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EPIGENETICS AND SYMBIOSES. ANALYSIS OF DNA METHYLATION PATTERN IN *Apis mellifera* AND *Varroa destructor* PARASITIC RELATIONSHIP.

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ABSTRACT

DNA methylation is an epigenetic mechanism which consist of the a addition of a methyle group to a cytosine in a DNA sequence. Such modifications are able to cause several changes in the regulation and expression of genes, allowing organisms to respond quickly to different kind of environmental variations.
In this study, the hypothesis that such epigenetic modification and, in particular DNA methylation, for its great simplicity, plasticity and magnitude can be involved in the regulation symbiont interactions. It is infact, well known that a lot of viruses and bacteria interact with their host also by interfering with the regulation of DNA methylation.
This work focused on the study of arthropod symbioses and in particluar on the case of *Apis mellifera*, a pollinator insect important both economically and genetically, especially since its genome has been sequenced, and *Varroa destructor*, an ectoparasitic mite that reproduces inside the bees' brood cells and have recently caused the disappearance of millions *A. mellifera* colonies.
Previous studies have shown that mite attack can cause extensive damages in bees, impairing in particular the immune system the immune system and cognitive abilities.
This work focused on a three levels analysis. First the genomic DNA methylation pattern was studied. Then the DNA pattern, with gene level resolution focusing on target genes direclty involved in the interaction between mite and bees, was analyzed.
Finally, all samples were screened for mite-transmitted virus presence.
Result show that in there are no differences between the genomic methylation level between parasitized and non parasitized bees, reflecting the genomic constitution of bees, which are characterized by very AT rich genomes, so poor methylable genome.
Gene resolution analysis shown that in every case, the DNA methylation levels are lower in parasitized non parasitized samples. Moreover, DNA methylation was shown to increase with the virus parasitization of the bees.
Taken together these result indicated that DNA methylation may play a role in the regulation of honeybee and parasites relationship. Moreover, this study also support DNA methylation as a good candidate for the interaction and communications of two symbiont in a symbioses, providing DNA methylation as a good target candidate for the control of parasitic symbionts.

1. INTRODUCTION

1.1 The concept of Epigenetics

The concept of epigenetics was originally established by Conrad Waddington in 1942 (Waddington, 1942). He proposed that environmental stimulus could be converted into an internal genetic factor by "canalization of development" (Waddington, 1942), explaining how complex phenotypes could form from the interaction between genes and environment.

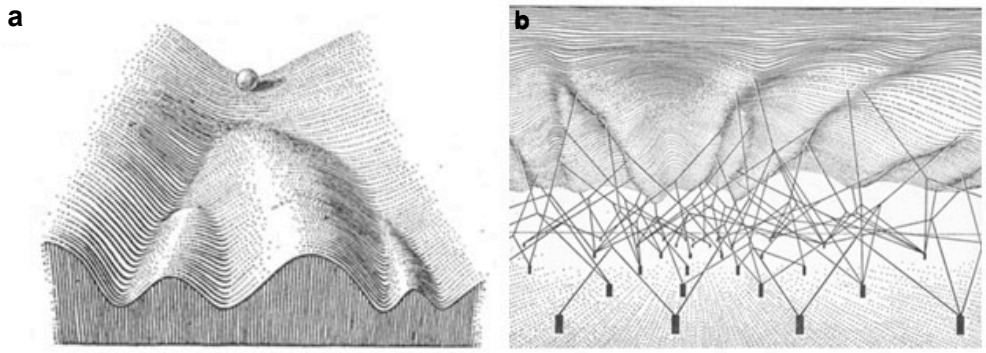

Figura 1.1 Waddington epigenetic landscapes. The picture is intended to represent the epigenetic landscape, the developmental pathways that could be taken by each cell of the embryo. **(a)** The ball represents a cell, and the bifurcating system of valleys represents the 'chreodes' or bundles of trajectories in state space. **(b)** A rare view behind the scenes of Waddington's landscape. Each valley in the landscape is formed by tension on guy ropes that are attached to complexes of 'genes', represented as pegs stuck in the ground. (Waddington, 1942).

Epigenetics focuses on DNA related information, heritable through both meiosis and mitosis, that does not involve the DNA sequence itself. Recently, an operational consensus definition of epigenetics was established (Berger *et al.*, 2009). In this context: "*An epigenetic trait is a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence*" (Berger *et al.*, 2009).

