

Review



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The role of epigenetics, particularly DNA methylation, in the evolution of caste in insect societies

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Eusocial insects can be defined as those that live in colonies and have distinct queens and workers. For most species, queens and workers arise from a common genome, and so caste-specific developmental trajectories must arise from epigenetic processes. In this review, we examine the epigenetic mechanisms that may be involved in the regulation of caste dimorphism. Early work on honeybees suggested that DNA methylation plays a causal role in the divergent development of queen and worker castes. This view has now been challenged by studies that did not find consistent associations between methylation and caste in honeybees and other species. Evidence for the involvement of methylation in modulating behaviour of adult workers is also inconsistent. Thus, the functional significance of DNA methylation in social insects remains equivocal.

This article is part of the theme issue ‘How does epigenetics influence the course of evolution?’

1. Introduction

Eusocial insects live in colonies with distinct queen and worker castes [1]. Queens produce most or all of the eggs, while workers are either totally or partially sterile. Workers reproduce either vicariously by rearing their sisters and brothers—the offspring of the resident queen—or, rarely, directly [2]. All termites (Blattaria: Isoptera) [3] and ants (Hymenoptera) [4] are eusocial, although some species have secondarily lost their worker or queen caste. A few bee and wasp (Hymenoptera) genera are eusocial [5], as are a few ambrosia beetles (Coleoptera) [6] and gall-forming thrips (Thysanoptera) [7].

Despite their importance and inherent biological interest, the very existence of social insects remains an evolutionary puzzle. First, their morphologically distinct castes arise from genetically identical eggs (polyphenism). How can alternate developmental trajectories from alike genomes stably result in such behaviourally and morphologically distinct creatures as a massive and hyper-fertile queen, a soldier army ant and a minor worker? Second, many insect workers change behaviourally over their lifetime, flexibly engaging in new roles as they mature. For example, a honeybee worker begins life engaged in nest-bound tasks such as cell cleaning and feeding larvae, and later progresses to foraging [8,9]. How these changes are orchestrated at the individual and colony level has not been fully elucidated. These proximate questions also need to be resolved in the evolutionary context: how did behavioural and morphological castes evolve?

In the literature of insect colonies, ‘caste’ has several meanings, which we need to clarify. In this review, we will recognize three kinds of caste: (i) morphologically distinct queens and workers of the ‘highly’ eusocial insects like termites, honeybees, stingless bees, vespine wasps and most ants. In these species, queens and workers arise as a consequence of distinct developmental trajectories that are irreversible. We refer to these castes as queen–worker

(Q-W) castes; (ii) in some species, there are morphologically specialized workers such as soldier ants and termites that are larger (especially in the head) and more armoured than normal workers [10,11]. These we refer to as worker subcastes; and (iii) finally, in species with only one worker caste, there are often behavioural castes such as reproductive and non-reproductive workers, or nurses and foragers. Behavioural castes are generally reversible and are morphologically identical. We refer to these as behavioural castes.

Our aim in this review is to discuss whether epigenetic mechanisms, particularly DNA methylation (hereafter 'methylation'), has played a role in the evolution of castes in social insects. In a companion paper, we discuss epigenetic inheritance in social insects, and its possible role in genomic conflict.

2. Queens and workers come from the same genome, so differential development must have an epigenetic basis

An animal's body is usually generated from a single cell, the zygote, and all of the somatic cells of the animal are a clonal lineage. The course of development is, therefore, dependent on epigenetic processes that permanently shut down some genes at particular time points, while allowing others to be expressed. These epigenetic changes are then faithfully transmitted to daughter cells, leading to the development of tissues and organs [12]. Similarly, queens, kings and workers, and the specialized worker castes of ants and termites, arise from more or less identical genomes. For the most part, any egg can develop as a queen/king or a worker, or, where they exist, morphologically specialized worker subcastes [13].

In termites, a combination of inhibitory factors produced by the king and queen, together with seasonal factors, seem to determine whether nymphs of both sexes mature as workers or as future reproductives [14,15]. In contrast with termites, in most Hymenopteran species, the direct cause of differential development is nutritional [16]. Queen-destined larvae are fed more [17] and different food [18–21], to worker-destined larvae. In some instances, these factors are thought to result in different reproductive and morphological castes via perturbations to the insulin-signalling and other growth-related pathways [22–24].

