



SYMPOSIUM

The Role of Methylation of DNA in Environmental Adaptation

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Synopsis Methylation of DNA is an epigenetic mechanism that influences patterns of gene expression. DNA methylation marks contribute to adaptive phenotypic variation but are erased during development. The role of DNA methylation in adaptive evolution is therefore unclear. We propose that environmentally-induced DNA methylation causes phenotypic heterogeneity that provides a substrate for selection via forces that act on the epigenetic machinery. For example, selection can alter environmentally-induced methylation of DNA by acting on the molecular mechanisms used for the genomic targeting of DNA methylation. Another possibility is that specific methylation marks that are environmentally-induced, yet non-heritable, could influence preferential survival and lead to consistent methylation of the same genomic regions over time. As methylation of DNA is known to increase the likelihood of cytosine-to-thymine transitions, non-heritable adaptive methylation marks can drive an increased likelihood of mutations targeted to regions that are consistently marked across several generations. Some of these mutations could capture, genetically, the phenotypic advantage of the epigenetic mark. Thereby, selectively favored transitory alterations in the genome invoked by DNA methylation could ultimately become selectable genetic variation through mutation. We provide evidence for these concepts using examples from different taxa, but focus on experimental data on large-scale DNA sequencing that expose between-group genetic variation after bidirectional selection on honeybees, *Apis mellifera*.

Introduction

The methylation of DNA in eukaryotes is a chemical modification that involves the addition of a methyl group onto the position 5 of a pyrimidine ring on cytosines (5mC), primarily within cytosine–phosphate–guanine (CpG) dinucleotides. DNA methylation can affect structural changes to chromatin by attracting protein complexes that modify the histone scaffolds holding the DNA coil. DNA methylation in promoter regions can induce a tightly packed form of DNA with attached proteins, called heterochromatin, that restrict the access of the transcriptional machinery. The outcome is the silencing of proximal gene expression (Klose and Bird 2006). In species that contain DNA methylation, the mechanism has been functionally linked to development, behavior, and phenotypic plasticity (Day and Sweatt 2010; Feng et al. 2010; Law and Jacobsen 2010; Boyko and Kovalchuk 2011; Lyko and Maleszka 2011).

DNA methylation is present in genomes across taxa and likely pre-dates the divergence of plants and animals. However, the amount and distribution of DNA methylation in the genome varies widely among species. For instance, >70% of CpGs are methylated in humans, whereas this number is ~18% in *Arabidopsis thaliana* and <1% in *Apis mellifera* (honeybee) (Flores and Amdam 2011). Moreover, some species have lost the enzymes necessary for DNA methylation despite possession of complex development, behavior, and expression of phenotypic plasticity. For example, the fruit fly *Drosophila melanogaster* has no CpG DNA methylation, but its molecular biology is similar enough to that of mammals to model development, behavior, human disease, and nutrition (Beckingham et al. 2005). In addition, the fly shares complex programs such as metamorphosis with the honeybee, in which methylation of CpGs contributes to developmental

outcomes (Kucharski et al. 2008). Thus, it appears that quite dramatic changes in interspecific amounts and distributions of DNA methylation are permitted—and, as we will argue, perhaps accommodated—by evolutionary processes.

Methylation of DNA is unique from other epigenetic mechanisms that affect the structure of chromatin because its usage by genomes carries an evolutionary ramification in the form of increased mutability. For example, the rate of C-to-T mutations is 10-fold to 50-fold higher in humans' methylated cytosines (Duncan and Miller 1980; Bulmer 1986; Britten et al. 1988; Sved and Bird 1990). Genomes with DNA methylation, overall, show a depletion of CpG dinucleotides that reflects the occurrence of mutations induced by DNA methylation in the germline (Flores and Amdam 2011). It is unclear whether such patterns of depletion include adaptive mutations or reflect neutral and tolerated genomic changes.

Here, we propose a four-stage mechanism that may explain how methylation of DNA can play a role in adaptive evolution: (1) environmental exposures contribute to variability in targeting of DNA methylation, (2) targeting that benefits reproduction and survival are perpetuated over generations when environmental exposures remain unchanged, (3) targeted genomic regions experience increased mutability, and (4) mutations can accommodate the phenotype achieved by methylation targeting and make it available to natural selection.

To arrive at this explanatory framework, we begin by discussing the functional roles of DNA methylation at the cellular and organismic levels. We then discuss studies that exemplify how changes in patterns in genomic DNA methylation can occur in response to environmental variability and the degree to which those changes are transferred to offspring. Thereafter, we build support for the evolutionary role of DNA methylation from genome-wide resequencing data of honeybees that were subject to 37 generations of bidirectional selection (Page and Fondrk 1995).

DNA methylation: targeting, gene regulatory functions, and programming

The *de novo* methylation of DNA in eukaryotic genomes is performed by the DNA methyltransferase DNMT3. The maintenance DNA methyltransferase DNMT1 carries out the methylation of the cytosine on the complementary strand subsequent to *de novo* methylation and during replication of DNA (Law and Jacobsen 2010). Although DNA methylation is

predominantly found on cytosines within CpG dinucleotides, it also occurs to a much lesser extent in the context of CHG and CHH sequences (H = A, C, or T) (Chan et al. 2005; Lister et al. 2008, 2009).