Moreover, Berger *et al* (2009) proposed that there are three categories of signals that culminate in the establishment of a stably heritable epigenetic state (Figure 1.2):

- a signal called the "Epigenator", which starts from the environment and triggers an intracellular pathway;

- an "Epigenetic Initiator" signal, which responds to the Epigenator and is necessary to define the precise location of the epigenetic chromatin environment;

- an "Epigenetic Maintainer" signal, which sustains the chromatin environment in the first and subsequent generations.

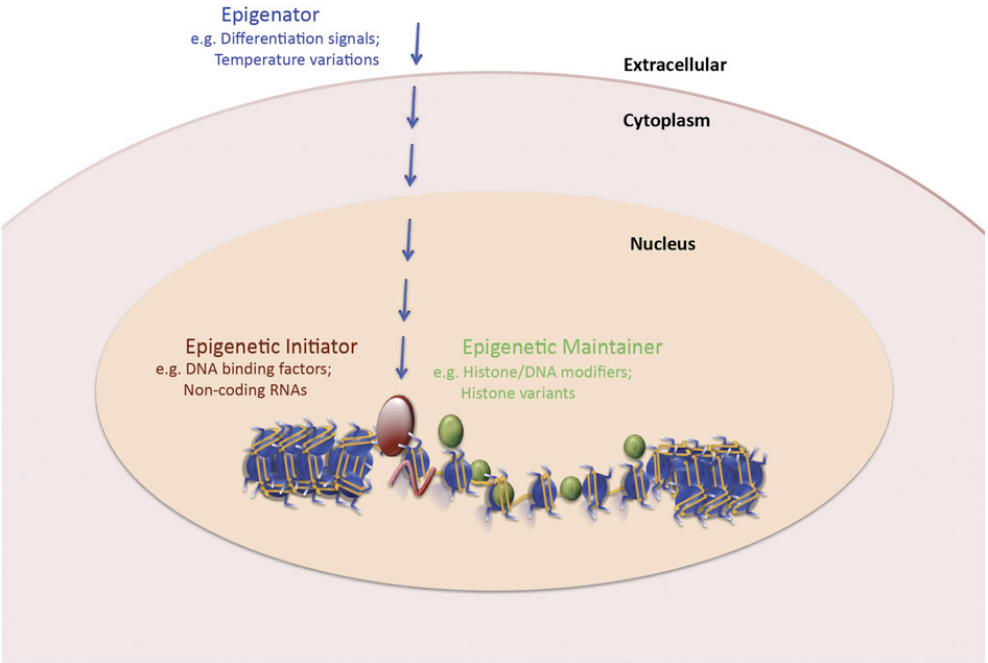

Figure 1.2 The epigenetic pathway. Three categories of signals are proposed to operate in the establishment of a stably heritable epigenetic state. An extracellular signal referred to as the "Epigenator" (shown in blue) originates from the environment and can trigger the start of the epigenetic pathway. The "Epigenetic Initiator" (shown in red) receives the signal from the "Epigenator" and is capable of determining the precise chromatin location and/ or DNA environment for the establishment of the epigenetic pathway. The "Epigenetic Maintainer" (shown in green) functions to sustain the chromatin environment in the initial and succeeding generations. Persistence of the chromatin marks may require cooperation between the Initiator and the Maintainer. Chromatin is depicted in blue. (Berger *et al.*, 2009)

Epigenator

The epigenetic phenotype is likely triggered by changes in the environment of the cell. Everything occurring upstream of the first event on the chromosome would be part of

the Epigenator signal, including an environmental cue or niche and the subsequent signaling pathways leading to the Initiator. Once an Epigenator signal is received, it is converted to an intracellular Epigenator pathway culminating in the ‘‘activation’’ of the Initiator. The Epigenator signaling pathway could appear in the form of a protein–protein interaction or a modification-based event that unleashes the latent activity of the Initiator. The Epigenator signal will be transient, remaining in the cell long enough to trigger the epigenetic phenotype but not necessary for subsequent events.

Epigenetic Initiator

The Initiator translates the Epigenator signal to mediate the establishment of a local chromatin context at a precise location. Following the priming of the Initiator by the Epigenator signal, the Initiator will define the location on a chromosome where the epigenetic chromatin state is to be established. The Initiator could be a DNA-binding protein, a noncoding RNA, or any other entity that can define the coordinates of the chromatin structure to be assembled. Consequently, some form of sequence recognition must be a feature of this signal. The Initiator will in general be a signal that requires self-reinforcement and self-renewal through positive feedback mechanisms. One operational characteristic of the Initiator is that it may be sufficient to start an epigenetic phenotype when introduced in a cell. Also, unlike the Epigenator, the Initiator may not dissipate after its action, but rather may persist with the Maintainer.