3. DNA methylation as a proposed mechanism underlying queen/worker polyphenism

Methylation involves the attachment of a methyl group to cytosines, often in a CG/GC (referred to as CpG) context so that the pair of adjacent cytosines across strands are both methylated [25]. Methylation is evolutionarily conserved from bacteria to eukaryotes [26,27]. In insects, methylation is restricted to gene bodies, especially at intron–exon boundaries and at the 5' end of genes [25,28,29]. There is a strong association between gene function and the degree of methylation across invertebrates, with 'housekeeping' genes showing the highest levels of methylation [30] and highly methylated genes tending to show greater sequence conservation across diverse taxa [30–32]. This suggests that gene

body methylation has function, though it is unclear what those functions may be. One proposed function is that methylation is involved in differential gene splicing and in regulation of expression [33–38].

Methylation is mediated by DNA methyl-transferase (Dnmt) enzymes. Dnmt1 is thought to be responsible for the faithful transmission of methylation states across cell divisions, while Dnmt3 is responsible for de novo methylation [39]. Most social insect species examined have methylation, as evidenced by the presence of *Dnmt*-coding genes in their genomes, and evidence of methylation in their DNA [40–42]. Therefore, methylation could potentially be involved in regulating differential caste development and behaviour in social insects.

In a landmark paper, Kucharski *et al.* [43] suggested that methylation is the primary mechanism by which Q-W dimorphism is orchestrated in honeybees. They proposed that the default developmental pathway is the queen phenotype, and that the more Spartan diet of workers (less volume, lower fatty acid content and, particularly, lower carbohydrate content) relative to queen larvae [19], leads to the methylation of genes whose expression is then changed to generate worker morphology. Support for this hypothesis was lent by the demonstration that the knockdown of expression of *Dnmt3* in young larvae (i.e. those that are capable of either developmental trajectory), using RNA interference, led to the development of the queen phenotype. This, it was argued, is a direct result of reduced methylation.

Following the Kucharski *et al.* [43] paper, several studies reported differences in methylation patterns between queen and worker castes in bees, ants and termites (table 1). In the honeybee, eight studies have supported such differences, whereas three did not. One of the studies that did not show differences was based on methylation-sensitive restriction fragment length polymorphisms [47], a technique that has low sensitivity. Another did not directly compare methylation patterns across castes [51], but the other [46] used gold-standard genomic procedures.

There is a trend, not always consistent, that in studies which have shown differentially methylated genes, methylation is higher in workers than in queens. Gene ontology studies of honeybee genes that are differentially methylated show that they tend to be related to metabolism regulators [36,44,58]. This finding points to a mechanism whereby the reduced feeding received by worker-destined larvae causes an increased level of methylation, and the development of the worker phenotype [59]. The most recent study of methylation levels between queen- and worker-destined larvae showed divergent methylation levels at 3 and 4 days of age, before equalizing at day 5.

4. Criticisms of the 'methylation is key to queen–worker caste polyphenism' paradigm

If methylation is key to Q–W caste differentiation in social insects, we can make three predictions: (i) methylation patterns should consistently differ between castes particularly at the larval/nymphal stage when differential development begins; (ii) methylation patterns should be in the same direction for the same genes across all or most individuals of a particular caste. For example, if we find that a gene is heavily methylated in workers and under-methylated in queens,

Table 1. Studies investigating differences in DNA methylation patterns between queen and worker castes in social insects. (WGBS, whole-genome sequencing of bisulfite-treated DNA; MeDIP, methylated DNA immunoprecipitation-sequencing; MSRELP, methylation-sensitive restriction fragment polymorphisms; RRBS, reduced representation bisulfite sequencing.)

