GB40405	8,6	422501	422700	6,3E-15	6,8E-11	18,19449554	uncharacterized protein MAL13P1.304(LOC409616)
GB54235	10,24	544001	544200	1,3E-09	1,7E-06	18,08069734	supervillin(LOC551361)
GB42824	9,1	1799801	1800000	1,7E-09	2,0E-06	17,99592653	zinc finger CCCH domain-containing protein 14(LOC724937)
GB54235	10,24	544101	544300	1,9E-09	2,3E-06	17,88629543	supervillin(LOC551361)
GB42781	9,1	811201	811400	2,5E-11	7,4E-08	17,84882077	protein bicaudal D(LOC412961)
GB43824	8,8	733801	734000	1,3E-09	1,7E-06	17,64303811	lysoplasmalogenase-like protein TMEM86A(LOC409538)
GB54288	10,24	565301	565500	1,2E-05	1,1E-03	17,56986807	nucleoprotein TPR(LOC412508)
NA	17,264	4301	4500	1,7E-06	3,4E-04	17,37508488	
GB43099	11,2	715401	715600	2,5E-06	4,2E-04	17,36422172	transcription elongation factor SPT6-like(LOC410274)
GB41886	8,9	1057101	1057300	6,3E-05	2,8E-03	17,11006781	protein transport protein Sec61 subunit alpha(LOC725571)
GB48706	2,3	84801	85000	2,2E-05	1,6E-03	16,98250168	uncharacterized LOC726760(LOC726760)
GB41803	8,9	565701	565900	2,9E-09	3,1E-06	16,95949662	chromodomain-helicase-DNA-binding protein Mi-2 homolog(LOC552031)
GB40911	5,8	462601	462800	7,0E-09	6,4E-06	16,86777527	E3 SUMO-protein ligase PIAS3(LOC551174)
GB40911	5,8	462701	462900	7,0E-09	6,4E-06	16,86777527	E3 SUMO-protein ligase PIAS3(LOC551174)
GB41188	15,3	275601	275800	1,1E-08	9,0E-06	16,74474648	ABC transporter G family member 20-like(LOC724865)
GB54425	15,9	273101	273300	6,2E-06	7,5E-04	16,68159427	cullin-associated NEDD8-dissociated protein 1(LOC409918)
NA	17,43	19601	19800	9,3E-05	3,5E-03	16,65786137	
GB43481	2,7	896301	896500	2,7E-06	4,5E-04	16,61327542	EF-hand calcium-binding domain-containing protein 2-like(LOC412759)
GB45259	11,18	4270501	4270700	1,4E-05	1,2E-03	16,53992062	zinc finger protein 91-like(LOC410108)
GB54939	1,41	479101	479300	2,8E-06	4,5E-04	16,50588071	WD repeat-containing protein 7(LOC412288)
GB44756	1,22	64001	64200	3,3E-07	1,1E-04	16,40905992	uncharacterized LOC727390(LOC727390)

Table S3. Statistical details of GLMM test of gene expression data comparing queenright and queenless social contexts.

		GLMM Main effects			Tukey's Post Hoc test - Queenright *vs.* Queenless	
Genes	Function	Social context	Tissue	Interaction	Brain	Ovary
Dnmt1a	log	0.25593	0.32016	**0.01036**	0.2589	**0.0146 ***
Dnmt1b	exp	**0.00789**	**1,98E-03**	**0.03380**	**0.0093**	0.7309
Dnmt2	sin	0.263029	0.857432	**0.005697**	0.2660	**0.0064 ***
Dnmt3	none	**0.035460**	0.173786	**0.002046**	**0.0383**	**0.0264**
GB42836	log	**1,18E-05**	**< 2.2e-16**	**0,003728**	**<.0001**	0,7802
GB40816	log	**1,34E-02**	**< 2e-16**	**0,0142**	**0,0153**	0,3225
GB46253	sin	0,2484	**< 2.2e-16**	**0,008925**	0.2515	**0.0127 ***
GB54664	log	**6,68E-03**	**5,78E-13**	**1,47E-05**	**0,008**	**0,001**
GB54425	log	0,1255	**<2e-16**	0,7134	0.1290	0.3140
GB49839	log	0,0518	**< 2.2e-16**	**0,0005149**	0.0550	**0.0039 ***
GB41852	log	0,0542	**< 2.2e-16**	**0,036199**	0,0573	0,3025
GB47277	none	0,2166	0,2653	0,06475	0.2198	0.1721
GB51802	log	0,0636	**< 2.2e-16**	**0,003017**	0.0668	**0.0215 ***
GB55278	log	**0,0367**	**< 2e-16**	0,08576	**0,0395**	0,7342
GB50784	log	**2,54E-06**	**< 2.2e-16**	**0,003449**	**0.0002**	0.4273