Epigenetic Maintainer

The Maintainer sustains the epigenetic chromatin state but is not sufficient to initiate it. This signal involves many different pathways, including DNA methylation, histone modifications, histone variants, nucleosome positioning, and others. Maintainers have the common property that they do not have absolute DNA sequence specificity. Consequently, they could operate at any chromosomal location to which they are recruited by an Initiator. Maintainers may function by carrying an epigenetic signal through the cell cycle or could maintain epigenetic landscapes in terminally differentiated cell types.

Epigenetic marks (Epigenetic Maintainers) maintain the heritable chromosome changes (Berger *et al.*, 2009). Currently, the best known marks are DNA methylation, post-translational histone modifications (hystone methylation and acetylation) and nucleosome positioning. These epigenetic marks affect gene expression (Jones *et al.*,

1998; Razin & Riggs, 1980) and have tissue-specific patterns (Eckhardt *et al.*, 2006) underlying tissue-specific gene expression of genes (Musco & Peterson, 2008). The various epigenetic marks interact resulting in a complex epigenetic machinery (Ikegami *et al.*, 2009; Ng & Bird, 1999) and its disruption often underlies the pathogenesis of many human diseases, such as cancer (Esteller, 2008).

The ability to produce contrasting phenotypes from the same genome in the absence of mutation is one of the key milestones in evolution. For example, the different cell types in the human body have the same genome, thus their very different cellular phenotypes are brought about by differential epigenetic events, largely occurring during embryogenesis. Moreover, human metabolism, aging and behavior are due to changes in epigenetic properties as a result of environmental signals and the responses of epigenomic receivers in different tissues and organs (Weaver *et al.*, 2007).

On the whole, today we know that epigenetics has a key role in the biology of cell: epigenetic information is an important, environmentally responsive mediator of the relationship between genotype and phenotype, and moreover, is the most important mediator between genotype and environment (Jaenisch & Bird, 2003; Kucharski *et al.*, 2008; Margueron & Reinberg, 2010), which results from mechanisms other than changes in DNA sequence (Berger *et al.*, 2009; Margueron & Reinberg, 2010). Nevertheless, such information is heritable: it is transmissible across mitotic, and meiotic, cellular divisions (Bonasio *et al.*, 2010).

Epigenetic information in the genome is not uniform, but is applied regionally, and it signals or preserves local activity states, such as gene transcription or silencing (Bird, 2007). The sum total of all epigenetic information in a genome is termed the ‘epigenome’. Unlike the genome, the epigenome is highly variable between cells and fluctuates in time according to conditions even within a single cell. There are therefore at least as many epigenomes as there are cell types.

The importance of epigenetics in evolution has recently been highlighted by Jablonka and Lamb (2005). In their works they defined epigenetics as one of the dimension in evolution. Indeed, Jablonka and Lamb argued that there is more to heredity than genes. They trace four dimensions in evolution, four inheritance systems, that play a role in evolution: genetic, epigenetic (or non-DNA cellular transmission of traits), behavioral, and symbolic (transmission through language and other forms of symbolic

communication). These systems, they argue, can all provide variations on which natural selection can act. The concept of evolution in four dimensions offers a richer, more complex view of evolution than the gene-based, one-dimensional view and it is nowadays always more supported (Jablona & Raz, 2009).

1.2 DNA methylation

1.2.1. DNA methylation introduction

In eukaryotic cells different epigenetic mechanism are present for example hystone methylation, hystone acetilation and DNA methylation. The most important form of epigenetic information among these is the methylation of DNA which consist in the addition of methyl group on a cytosine base in the DNA sequence by a covalent modification.

The modified base 5-methylcytosine (m5C) is present in the DNA of all animals and plants, some fungal and protist taxa and many bacterial species: cytosine methylation is common to all large-genome eukaryotes but is present in only some small-genome eukaryotes. Cytosine methylation is mediated by a conserved group of proteins called DNA methyltransferases (Goll & Bestor, 2005).