Mechanisms targeting DNA methylation

The targeting of DNA methylation both in plants and mammals is controlled by an RNA-directed mechanism that allows different genomic sites to be independently methylated in response to growth and developmental, and environmental cues (Mette et al. 2000; Aravin and Hannon 2008; Kuramochi-Miyagawa et al. 2008; Morris 2009; Mahfouz 2010). This process involves members of the family of PAZ Piwi domain proteins that are capable of binding to 24- to 26-nt-long RNAs transcribed from non-coding regions, called Piwi-interacting RNAs (piRNAs). The PIWI/piRNA complex is guided to specific sequences in the genome by RNA–DNA or RNA–RNA pairing recognition (Wassenegger et al. 1994; Pélissier and Wassenegger 2000). This PIWI complex then attracts DNMT3 to perform *de novo* methylation. It is possible that the PIWI/piRNA mechanism of directed DNA methylation may affect the placement of other epigenetic modifications such as H3K4 demethylation, which then attract *de novo* DNA methyltransferases, but it has been shown that the PIWI/piRNA pathway is at least upstream of *de novo* DNA methylation (Aravin et al. 2008). Recently, it has also been shown that other small RNAs similarly mediate *de novo* DNA methylation by associating with PIWI proteins in plants. In these instances, siRNAs or miRNAs that arose from miRNA-coding regions guided DNA methylation at some of their generation sites and *in trans* at their target sites (Chellappan et al. 2010; Wu et al. 2010).

The genomic functional roles of DNA methylation

DNA methylation is known to affect transcriptional silencing when it occurs in gene-promoter regions, transposons, and repeats. In contrast, intragenic DNA methylation (inside gene bodies) is frequently associated with actively transcribed genes, suggesting that the precise role of DNA methylation in transcriptional regulation may vary between promoter and intragenic regions and between genes (Zhang et al. 2006; Hellman and Chess 2007; Zilberman et al. 2007; Ball et al. 2009; Rauch et al. 2009). Although the conserved regulatory function(s) of intragenic DNA methylation remains elusive, an emerging theory congruent with these findings is that one conserved function of exon methylation is the regulation alternative splicing (Laurent et al.

2010; Lyko et al. 2010; Park et al. 2011). This is especially relevant for the honeybee because over 80% of the DNA methylation in its genome was found to be located within exons (Lyko et al. 2010), and DNA methylation distinctly ends at intron–exon boundaries (Flores and Amdam 2011).

Programming of DNA methylation

The targeting of DNA methylation in the genome is both internally regulated, and as discussed in the next section, sensitive to extrinsic signaling. The internal regulation constitutes a program of DNA methylation which interacts with other dynamic molecular processes, such as transcription. Programmed changes in DNA methylation are thought to help regulate cellular differentiation during development by inducing stable alterations in gene expression (Monk et al. 1987; Kafri et al. 1992; Reik 2007; Sasaki and Matsui 2008; Cedar and Bergman 2009). Recent studies of genome-wide DNA methylation support the tenet that programmed locus-specific changes in DNA methylation correlate with changes in cell phenotype (Lister et al. 2009; Laurent et al. 2010; Li et al. 2010). Experimental perturbation of the intrinsic developmental program of DNA methylation can cause drastic changes in phenotype or be lethal to organisms across eukaryotic taxa, including plants (Lindroth et al. 2001; Cao and Jacobsen 2002a, 2002b; Kankel et al. 2003; Xiao et al. 2006), vertebrates (Li et al. 1992; Okano et al. 1999; Stancheva et al. 2001; Li 2002), and invertebrates (Kucharski et al. 2008; Shi et al. 2011).

Besides organismal development, programmed changes in DNA methylation are also essential to regulation of synaptic plasticity in memory and of stress-induced behavior (Miller and Sweatt 2007; LaPlant et al. 2010; Miller et al. 2010). For example, various locations in the mouse brain undergo dynamic changes in DNA methylation in connection with neuronal activity (Guo et al. 2011), while inhibition of DNMT enzymes after associative learning in honeybees can interfere with the consolidation of memory (Lockett et al. 2010). Programmed changes in locus-specific DNA methylation also occur in the bee brain during behavioral transitions that are essential for colony fitness (Herb et al. 2012) (Fig. 1). These data suggest that the functional role of the programming of *de novo* DNA methylation in the brain is conserved between vertebrates and invertebrates.

Changes to the epigenetic code, such as differences in the programming of DNA methylation, can impact fitness by inducing alternative developmental

or behavioral phenotypes similar to genetic mutations. Changes in the developmental program of DNA methylation could lead to differences in cellular differentiation and be causal to differences in post-developmental physiology. Changes in the program of neuronal DNA methylation could elicit novel behaviors or behavioral responses to the environment. In the next two sections, we discuss how the environment affects variability in DNA methylation and the epigenetic mechanisms that could transmit such variable DNA methylation to offspring.

The environment as a modifier of DNA methylation

There is increasing evidence that environmental variability can cause variation in the program of DNA methylation in developing offspring. Because DNA methylation also plays a functional role in transcriptional regulation, it is possible that the altered patterns of DNA methylation signaled by the environment may, in turn, signal changes in gene expression. Thereby, variations in DNA-methylation induced by environmental changes may be functional and allow a population to display phenotypic variability despite being genetically homogeneous.

Recent studies in several plant species show that alternative phenotypes can occur in populations with little or no genetic variation, but instead correlate with increased variation in DNA methylation (Lukens and Zhan 2007; Gao et al. 2010; Lira-Medeiros et al. 2010). In the dandelion, *Taraxcum officinale*, such variability may be induced by environmental stress (Verhoeven et al. 2010). Other data support the view that DNA methylation is required for phenotypic responses to environmental exposures. For example, mutations in the targeting pathway of DNA methylation in *A. thaliana* can reduce global genomic DNA methylation along with changes in the plant's adaptive responses to heat, cold, salt, drought, and flood (Boyko et al. 2010). In animals, moreover, environmental factors such as the maternal diet (Lillycrop et al. 2005, 2007), neonatal diet (Plagemann et al. 2009), rearing behavior (Weaver et al. 2004) and folic acid supplementation (Wolff et al. 1998) can alter *de novo* programming of DNA methylation during development of the offspring. For example, feeding a protein-restricted diet to pregnant rats results in gene-specific hypomethylation in the offspring. These differences in DNA methylation in the offspring correlate with changes in their adult phenotype, such as alterations to glucose production in response to stress (Lillycrop et al. 2007) and an increase in systolic blood pressure