Table S4. Statistical details of GLMM test of gene expression data comparing different colonies.

		GLMM Main effects			Tukey's Post Hoc test: Brain			Tukey's Post Hoc test: Ovary		
Genes	Function	Colony	Tissue	Interaction	Colony A *vs.* Colony B	Colony A *vs.* Colony C	Colony B *vs.* Colony C	Colony A *vs.* Colony B	Colony A *vs.* Colony C	Colony B *vs.* Colony C
GB42836	log	**0,00763**	**< 2e-16**	0,86222	**0,0179**	0,9855	**0,0277**	**0,0086**	0,6289	**0,0911**
GB40816	log	3,47E-01	**5,49E-08**	0,2245	N/A	N/A	N/A	N/A	N/A	N/A
GB46253	none	**5,38E-05**	**< 2.2e-16**	**0,00216**	0,8099	**0,0003**	**0,0021**	**0,0001**	0,6104	**<.0001**
GB47879	log	0,1586	**<2e-16**	0,5349	N/A	N/A	N/A	N/A	N/A	N/A
GB54664	log	0,75883	0,06077	0,24466	N/A	N/A	N/A	N/A	N/A	N/A
GB54425	log	**2,41E-02**	**< 2e-16**	0,57776	**0,0207**	0,3639	0,3637	0,0331	**1**	0,0332
GB49839	log	**8,74E-03**	**< 2.2e-16**	**1,29E-06**	**0,7397**	**0,0109**	0,0737	<.0001	0,0001	0,0038
GB50283	log	**< 2.2e-16**	**0,0004304**	**8,48E-12**	**0,0015**	**<.0001**	**<.0001**	**0,0046**	**<.0001**	**<.0001**
GB41852	log	**0,02046**	**< 2e-16**	**0,01819**	**0,0196**	0,5962	0,183	0,4323	0,7343	0,8758
GB42770	none	**0,0005485**	**< 2.2e-16**	0,0868629	**0,0013**	0,7878	**0,0104**	0,436	0,4616	0,999
GB53043	log	0,26383	**< 2e-16**	0,9825	N/A	N/A	N/A	N/A	N/A	N/A
GB55711	log	**7,35E-05**	**< 2.2e-16**	**0,04141**	**0,0017**	**0,0004**	0,9185	0,2698	0,9154	0,4825
GB47277	none	**3,64E-03**	0,258242	**2,77E-02**	0,9459	0,0084	0,0206	0,4323	0,7343	0,8758
GB51802	log	**0,02685**	**< 2.2e-16**	0,12508	**0,0274**	0,7041	0,1676	**<.0001**	0,5769	**0,0002**
GB55278	none	0,6912	**<2e-16**	0,3487	N/A	N/A	N/A	N/A	N/A	N/A
GB50784	log	**8,07E-05**	**< 2.2e-16**	0,2969	**0,0004**	**0,002**	0,8892	**0,0003**	0,2326	**0,0465**

Table S5. List of primers used in RT-qPCR and amplicon sequencing. The amplification temperature and references are given. Underlined nucleotides represent the sequence of nextera adaptors.