species	queens and workers strongly morphologically divergent?	differences found	kind of study	biological replication	reference	year
honeybees	yes	yes	bisulfite sequencing of a single gene, experimental manipulation	n/a	[43]	2008
<i>Apis mellifera</i>	yes	yes	bioinformatic study of existing datasets. Genes that are differentially expressed between queens and workers show evidence of methylation over evolutionary time	four 'caste specific' genes showed differential methylation	[44]	2009
<i>Apis mellifera</i>	yes	yes	WGBS of 50 pooled queen brains and 50 pooled worker brains	none at individual level	[45]	2010
<i>Apis mellifera</i>	yes	no	WGBS and an array-based system	five adult queens and workers	[46]	2012
<i>Apis mellifera</i>	yes	yes	WGBS of 96 pooled queen and 96 pooled worker larval heads	none at individual level	[36]	2012
<i>Apis mellifera</i>	yes	no	MSRELP	five each: adult and pupal queens and workers	[47]	2013
<i>Apis mellifera</i>	yes	yes	MeDIP	nil at three ages	[48]	2013
<i>Apis mellifera</i>	yes	yes	amplicon bisulfite sequencing of two genes	three sets of five brains from queens and workers	[49]	2017
<i>Apis mellifera</i>	yes	yes	MeDIP	pools of 10 larvae, three queen and three worker	[50]	2017
<i>Apis mellifera</i>	yes	no, but no direct comparison across castes	WGBS and RNA sequencing	two biological replicates and multiple tissues	[51]	2019
<i>Apis mellifera</i>	yes	yes	WGBS of queen-destined and worker-destined larvae at three ages	tree replicate colonies but samples pooled across colonies	[52]	2020
bumblebee	size only	no	MSRELP	five each: adult and pupal queens and workers	[47]	2013
<i>Bombus impatiens</i>						

(Continued.)

Table 1. (Continued.)

ants	species	queens and workers strongly morphologically divergent?	differences found	kind of study	biological replication	reference	year
	<i>Camponotus floridanus</i>	yes	yes	WGBS of whole individuals	two biological replicates but queens and workers harvested from different colonies	[37]	2012
	<i>Harpegnathos saltator</i>	minimal	yes	WGBS of whole individuals	two biological replicates	[37]	2012
	<i>Pogonomyrmex barbatus</i>	yes	yes	MSRFLP	adult virgin queens ($n = 9$) versus workers (72)	[53]	2012
	<i>Ooceraea biroi</i>	no	no (between reproductive phases)	WGBS of adult brains	four replicates of pools of 20 brains	[54]	2016
	<i>Dinoponera quadricreps</i>	no	no (between gamergate and worker)	WGBS of adult brains	three biological replicates	[55,56]	2015
	<i>Formica exsecta</i>	yes	yes	RRBS	comparisons of 5–7 old and young workers and queens randomly selected from 19 colonies	[57]	2019
wasps	<i>Dolichovespula maculata</i>	size only	no	MSRFLP	five each: adult and pupal queens and workers	[47]	2013
	<i>Polistes dominula</i>	no	yes	MSRFLP	five each: adult and pupal queens and workers	[47]	2013
	<i>Polistes canadensis</i>	no	no	WGBS of adult brains	three biological replicates	[55,56]	2015
termites	<i>Zootermopsis nevadensis</i>	yes	yes	WGBS of male and female workers and alates	replicate individuals from a single colony	[33]	2016

suggesting that methylation of this particular gene is causal of caste differentiation, we would not expect too many exceptions to this. In particular, we would not expect to find queens in which this target gene is heavily methylated, or workers where it is not. Furthermore, we would expect methylation patterns of these differentially methylated genes to be correlated with caste and to be in a consistent direction regardless of genotype or colony origin; and (iii) DNA methylation should affect gene expression and/or gene splicing. That is, to show a causality, we would like to see that the differential methylation of the gene that we think has something to do with caste differentiation actually changes expression in response to methylation.

How well does the evidence stack up against these predictions? Libbrecht *et al.* [54] have argued that evidence for caste differences in methylation patterns may be artefacts of insufficient biological replication. If queens and workers are unevenly sampled from different colonies, genetic backgrounds (e.g. patriline) or time points, this can give the appearance of caste-specific methylation where none exists. In studies where there has been adequate biological replication (i.e. replicated colonies are considered), it is often the case that genes are differentially methylated between workers and queens in different directions in different colonies [51,60]. Strikingly, many whole-genome sequencing studies that have reported large differences in methylation between queens and workers lacked biological replication [36,45] or pooled biological replicates [52]. By contrast, the only well-replicated study [46] did not find any differences between queen and worker castes.