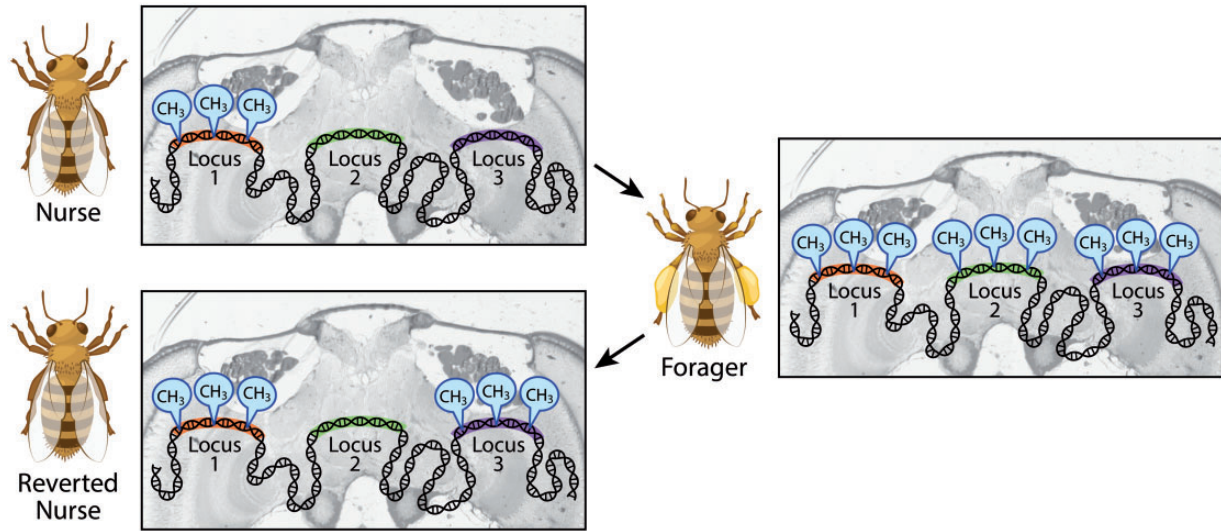


Fig. 1 DNA methylation in the honeybee brain is dynamic and associated with an individual's life history. Honeybee workers usually progress with increasing age from tasks in the nest, such as nursing the brood, to foraging outside the nest. This behavioral switch is essential for colony fitness because it regulates the allocation of workers dedicated to resource-harvesting in response to environmental conditions. When forcing foragers to assume nursing tasks, some, but not all of the patterns of DNA methylation characteristic of foragers will revert to patterns characteristic of nest bees. The patterns of DNA methylation in the brains of nurse bees and foragers differ (Herb et al. 2012).

that may ultimately lead to hypertension (Bogdarina et al. 2007).

Honeybees are an invertebrate species for which the sensitivity of DNA methylation to the environment is conserved. This sensitivity has been socially co-opted to regulate caste fate in female larvae. These larvae can develop into either reproductive queens or sterile workers depending on the diet they receive. The diet is tightly controlled by the larvae's adult sisters that are nurse bees within the hive. If the rearing of larvae is perturbed, the process of caste differentiation, including the developmental program of DNA methylation is altered, and this process involves changes in the expression of DNMT3 and the locus-specific placement of DNA methylation (Shi et al. 2011). The role of DNA methylation in caste fate was further cemented by results showing that queens can develop from larvae that are artificially reared on a combination of a worker's diet and silencing DNMT3 with double-stranded RNA (Kucharski et al. 2008).

These studies suggest that DNA methylation is a conserved molecular mechanism in plants, vertebrates, and invertebrates that can be used to convert environmental heterogeneity into phenotypic differences. Thus, we may gain a better understanding of how phenotypic variability arises in populations by studying how the cellular pathways that regulate the genomic targeting of DNA methylation are signaled by environmental change (Fig. 2). Studying these

pathways may also allow us to determine how patterns of DNA methylation evolve because genetic changes to these pathways could lead to differences in targeting of DNA methylation and in developmental programming.

The evolutionary role of environmentally-induced phenotypic heterogeneity mediated by DNA methylation

The capacity for the pathway of *de novo* DNA methylation to transduce spatial or temporal environmental variation into phenotypic variation implies a potential role for DNA methylation in adaptive evolution. In honeybees, phenotypic variation that is signaled by the environment and mediated by DNA methylation may affect fitness of the colony. Phenotypic heterogeneity among the honeybee workers underlies division of labor within the hive, and an increase in division of labor can increase colony fitness (Waibel et al. 2006; Oldroyd and Fewell 2007). It has been proposed that DNA methylation could be involved in processing the internalization of variations in micro-environments of larvae during development, of workers, thereby helping generate phenotypic heterogeneity in the worker population despite genetic homogeneity (Flores and Amdam 2011). DNA methylation is known to have a functional role in transducing differences in the composition of the diet of larvae between queens and workers (Kucharski et al. 2008). It is possible that