Aplication	Gene/GenBank accesss	Forward/Reverse	Sequence	Amplification temperature (ºC)	Reference (DOI)
Gene expression analysis (RT-qPCR)	*Dnmt1a* (GB47348)	F	CGAGTAGTAAGCGTGCGTGAA	60	10.1016/j.ibmb.2017.11.005
		R	CAAGTGGTGGAGGAACTGC		
	Dnmt1b (GB48403)	F	GAAATTACATGGGTGGGAGAA	60	10.1038/srep16223
		R	GTCACTGCCTCTTCGAAACC		
	Dnmt2 (GB54141)	F	TGAGTCCTCCATGTCAACCTT	60	10.1038/srep16223
		R	GCCAAATTGACAAGGGCTTA		
	Dnmt3 (GB55485)	F	CAGCGATGACCTGCGATCGGCGATA	60	10.1097/WNR.0b013e32833ce5be
		R	TACAGGG TTTATATCGTTCCGAAC		
	Rp49 (GB47227)	F	CGTCATATGTTGCCAACTGGT	60	10.1051/apido:2008015
		R	TTGAGCACGTTCAACAATGG		
	Ef1 ∝ (GB52028)	F	TGCAACCTACTAAGCCGATG	60	10.1051/apido:2008015
		R	GACCTTGCCCTGGGTATCTT		
	GB40816	F	TGGTTCTTCGGGTGTTCC	63	This study
		R	TGGTGGTGGTCGTTGGCTAA		
	GB41852	F	GGAGATGGTGGTTTCGATGGA	60	This study
		R	ACAGCAATCCCATGCGGTTTAT		
	GB42770	F	CGGTGTAACGTTGAGAATACC	60	This study
		R	GGCACCATAATAGGTAGAAGGA		
	GB42836	F	CCGGAGATATCGTACTTCACGA	60	This study
		R	CGATTGTGTAGTTATTCTGAGCTG		
	GB46253	F	GATTGAAATCACCAGACTCGGA	60	This study
		R	CGTAAAAACTGGAGGTGGTGAA		
	GB47277	F	CCTACTCCACCCGTCCTG	60	This study
		R	GGAACGACCTTGGAAACTCTAT		
	GB47879	F	ATTGGGTTCAAGTACGGCAAC	63	This study
		R	TGGCACGTTTACCTTCTAATCC		
	GB49839	F	CAAGGCGTGATACGAGGAAC	60	This study
		R	ATGAGCATGAACGAGTTGCGTT		
	GB50283	F	AACGACCAACCAAACCGAGCA	63	This study
		R	CGATGACAGGGCGAAAGGC		
	GB50784	F	CCTCCTATGATATGGGAACTTC	60	This study
		R	CATCTGTAGTGCTTGTGCTCG		
	GB51802	F	GCAAACGTATTGTACTTAGCCC	56	This study
		R	GTTGAGCCGGTACTTCATGC		
	GB53043	F	ACAACCAAATAGCGGAACAGTG	56	This study
		R	ATGGGAAATCAGCCTTACTCTC		
	GB54425	F	GCATTAAGAGCTGTAGCAGCAT	60	This study
		R	TTGTGGATGCTTAGCTAACAGC		
	GB54664	F	CAGCAGGATGGGTTGGTG	60	This study
		R	CCATTTTGTGGATTGCAGGC		
	GB55278	F	ACCTGATTCTGAGCATGACCAA	56	This study
		R	CCACATGCCGATTCAGACTAG		
	GB55711	F	GGAATATAACACCTGAGAGAGC	60	This study
		R	TGACCGCTATAGGAATCATCAC		
Amplicon sequencing	GB54664	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTATGTATTATTATTATATTTTGTTAGG	55	This study
		R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCTTACCATTTTATAAATTACAAACAAC		
	GB46253	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGAAATAATTTGTTTTAGAAAATG	55	This study
		R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTTACAAATTTTAAAAAATAAAATCTC		
	GB41147	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGATTTTATAAAGTTATTTGTATAGTATAG	55	This study
		R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAATCAATATCTACAAACATCCTC		
	GB41852	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGATTTATATTTGAATAGTATTATT	55	This study
		R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCAACATCTTTTCCAATAAAAATACCC		
	GB42836	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTAATTTATATTTTTATTATTTTTTTTGTAG	55	This study
		R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCAATAACAAATAATTAAAAATTTTCAAACC		
	GB47879	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTAGATTTATGAAGGTATAATGTAAATG	55	This study
		R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTTCTATTTCACCTTAAAAATTTACTAC		
	GB49839	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTAAGTAGTTAGAGAGGAAATAATGG	55	This study
		R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTTAAAACTATATATAAATCATATACC		
	GB50283	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGATTATTTATATTTTGTGTAGGTATTTTG	60	This study
		R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCACATACCTAAATAATAAAATCACC		
	GB53043	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTTATGGTTGTAAATAATGGTAAATGTG	55	This study
		R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCTTTTAAAAAAAATCCAAAATTAAATCC		
	GB54425	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAAAGATTAATTTGTTATTGTAAGATG	60	This study
		R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCAATATAATAAATCAAAACTAAAATATC		
	GB55711	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGAATAAGAATTTTTTTGGGGATAAGATG	55	This study
		R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCATTATTTACCCTTAATAATTCTCCTTC		
	GB51802	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGATTGGAAATAATGAAAATTTTAATG	55	This study
		R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCTTTAATACATCTTTAAAAAATTCATC		
	GB55278	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGAGTTTATTGGAATGTAATATTTG	55	This study
		R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTTTTTCCCTAAATACTACAAAACATAC		
	L516 (lambda amplification)	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGGAAGAGGATGGTGTAGTAATTAATAAGA	55	This study
	L517 (lambda amplification)	R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAACAAAACTACATCCACACTTTCACTC		