Other studies have indeed found caste-specific methylation patterns across replicate colonies in honeybee larvae. Two of these studies used methods that detected only a small fraction of methylated sites. Welsh *et al.* [49] focused on two genes, *cabin-1* and *nadrin-2*, that were previously identified as being differentially methylated. Shi *et al.* [50] used methylated DNA immunoprecipitation-sequencing, a procedure that detects only a small proportion of methylated cytosines, and in a biased way [61]. In Shi's experiment, methylation appeared to be strongly biased towards intronic sequences. Because most DNA methylation occurs in gene bodies in insects [38,45,52], the significance of low levels of differential methylation in introns is unknown.

Finally, there remains the question of function. Gene body methylation is generally thought to regulate gene transcription and to affect alternative splicing [38,45,62]. However, recent studies have convincingly shown that there is no overall link between methylation, gene expression and alternative splicing in social insects [51,55,63]. Indeed, mounting evidence indicates that methylation patterns are conserved during development in honeybees [51,64,65]. These results further question the role of methylation in caste determination.

We note that some of the above criticisms remain equivocal. For instance, the honeybee studies that have not found differences between castes (table 1, [46,47,51]) have compared samples from the adult stage and not the larval stage during which the alternate queen versus worker developmental trajectories are laid down. It is thus possible that queens and workers harbour differential methylation at the larval stage and that this causes differential gene expression [43,52], but this differential methylation is lost during the transition to the adult moult. Further, several of the studies that have not found differential methylation between queen and worker castes (table 1) were done in species that lack

morphologically different castes [54,55]. These findings in caste-free species may be irrelevant to species like honeybees with irreversible morphological queens and workers.

In the light of these criticisms (and the criticisms of the criticisms), we conclude that the jury is still out on whether DNA methylation plays a role in Q–W caste differentiation. Resolving this question will require whole-genome bisulfite sequencing of DNA from queen and worker larvae with good sequencing depth and replication across colonies. If it can be shown that particular genes are consistently differentially methylated in a caste-specific manner, and this methylation affects gene expression, then this will be conclusive evidence that methylation is involved in caste differentiation. If not, then we should discount a role for methylation in caste determination.

Given the currently equivocal role of methylation in Q–W caste determination, how can we interpret the fact that experimental knockdown of the *de novo* methylation system in honeybees reliably produces the queen phenotype in young larvae [43]? *Dnmt* genes probably have functions beyond methylation because knockdown of *dnmts* is lethal in the milkweed bug where methylation is absent [66,67]. Thus, it is possible that *dnmt3* is indeed involved in caste determination [43], whereas methylation is not. A decisive experiment might involve using RNA interference to knock down expression of *dnmt3* in honeybee larvae, followed by whole-genome bisulfite sequencing and confirmation that knockdown generates the queen phenotype. However, if *dnmt3* plays a direct role in caste determination, unrelated to methylation, then this would add a confounding factor.

5. Small RNAs and chromatin modification

Small RNA (s-RNA) molecules are a class of short (18–50 bp) non-coding RNAs that are important for the regulation of gene expression in eukaryotes via post-transcriptional modifications to messenger RNAs, and for direct regulation of the rate of translation at the ribosome [68,69]. Because queens and workers are very different animals with significantly different gene expression profiles [70–72], it is unsurprising that a class of molecules like s-RNAs that regulate gene expression should also differ in abundance and kind between queens and workers [73–75] and between worker castes [76]. Unclear at this time is whether s-RNAs are the 'first responders' to larval diet that establish caste-specific developmental patterns, or are a downstream response to other triggers such as methylation or hormonal regulation [77,78].