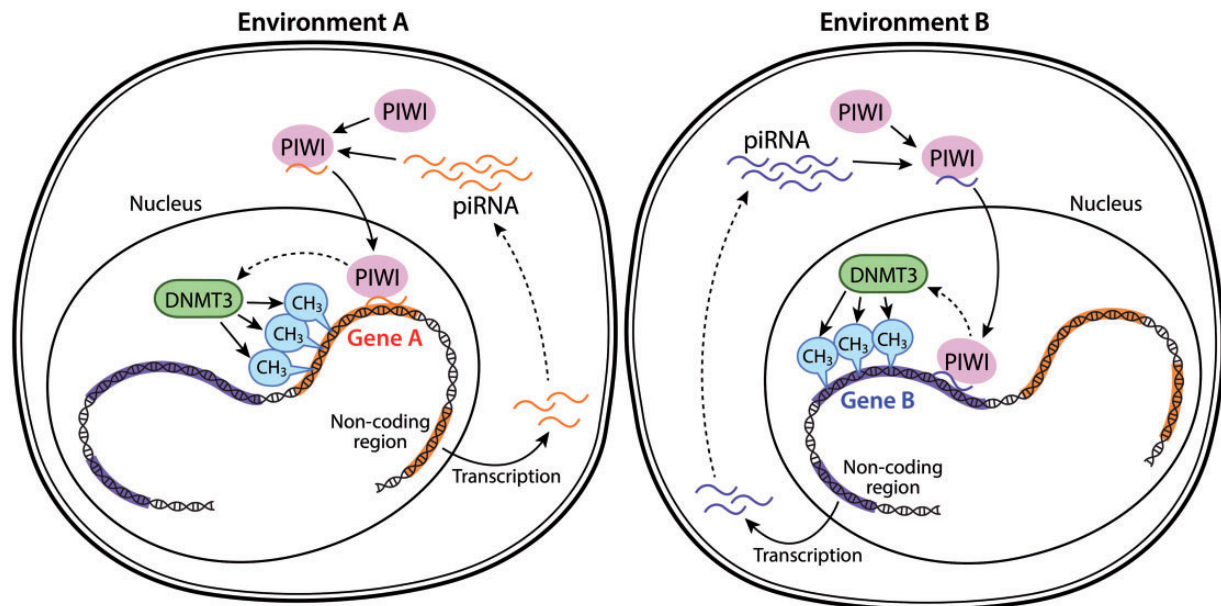


Fig. 2 Environment and RNA-dependent mechanisms act to influence the methylation of DNA. Patterns of DNA methylation depend on the environment and are directed by piRNAs and the RNA-binding protein PIWI. For simplification, only one gene is shown to be affected under each environment. Under environmental condition A, genomic regions that do not encode a gene (non-coding red part of the DNA) produce piRNAs that bind to PIWI and then attract PIWI to genes that bear near 100% homology on the piRNA sequence. Subsequently, DNMT3 is recruited and the respective gene is methylated. Under environmental condition B, piRNAs derived from another genomic region are produced and a different gene is methylated, leading to an alternate molecular phenotype.

DNA methylation may have a similar function among the worker caste, in which altering the larval diet has the capacity to modulate physiological traits that regulate the worker's heterogeneity, such as the number of ovarioles (Kaftanoglu et al. 2011). Similarly, variability in the environment of queen larvae could signal changes in the queen's developmental program of DNA methylation. This could lead to an increase in the phenotypic heterogeneity of the worker population that the queen produces if those changes in DNA methylation affect her germline. Alternatively, environmentally signaled DNA methylation could mediate biological effects that are manifest over the lifetime of individual bees, also leading to phenotypic heterogeneity.

Further studies are needed to test whether variability in the environments of larvae or adults (e.g., diet or temperature) could imprint differences in patterns of DNA methylation in workers' brains, thereby altering behavioral regulation that is critical for division of labor, such as the transition from nurse to forager.

Inheritance of DNA methylation

The capacity to transmit environmentally-induced DNA methylation marks from parent to offspring can be evolutionarily advantageous because it may

prepare the offsprings' phenotype for the environmental stress that the parent(s) may have endured (Mousseau and Fox 1998; Jablonka and Lamb 2005; Youngson and Whitelaw 2008; Jablonka and Raz 2009). A genome-wide erasure of DNA methylation during development would prevent the transfer of DNA methylation marks, and any phenotypic traits caused by them, from parent(s) to offspring. Patterns of DNA methylation are reprogrammed genome-wide during plant and mammalian development, thereby limiting the capacity for transgenerational inheritance of DNA methylation, at least on a genome-wide scale.

DNA methylation reprogramming during plant and mammalian development

The degree to which DNA methylation is erased and then re-established during development differs between plants and mammals; evidence for DNA demethylation is lacking in insects. The erasure of DNA methylation in plants is carried out by a demethylation pathway, which includes the DNA glycosylases and AP lyases ROS1 (repressor of silencing 1), DME (demeter), DML2 (Demeter-like 2), and DML3 (demeter-like 3), to excise cytosine's that are methylated. The nucleotide gap in DNA is then

presumably filled by DNA repair polymerase and ligase enzymes (Zhu 2009). This demethylation pathway affects genome-wide hypo-methylation in *Arabidopsis* endosperm, especially within transposable elements (Gehring et al. 2009), and mutation of DME partially restores endosperm DNA methylation to the amount found in other tissues (Hsieh et al. 2009). The expression of DME in maternal-specific cells of the endosperm results in the demethylation, and consequently changes in expression, at specific genes, i.e., maternal allele-specific imprinting (Huh et al. 2008).

In mammals, direct transmission of DNA methylation marks from parent(s) to offspring is limited to specific loci because of the waves of genome-wide demethylation, followed by the re-establishment of DNA methylation that occurs during gametogenesis in primordial germ cells and in the embryo immediately following fertilization (Reik 2007; Surani et al. 2007; Sasaki and Matsui 2008; Hemberger et al. 2009). Demethylation in the embryonic stage has the additional complexity that the paternal genome from the sperm is demethylated, but the maternal genome is not and may be protected from demethylation (Mayer et al. 2000; Oswald et al. 2000; Santos et al. 2002; Nakamura et al. 2007). Similarly, differences in the timing of remethylation of DNA occur between the male and female germs cells, in which maternal-specific methylation is established after the male germ cells are initially methylated (Bartolomei and Ferguson-Smith 2011). A combination of active and passive mechanisms of demethylation may contribute to genome-wide demethylation in mammals. Passive demethylation involves the loss of DNA methylation through the lack of maintenance through cell division, resulting in hemi-methylated substrates during the G₂ phase of the cell cycle. Several molecular mechanisms have been proposed for active demethylation in mammals, including 5mC modification enzymes, DNA deaminases, and the base excision repair pathway (Hajkova et al. 2010; Popp et al. 2010). It still remains unclear how extensively the active or passive demethylation pathways contribute to the genome-wide erasure of DNA methylation; however, it has been demonstrated that some parental DNA methylation marks can be transmitted to offspring (Richards 2006; Hitchins et al. 2007).