Final considerations

4. Final considerations

Honeybees exhibit an active and functional epigenetic system for DNA methylation and histone modification [68,69,91]. Here, we studied the role of DNA methylation in the context of the "reproductive *vs.* longevity trade-off", a central factor in metazoan life histories, but which is clearly violated in honeybees and other social insects [46,47]. For insights into the regulation of DNA methyltransferase 3 (DNMT3), a key determiner of epigenetic reprograming via DNA methylation, we studied its regulation at the transcriptional, post-transcriptional and post-translational levels during a major transition in the adult life cycle of worker bees, the transition from brood care to foraging behaviour. Moreover, we investigated the expression of DNMTs and histone-modifiers encoding genes in response to environmental cues, such as queen pheromones and the presence of larvae or young adults in a colony. Finally, we generated whole methylome sequencing data at single base-pair resolution of brains and ovaries of workers subjected to different social contexts, especially the presence or absence of a queen, a situation that naturally occurs in the life history of honeybees.

In the first chapter we report that the *Dnmt3* gene is upregulated in the abdomen of old honeybee workers, suggesting a role for DNA methylation in aging. Inhibition of the enzymatic activity of DNMTs caused genomic hypomethylation, increased the lifespan of workers and an upregulation of vitellogenin, a protein synthesized by the fat body that is associated with behavioural maturation in workers and reproduction in the queen [92–94]. These results suggest that DNA methylation participates in aging processes of fat body cells. However, in Chapter V we show that, contrary to expectation, the methylomes of brains and ovaries are highly similar, despite the major functional difference between the two tissues. This strongly indicates that DNA methylation does not control differential gene expression in honeybees. Therefore, we now suggest that the effects of DNMT inhibition on the lifespan of workers are not directly linked to DNA methylation. Instead, we propose that DNMT enzymes are likely to have other, previously unreported functions, and one of them would be the regulation of the workers' lifespan.

We have also observed that the majority of *Dnmt* genes is differently regulated by several environmental factors that affect aging in workers. For example, in Chapter II we provide evidences that the *Dnmt1a*, *Dnmt2* and *Dnmt3* genes dynamically change their expression over different time points of the yearly colony cycle, in response to the presence/absence of brood and young workers.

In addition to differential regulation of histone-modifier genes, dynamic responses in the expression of *Dnmt* genes were also observed in the results presented in the Chapter III. In the experimental design for this chapter, the expression of *Dnmt* genes was found altered in the brains of workers in response to the queen mandibular pheromone, a key regulator of colony social cohesion.