Chromatin is the DNA–protein complex of which chromosomes are comprised. The protein component provides a supporting structure for the DNA. Post-translational modifications to the histones of chromatin influence the degree to which it is contracted or expanded, and this in turn influences the expression of genes in the region [79]. Chromatin states are usually propagated across cell divisions [80]. Chromatin modifications often reflect the methylation state of the CpG sites in the associated DNA, that is, methylation directs chromatin modifications and *vice versa* (the piggy-back model) [81,82]. Therefore, any link between methylation and gene expression may be via histone modification [83–86]. Queen- and worker-honeybee larvae show caste-specific chromatin states across thousands of genes and these are correlated with caste-specific gene expression [87]. We are unaware of any study that has related methylation pattern to chromatin state to gene expression.

6. DNA methylation and behavioural castes

Another phenomenon characteristic of insect colonies that demands both evolutionary and mechanistic explanations is the ability of individuals to undergo profound behavioural and physiological changes that do not include changes in morphology. These changes can be as normal as the transition from nest-bound work to foraging that most workers undergo as they age [9,88], to the transition from worker to mated reproductive that occurs in some ant [89], wasp [55] and termite [90] species. These behavioural changes are associated with significant rearrangements of the worker's physiology and gene expression [91–95]. Could methylation play a role in regulating these transitions?

In the honeybee, behavioural castes can be experimentally created by simple manipulations of colony demography. For example, precocious foragers can be created by removing all the natural ones, which causes rapid maturation of nurse workers to foraging tasks. Herb *et al.* [46] used an array-based technology, verified by whole-genome bisulfite sequencing, to examine the methylation patterns in same-aged nurse workers and foragers, including foragers that had been forced to revert to nursing. Each behavioural phenotype was associated with its own specifically methylated regions, and these regions were enriched for gene-regulatory functions. Importantly, foragers that had reverted back to nursing showed methylation patterns more similar to nurses than to foragers. Similar findings were shown for a set of eight genes in which methylation levels were more associated with behavioural task than with chronological age [96]. Most recently, ultra-deep sequencing has shown that three genes, *dynactin*, *nadrin* and *pcb1*, show small but significant differences in methylation patterns in honeybees of different ages that perform different tasks (newly emerged, young, nursing, foraging) [97].

In bumblebees, there are genome-wide methylation differences between queenless (reproductively active) workers and sterile workers with a queen [34,98]. By contrast, there is no association between methylation level and reproductive–non-reproductive phase in clonal raider ants [54]. In raider ants, methylation patterns are consistent over life, and there is no association between methylation and gene expression [54].

In summary, some of these experiments suggest that an ancient mechanism, methylation, is used in at least some social insects to allow rapid, environmentally responsive changes in gene expression and behaviour in workers [46,59,99]. However, there is mounting evidence against the involvement of methylation in these processes [51,54,55], and it is now far from certain that methylation is involved in the regulation of behaviour.

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7. Associations between DNA methylation and degree of sociality

If methylation plays a major role in sociality by providing a mechanism by which morphological and behavioural castes can be generated from similar genomes, then one might predict an association between the level of sociality and methylation. 'Sociality' in this context is the continuum [100] between solitary reproduction by females (e.g. in saw flies) through small family groups with facultative reproductive skew towards one or more females (as in paper wasps), to colonies in which large numbers of workers support a single morphologically distinct queen who is the mother of all individuals in the colony, as in sugar ants (*Camponotus*).

This question has been addressed in five studies using increasingly sophisticated genomic techniques and better taxon sampling. Overall, we think that the major conclusion from these studies [47,63,101–103] is that there is no clear relationship between the level of sociality and the extent of methylation. The strongest association is across the phylogeny of bees [103], but the relationship is not clear cut when wasps and ants are added to the picture [102]. In general, the Hymenoptera have low levels of methylation relative to other insect orders in which there are no social species [41,42]. In summary, these phylogenetic analyses emphasize that the evolutionary paths to eusociality have followed different routes [103]. All involve increasingly complex gene networks and regulatory systems, but the mechanisms by which this complexity is regulated vary across clades.

8. Conclusion

In this review, we have discussed the enigma of caste determination, and the progress that has been made in determining how environmental signals, mostly nutritional, are translated into developmental changes via epigenetic mechanisms. There is now confirmation that methylation levels differ between honeybee queens and workers at the early larval stage and that differentially methylated genes are related to reproductive, morphological and vision systems [52]. The remaining open question is whether the methylation differences are functional.

Data accessibility. This article has no additional data.

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