In any case, DNA methylation patterns of the parents could be re-established after demethylation has occurred. This could be facilitated by other epigenetic mechanisms, such as the differential inheritance of DNA-binding proteins, including modified histones, which have the capacity to mediate DNA

methylation (Cedar and Bergman 2009). Molecules involved in the targeting of *de novo* DNA methylation, such as piRNAs, could be passed through the germ line and lead to *de novo* DNA methylation at specific loci during development in offspring. It has also been demonstrated that DNA methylation imprinted during the development of offspring can be transgenerationally inherited through recapitulation of maternal traits. For example, an increase in the licking and grooming (LG) of pups and arched-back nursing (ABN) by rat mothers alters the pattern of DNA methylation (compared with low-LG-ABN mothers) in the promoter region of the glucocorticoid receptor (GR) in the offspring's hippocampus. The GR gene regulates the hypothalamic-pituitary-adrenal (HPA) axis and stress response in the hippocampal neurons, and the offspring of high-LG-ABN mothers have a reduced reactivity and anxiety. These more relaxed offspring are then more likely to adopt the same approach to the rearing of young as did their mothers, thereby perpetuating their GR DNA methylation patterns in the next generation (Weaver et al. 2004; Diorio and Meaney 2007).

Evidence for transgenerational inheritance of DNA methylation

Transgenerational inheritance of DNA methylation is more plausible in plants due to the possibility for asexual vegetative reproduction and because in sexual reproduction gametes are derived from almost fully matured vegetative tissue. Transmission of DNA methylation from parent(s) to offspring may also be more adaptive in plants than in animals, since gametes and vegetative offspring are derived from tissue that has been subject to the environmental stress that occurred during almost the entire life history of the parent generation. The exposure to environmental stress has been shown to induce phenotypic changes that can persist to the next generation in plants and animals (Pembrey et al. 2005; Grant-Downton and Dickinson 2006; Koturbash et al. 2006; Molinier et al. 2006) and DNA methylation may play a critical role in the transgenerational perpetuation of such environmentally-induced phenotypes (Mirouze and Paszkowski 2011). For example, in *A. thaliana*, transgenerational inheritance of stress-induced responses is dependent on *de novo* DNA methylation (Boyko et al. 2010). A similar study in the dandelion *T. officinale* found that environmental stress, specifically chemical induction of defenses against herbivores and pathogens, induces differences in DNA methylation and that most of

these differences are inherited by the offspring (Verhoeven et al. 2010).

A key feature of the study of dandelions was that it involved a species with asexual reproduction, allowing the variability in DNA methylation to be associated with variability in environments of the parents instead of with genetic variation. DNA methylation may play a role in evolutionary adaptation by providing an epigenetic layer of inheritance on top of genetic inheritance. However, substantiating this concept will require further studies that control for, or accurately measure, genetic variation on a genome-wide scale in order to negate the possibility that observed heritable changes in DNA methylation are caused by genetic mutations.

DNA methylation increases mutability

Genomic regions that are methylated, either due to programmed or environmental signaling, are subject to an increased mutation rate because methylated cytosines spontaneously deaminate to thymine, which is then followed by replacement of guanine by adenine on the opposite DNA strand due to the

mismatch repair of DNA (Duncan and Miller 1980). Evidence for these DNA-methylation-induced mutations (DMIMs) in the form of CpG depletion is found at genomic loci that have presumably been methylated over evolutionary time, i.e., across multiple generations, in germ line cells. For example, approximately half of all honeybee genes are methylated, leaving a pattern in which half of all genes have less CpGs than expected (Elango et al. 2009) (Fig. 3 [top]). This pattern of depletion of CpG in honeybees' genome is also apparent at the exon level, in concordance with the observation that honeybees' DNA methylation is largely targeted to exons (Figs. 3 [bottom] and 4). Similar patterns of CpG depletion are found in the genomes of *Acyrtosiphon pisum*, *Ciona intestinalis*, and humans, but are absent from species that do not show any significant levels of CpG methylation, such as *D. melanogaster* (Flores and Amdam 2011).

Because the developmental program of DNA methylation is sensitive to the environment, the level of methylation in some genomic regions in offspring may be a probabilistic function of the environment. In genomic regions at which methylation