Taken together, we propose that alterations in the expression of *Dnmt* genes are not directly linked to the reprogramming of gene expression via alteration in epigenetic marks, such as differential DNA methylation. Rather, the differential expression and regulation of *Dnmt* genes indicates their participation in important social contexts experienced by a honeybee worker; however, independent of alterations in DNA methylation marks. This conclusion is also reinforced by our gene expression, biochemistry, and *in silico* analyses of DNMT3 properties presented in Chapter IV. Interestingly, here we found that the DNMT3 protein predominantly localizes inside lipid vesicles of the cytoplasm, not in the nucleus as expected based on its DNA methylating enzymatic activity [91]. This unexpected subcellular location indicates that the primary function of DNMT3 is probably not to methylate the nuclear DNA, as this enzyme was only rarely found inside the nucleus of fat body cells of honeybee workers. Thus, a reinterpretation is needed for the previously published reports showing that the functional knockdown of Dnmt3 via RNA interference in larvae and adult honeybees, resulted in a queen-like phenotype and altered alternative splicing in fat body tissue, respectively [30,44].

In line with our findings for DNA methylation in honeybees, it has been shown for clonal raider ants, which reproduce asexually and therefore, have a genetically homogeneous colony composition, that DNA methylation is not associated with differential gene expression [55,95]. Instead, the authors proposed that there is a strong "colony effect" in the previous reports [36,96], attesting that previous reports on DNA methylation patterns neglected this colony effect in their experimental design, leading to the erroneous interpretation that DNA methylation is a key regulation in caste differentiation and social regulation [55]. Thus, the data from the clonal raider ant and our data indicate that DNA methylation is more likely sequence-specific [56,97] rather than dynamic in response to environmental changes. By controlling the genetic background of workers, as done in the experimental design for the methylome analyses, we provide evidence that DNA methylation does not control gene expression, but is rather colony-specific in honeybees, and possibly so also in other social insects. Thus, we show for the first time in honeybees that DNA methylation is not a major regulator of gene expression. Nonetheless, the results of our other experiments suggest that DNMTs might regulate gene expression in ways other than via DNA methylation.

In conclusion, the results generated in this work shed light on important aspects of the epigenetic machinery, its regulation, and its roles in the life cycle of adult honeybees. These data change our interpretation of epigenetic systems and their role in invertebrate genomes, adding important pieces to the epigenetic puzzle of invertebrates, and requiring major reinterpretations of the results from previously reports in the literature.

Conclusions

5. Conclusions

This study focused on the regulatory roles of epigenetic mechanisms underlying several aspects of the social life of honeybees, including aging, reproduction, and social communication. We found that the expression of *Dnmt* genes changes over time and responds to diverse social clues, such as pheromones and the presence of larvae or young adults in the colony.

We demonstrated that the inhibition of DNMT activity increases the workers' lifespan. This is likely due to an effect on vitellogenin expression, which is activated upon genomic demethylation. Next, we showed that the expression of *Dnmt* genes is regulated at different time points and tissues in response to the presence/absence of the queen, larvae, or young adult worker bees. All these social factors influence aging in honeybee workers, suggesting a critical role of DNMTs in behavioural maturation.

Surprisingly though, we found that DNA methylation itself is not as dynamic as the expression of DNMT encoding genes. While the expression of *Dnmt* genes are highly responsive to social cues, the methylomes of brains and ovaries tissues did not show significant alterations in response to the queen's presence or absence, even though her presence has a major effect on gene expression in workers. Thus, we conclude that DNMTs may have functions additional to DNA methylation. Also, we propose that DNA methylation in honeybees (and possibly in other social insects) does not control gene expression to the extent that was previously hypothesized. Our data, which revealed a strong colony effect in the worker brain and ovary methylomes, emphasize that more attention is needed in the design of experiments on DNA methylation, and call for a careful assessment of the results, taking into account the methylation profile specific for each colony.