Even though DNA methylation researches are largely focused on mammalian model systems, it is important to notice that this mechanism has been widely conserved during evolution (Schaefer & Lyko, 2007). Infact, cytosine DNA methylation was first described in the context of bacterial restriction modification systems where it is necessary to protect the host genome against the activity of exogenous restriction enzymes (Goll & Bestor, 2005). In light of the strong sequence conservation between bacterial modification enzymes and human DNA methyltransferases, it seems likely that these bacterial enzymes represent the evolutionary origin of the DNA methyltransferases in higher eukaryotes (Schaefer & Lyko, 2007). Consequentially, DNA methylation is present in most fungal, animal and plant genomes. The only model organisms for which DNA methylation could be excluded so far are the yeast *Saccharomyces cerevisiae* and the nematode *Caenorhabditis elegans*. It has been argued that DNA methylation has

been lost relatively recently during nematode evolution (Gutierrez & Sommer, 2004), and the forces that select against DNA methyltransferases are still under discussion (Goll & Bestor, 2005).

1.2.2. How to study DNA methylation

In epigenetic research field, several methods are available to assess DNA methylation at individual cytosines. The most popular method involves treating the DNA sample with sodium bisulfite. This converts unmethylated cytosine into uracil while methylated cytosine remains intact (Zilberman & Henikoff, 2007). Following this, the amount of conversion and thereby methylation is measured by performing either polymerase chain reaction (PCR) with targeted primers for methylated and unmethylated DNA (bisulfite PCR) (Fraga & Esteller, 2002), hybridization of the sample onto microarray or direct sequencing (bisulfite sequencing) (Zilberman & Henikoff, 2007). Another popular method, methylated DNA immunoprecipitation (MeDIP), involves isolating methylated DNA with antibody specific for methylated DNA (Sørensen & Collas, 2009). After immunoprecipitation, the methylated and unmethylated part of the sample can be differentially labeled and applied to microarray (MeDIP-chip) or sequenced (MeDIP-seq) (Sørensen & Collas, 2009). Several methods are also available to measure global methylation of DNA. These include assays based on high-performance liquid chromatography (HPLC) (Armstrong et al., 2010) and mass spectrometry (Rocha et al., 2010), in addition to methods measuring the amount of cut by methylation-sensitive restriction endonucleases (Karimi *et al.*, 2006).

1.2.3 Methyltransferases

Methylation of the vinyl carbon at the 5 position of cytosine residues in neutral aqueous solution has been termed a chemically improbable reaction (Chen *et al.*, 1991). Cytosine methyltransferases overcome the low reactivity of the C5 cytosine by means of a covalent catalysis mechanism (Figure 1.3 a and b) that is similar to that of thymidylate synthetase. The cysteine thiolate of a conserved prolylcysteinyl (PC) dipeptide in motif IV forms a covalent bond with the C6 of cytosine, as proposed by Santi (1983) and colleagues and modified by Verdine and colleagues (Bestor & Verdine, 1994). This cysteine is invariant in eukaryotic cytosine methyltransferases, and its substitution has been shown to result in a loss of activity by bacterial restriction methyltransferases and

by the eukaryotic cytosine methyltransferases Dnmt3A and Dnmt3B (Hsieh, 1999). Eukaryotic cytosine methyltransferases share the 10 sequence motifs (Figure 1.3 c) that are conserved within the bacterial (cytosine-5) methyltransferases (Goll & Bestor, 2005).