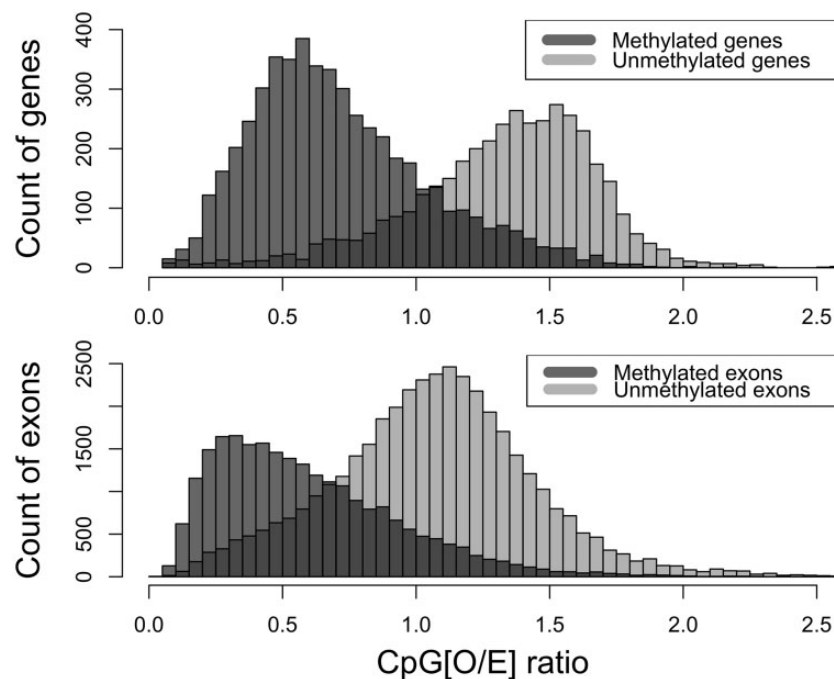


Fig. 3 Methylation correlates with depletion of CpG in honeybees' genes and exons. The CpG[O/E] ratio is used as a measure of the depletion of CpG in a genomic region; it is calculated as the number of observed CpGs divided by the number of expected CpGs based on GC content (Elango et al. 2009; Flores and Amdam 2011). (Top) Honeybees' genes are separated into methylated and unmethylated categories and the distributions of CpG[O/E] ratios is shown for each category. Methylated genes are more depleted of CpGs than are unmethylated genes, likely because of the increased rate of C/T transitions due to deamination of nucleotides. (Bottom) Honeybees' exons are separated into methylated and unmethylated categories and the distributions of CpG[O/E] ratios is shown for each category. Methylated exons are more depleted of CpGs than are unmethylated exons. Data on DNA methylation were obtained from bisulfite-sequencing of queens and workers [31].

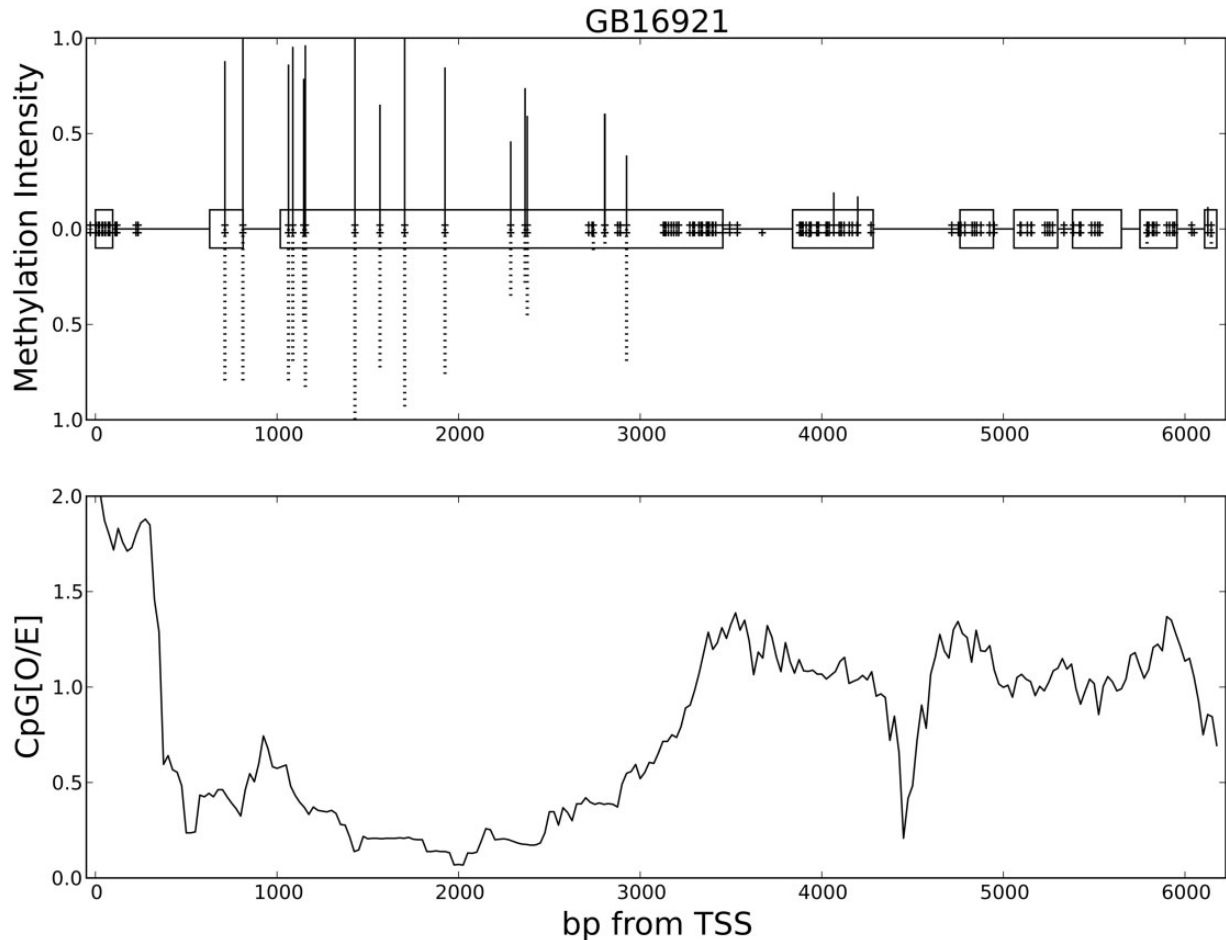


Fig. 4 Exon-specific depletion of CpG reflects exon-specific DNA methylation in the honeybee gene GB16921 (homolog of *Ptip*, part of the H3K4 methyltransferase complex). (Top) Base-pair resolution of the intensity of methylation is shown for the length of the gene GB16921. Solid (dotted) lines above (below) the *x*-axis indicate the intensity of methylation in the queen (worker). The nine exons (1–9 from left to right) for this gene are shown on the *x*-axis as open boxes. The *x*-axis is labeled as base-pairs (bp) from translation start site (TSS). Plus signs just above (below) the *x*-axis indicate CpG coverage from bisulfite-sequencing data in queens (workers). Exons 2 and 3 are heavily methylated in both queens and workers. (Bottom) The CpG[O/E] ratio is calculated with a 200-bp sliding window along the length of the gene (see Fig. 3 for discussion of CpG[O/E]). These data show that depletion of CpG in GB16921 is confined to exons that are heavily methylated (compare with top panel). Data on DNA methylation were obtained at base-pair resolution from genome-wide bisulfite-sequencing of honeybee queens and workers [31].

only occurs during an environmental change, mechanisms of inheritance of transgenerational DNA methylation may help to perpetuate methylation in these genomic regions for several generations after the environmental change has occurred. If this methylation is causal to a phenotype that has a selective advantage during a period of environmental change, then DMIMs could allow that phenotype to become fixed (i.e., genetically programmed). The fixation of phenotypes may be caused by DMIMs that emulate the function of the DNA methylation that was induced by the environmental change. One way this might occur is if DMIMs alter the propensity for a genomic region to be methylated, causing a locus-specific change in DNA methylation (Fig. 5).