A deep investigation of the regulators of DNMT3 and its biochemistry and molecular properties also reinforces the hypothesis raised in this study that DNMTs protein might possess multiple functions associated with social behaviour in a mode independent of DNA methylation events. Importantly, we found that two epigenetic mechanisms (*Dnmt3* and its regulation by the microRNA 29b, an epi-microRNA) form a regulatory circuitry. Therefore, we conclude that the presence of an active and efficient epigenetic system, operating within multiple aspects of the social life of honeybees, is an essential component in orchestrating the adult life cycle of queens and workers. Our data highlight for the first time that there is a discordance between the expression of *Dnmt* genes, the DNA methylation status, and its control on differential gene expression.

List of articles

6.1 Publications in peer-reviewed scientific journals

- ARAUJO, NAIARA*; **CARDOSO-JUNIOR, CARLOS A. M.*;** SILVA, E. H.; UEIRA-VIEIRA, C.; BELETTI, M. E.; FRANCO, M. B.; BEZERRA-LAURE, M.; ARAUJO, G.R.; TRAVENÇOLO, B.A.N.; BONETTI, A.M. Three-dimensional reconstruction of *corpora allata* nucleus reveals insights into epigenetic mechanisms of caste differentiation in *Melipona scutellaris* stingless bees. APIDOLOGIE v. 50, p. 330-339, **2019.** *** Equal first authorship contribution**.

- **CARDOSO-JÚNIOR, CARLOS A.M.**; FUJIMURA, PATRÍCIA TIEME; SANTOS-JÚNIOR, CÉLIO DIAS; BORGES, NAIARA ARAÚJO; UEIRA-VIEIRA, CARLOS; HARTFELDER, KLAUS; GOULART, LUIZ RICARDO; BONETTI, ANA MARIA. Epigenetic modifications and their relation to caste and sex determination and adult division of labour in the stingless bee *Melipona scutellaris*. GENETICS AND MOLECULAR BIOLOGY. v. 40, p. 61-68, **2017.**

- **CARDOSO-JÚNIOR, CARLOS A. M.**; SILVA, RENATO PEREIRA; BORGES, NAIARA ARAÚJO; DE CARVALHO, WASHINGTON JOÃO; WALTER, S. LEAL; SIMÕES, ZILÁ LUZ PAULINO; BITONDI, MARCIA MARIA GENTILE; UEIRA VIEIRA, CARLOS; BONETTI, ANA MARIA; HARTFELDER, KLAUS. Methyl farnesoate epoxidase (*mfe*) gene expression and juvenile hormone titles in the life cycle of a highly eusocial stingless bee, *Melipona scutellaris*. JOURNAL OF INSECT PHYSIOLOGY. v. 101, p. 185-194, **2017**.

- CERVONI, MÁRIO S.; **CARDOSO-JÚNIOR, CARLOS A. M.**; CRAVEIRO, GIOVANA; SOUZA, ANDERSON DE O.; ALBERICI, LUCIANE C.; HARTFELDER, KLAUS. Mitochondrial capacity, oxidative damage and hypoxia gene expression are associated with age-related division of labour in honey bee, *Apis mellifera* L., workers. JOURNAL OF EXPERIMENTAL BIOLOGY v. 220, p. jeb.161844-4046, **2017**.

6.2 Manuscripts under review

- **CARDOSO-JUNIOR, CARLOS A. M.;** OLDROYD, BENJAMIN; RONAI, ISOBEL. Vitellogenin expression in the ovaries of adult honeybee workers provides insights into the evolution of reproductive and social traits. (BioRxid DOI: 10.1101/547760). **2020**.

- HARTFELDER, KLAUS.; …; **CARDOSO-JUNIOR, CARLOS A. M.;** …; PINHEIRO, DANIEL. The nuclear and mitochondrial genomes of *Frieseomelitta varia* – a highly eusocial stingless bee (Meliponini) with a permanently sterile worker caste. **2020.**

6.3 Manuscript in preparation

- TIBERIO, GUSTAVO.; **CARDOSO-JUNIOR, CARLOS A. M.**; LAGO, DENYSE.; HARTFELDER, KLAUS. Epigenetic regulation of ovary developmental plasticity in honeybee larvae.

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