Alternatively, DMIMs could fix an adaptive phenotype by changing the sequence of a gene-coding region, such as an exon if a DMIM is caused by exon-targeted DNA methylation, or a gene regulatory sequence, such as a transcription factor-binding site if a DMIM is caused by promoter DNA methylation.

Circumstantial evidence for the evolutionary role of DNA methylation

DMIMs could ultimately circumvent the demethylation waves in the animal germ line and lead to a stable transmission of differences. Experimental evidence supporting the idea of DMIMs comes from studies of the honeybee. Honeybees can be

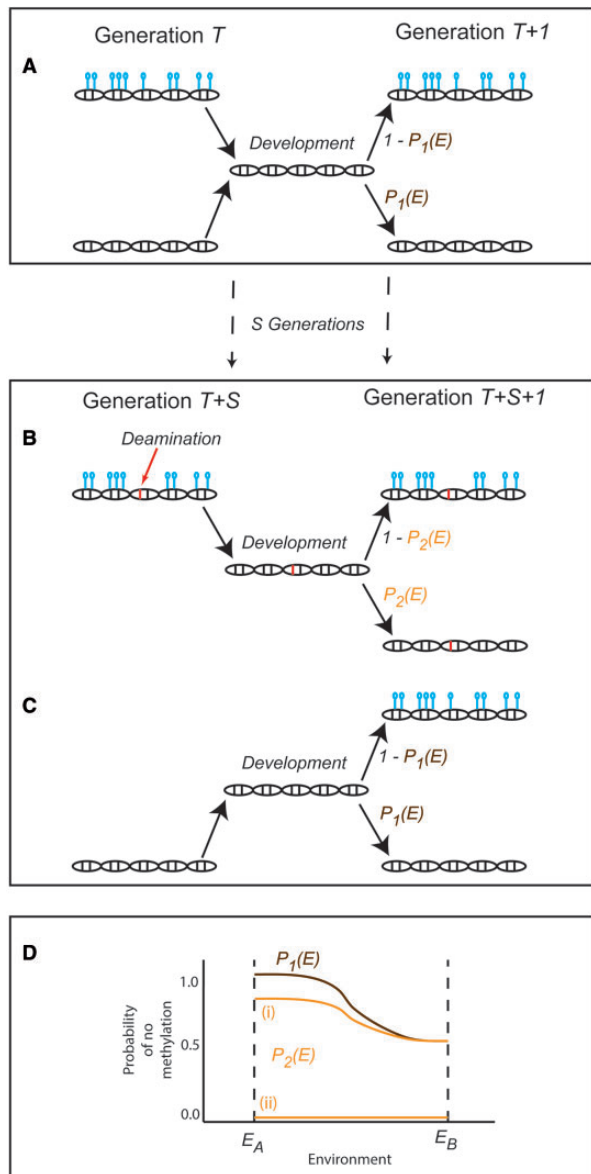


Fig. 5 The effect of DNA methylation induced mutations on environmentally sensitive methylation patterns. **(A)** Methylation patterns (open circles attached to lines) between the parent (Generation T) and offspring (Generation T+1) are erased and then re-established during development whether or not the parent generation is methylated. The mechanism that re-establishes methylation patterns can be sensitive to the environment. Here, E_A and E_B represent the normal and new environmental conditions, respectively. The probability function $P_1(E)$ describes a genotype in which there is no methylation when the environment is E_A , and the chance of methylation is random (probability = 0.5) in environment E_B . After a number of generations S, deamination in the germline may **(B)** or may not **(C)** occur, resulting in a genetic mutation (C → T transition) that changes the probability of methylation during development to a different function $P_2(E)$. **(D)** Scenario (i) represents a case in which $P_2(E)$ is more sensitive to a new environment, leading to a higher chance of methylation with less environmental change. Scenario (ii) represents a case in which $P_2(E)$ is completely insensitive to the environment and methylation always occurs.

bidirectionally selected for the amount of pollen (source of protein) versus nectar (source of carbohydrate) that is stored by colonies. This colony-level selection for high and low pollen-hoarding was first described by Hellmich et al. (1985) and was subsequently perfected by Page and Fondrk [14]. Page and Amdam (2007) described differences between the resulting genotypes in several traits such as workers' lifespan, sucrose sensitivity, and ovariole number. Interestingly, in wild-type (unselected) honeybees, similar suites of differences in traits are distributed between sister worker bees that share 0.75 genetic identity (Page and Amdam 2007). This suggests that the bidirectional selection on honeybee food-hoarding captured phenotypic variability that normally is expressed as heterogeneity by genetically similar individuals.

An analysis of quantitative trait loci that differentiate between high and low pollen-hoarding genotypes showed that epigenetic modulators such as histones, mSin3A (a core component of a large multiprotein complex that displays histone deacetylase activity), and a PIWI protein could be, at least partly, responsible for their physiological and behavioral divide (Hunt et al. 2007). We predicted that the two genotypes could differ in their patterns of DNA methylation. Indeed, an analysis of genome-wide DNA-methylation levels showed differences between the two genotypes with respect to their patterns of DNA methylation in the brain (Herb et al., unpublished data). In order to determine whether the two genotypes also show evidence for DMIMs, which would support a heritable difference in epigenetic effects, we analyzed the genome sequences of four biological replicates per genotype by testing for an enrichment of C/T transitions at cytosines within CpG dinucleotides versus all other cytosines. We found a significant enrichment of C/T transitions within CpG dinucleotides that was approximately equal (=2.59-fold) in both the high and low pollen-hoarding genotypes (Table 1). We also observed that there is a 38.7% GC content in third codon positions (3GC) in the honeybee reference genome, indicating a directional mutational pressure in the GC to AT direction (Sueoka 1988; Khurstalev and Barkovsky 2009). However, 81.8% of cytosines in the third codon position were not contained in CpG dinucleotides. Thus, it is unlikely that factors causal to the AT-pressure in the honeybee genome, besides nucleotide deamination, contributed to the enrichment of C/T transitions within the CpG dinucleotides that we found. These observations suggest a connection between DNA methylation and mutations in the two selected genotypes.

Table 1 CpG dinucleotides are enriched for C/T transition in bidirectionally selected strains of honeybees

	High pollen-hoarding strain		Low pollen-hoarding strain	
	C/T	no C/T	C/T	no C/T
C not within CpG	22,410	63,758,382	17,381	63,763,411
C within CpG	9148	10,020,985	7102	10,023,031

Compared with the number of C/T transitions at cytosine's not contained in CpG dinucleotides, there are ~ 2.59 times more C/T transitions at CpG dinucleotides in honeybee genotypes that were artificially selected for high or low pollen-hoarding behavior [14]. This enrichment of C/T transitions is statistically significant by Fisher's exact test in each of the two genotypes ($P < 2.2e-16$). We re-sequenced the whole genomes of four individuals from each of the low and high pollen-hoarding strains of honeybees, using deep sequencing (HiSeq 2000, Illumina, San Diego, CA). Reads generated from deep sequencing were aligned to *Amel_2.0* with Bowtie (version 0.12.7) with default options (Langmead et al. 2009). To determine the presence of genetic transitions between the high and low strains, we performed a standard case/control association analysis using PLINK (version 1.07) (Purcell et al. 2007; Purcell 2010); default options and a P -value cutoff of 0.05 were used to infer the presence of a significant genetic difference. This resulted in a total of 401,804 significant genetic differences. For each genotype, we tallied the number of C/T transitions by counting the number of significant genetic differences in which the genotype was called T and the reference genome was C. Similarly, C/T transitions were counted on the opposite strand if a G/A transition was found on the positive strand.

Remarks and future work

Phenotypic plasticity is a ubiquitous property in plants and animals that enables a population to achieve phenotypic variability with respect to environmental change despite genetic uniformity (West-Eberhard 2003). Epigenetic mechanisms used in the developmental program of an organism can be sensitive to the environment; hence epigenetic variation is expected to occur when phenotypic plasticity is manifest. Epigenetic mechanisms such as DNA methylation provide a means of extending the flexibility of the genome by affecting changes to the transcriptome, and to thus increase phenotypic plasticity. Here, we suggest that, by causing increased mutability, DNA methylation links this flexibility with evolutionary processes, culminating in selectable genetic variability. Interestingly, recent work has also shown that DNA hypo-methylation is associated with a higher frequency of homologous recombination and genomic instability (Li et al. 2012). Thus, it is possible that environmentally-induced hyper-methylation or hypo-methylation of DNA could lead to a higher mutation rate.

The evolutionary role of phenotypic plasticity mediated by DNA methylation remains unclear because patterns of DNA methylation mostly are reset in the gametes of plants and mammals, and in the primordial germ cells (PGCs) of mammals (Feng et al. 2010; Law and Jacobsen 2010). However, despite this limitation, we reviewed several mechanisms whereby methylation patterns are transferred transgenerationally. The assessment of these mechanisms will be facilitated by a clearer understanding of how piRNAs are generated, direct the placement of DNA methylation, and whether they are transferred to eggs or embryos. The transgenerational transference of functional DNA methylation has the potential for

contributing to short-term adaptation to environmental changes that cause variation in the methylomes of offspring. This effect can vary ever more dramatically in a population that exhibits genetic variance in master regulators of the machinery of DNA methylation and thus shows a broad range in sensitivity toward environmental perturbations. The resulting variable epigenetic response could confer positive fitness effects with respect to environmental change if it increases the rate at which alternative phenotypes that are only manifested during periods of environmental change become genetically fixed (West-Eberhard 2003). For example, in the genotypes of pollen-hoarding honeybees, it is possible that genetic differences in key epigenetic regulators, such as PIWI proteins, could have led to a difference in overall DNA methylation and thereby to variability in phenotypes such as pollen-hoarding behavior. In this scenario, a difference in behavioral phenotype is then repeatedly selected upon over generations and, over time, leads to DMIMs.

It remains to be uncovered precisely how DMIMs aid in the adaptability of an organism to its environment. We argue that DMIMs could decouple the developmental response from the environment by changing the likelihood that the functional effect of DNA methylation will occur without environmental extremes or by fixing genetic changes that replace the effect of DNA methylation. Thereby, DNA methylation may play a role in evolutionary adaptation due to the increased mutability induced in genomic regions where it is used.

Future research targeted at the fixation and reversibility of DNA methylation will be needed in order to shed more light on the question of how changes in the environment relate to epigenetic patterns and adaptability of organisms. Because of their

comparably small genomes and their almost exclusive restriction of DNA methylation to exons, insects provide a good model system to study these highly complex relationships. The advent of widely available and increasingly affordable bisulfite-sequencing of entire genomes is bound to help advance our knowledge in these areas. It will be highly instructive to analyze variations of DNA methylation and C/T transitions in wild-type populations and follow their associations throughout controlled breeding programs, such as the bidirectional selection of pollen hoarding in honeybees, in order to better elucidate the connections between DNA methylation and mutation. Furthermore, it will be important to investigate details of the DNA-targeting mechanism in order to understand how the same regions in the genome get consistently methylated, eventually leading to an increased mutation rate for C/T transitions in these areas, but not in others.

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