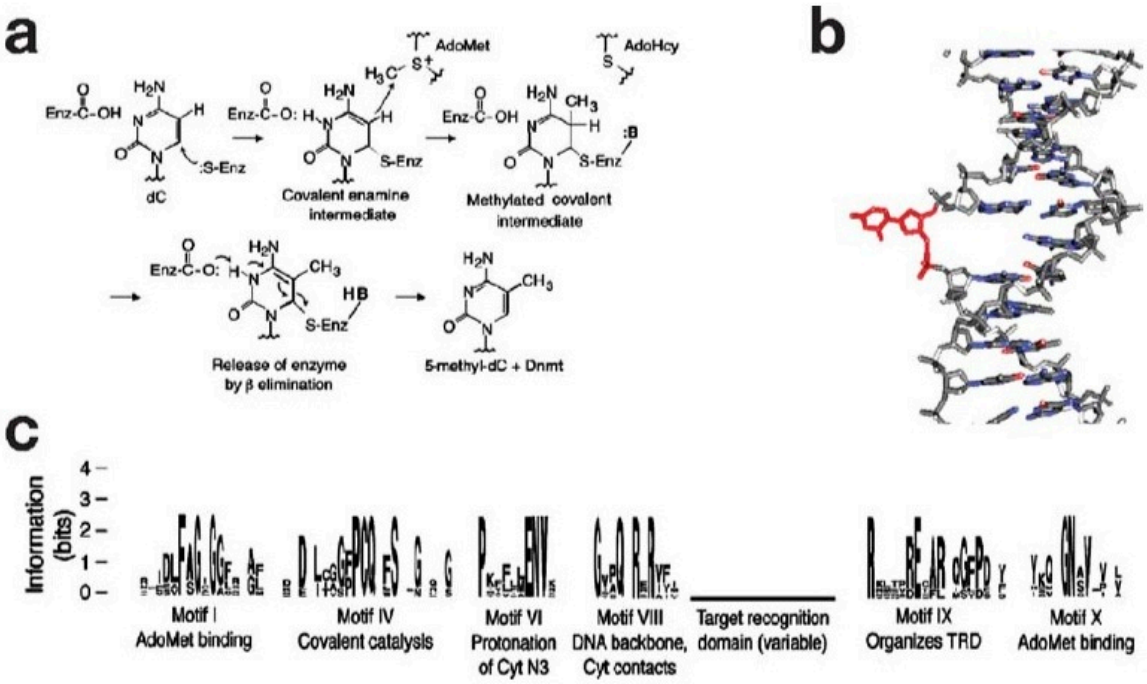

Figure 1.3 Catalytic mechanism and conserved motifs in DNA (cytosine-5) methyltransferases. (a) Catalytic mechanism: covalent addition of an enzyme nucleophile to the cytosine-6 position and protonation of the N3 position produces the 4,5 enamine intermediate that attacks the methyl group of S-adenosyl L-methionine (AdoMet). Following methyltransfer, an unidentified enzyme base or water molecule abstracts a proton from the cytosine-5 position, which allows release of free enzyme by beta elimination. **(b)** Eversion of the target cytosine from the DNA during catalysis. The DNA shown is from the M.HhaI-DNA-AdoHcy cocrystal structure. **(c)** Conserved motifs in DNA (cytosine-5) methyltransferases; functions of each motif are given at bottom. (Goll & Bestor, 2005)

These shared 10 sequence motifs are conserved within the bacterial (cytosine-5) methyltransferases (Posfai *et al,* 1989) and have high predictive value in the identification of new DNA cytosine methyltransferases. Indeed, almost all of the eukaryotic cytosine methyltransferase homologues were initially identified by the content of these motifs (Santi *et al*., 1983; Yoder & Bestor, 1998; Okano *et al.,* 1998). The functions of all 10 motifs are known from crystallographic studies of transition-state intermediates and from mutagenesis studies (Trautner *et al*., 1988). A region between motifs VIII and IX makes sequence-specific contacts with base edges in the major

groove and confers sequence specificity to bacterial cytosine methyltransferases (Wilkie *et al*., 1988). This region has been termed the target recognition domain.
The general architecture consists of a strongly conserved large domain, which includes the binding site for the cofactor AdoMet and the active site motifs, and a small domain, which is poorly conserved and is largely represented by the target recognition domain. Bacterial DNA (cytosine-5) methyltransferases have defined recognition sequences of 2 to 8 nucleotides, and all cognate sequences in the host genome are normally methylated. As discussed below, target selection by eukaryotic cytosine methyltransferases is not a function of innate sequence specificity (Cao *et al*., 2000).

Most cytosine methyltransferases can be grouped into four distinct families based on sequence homology within their C-terminal catalytic domains, although the fungal enzymes show greater divergence (Figure 1.4). All organisms that possess proteins from the DNA methyltransferase-1 (Dnmt1) family appear to also have at least one Dnmt3 homologue (Figure 1.5). Dnmt2 homologues are present in all organisms known to contain Dnmt1 and Dnmt3 homologues as well as in a number of additional organisms in which Dnmt2 is the only cytosine methyltransferase homologue (Lauster *et al*., 1989). The chromomethylase family is unique to the plant kingdom. Some eukaryotes (notably *C. elegans* and *Saccharomyces cerevisiae*) lack detectable cytosine methylation in their genomes and have no sign of any cytosine methyltransferase coding sequence. Other organisms, such as *A. thaliana*, have 10 or more cytosine methyltransferase homologues (Kumar *et al*., 1994).

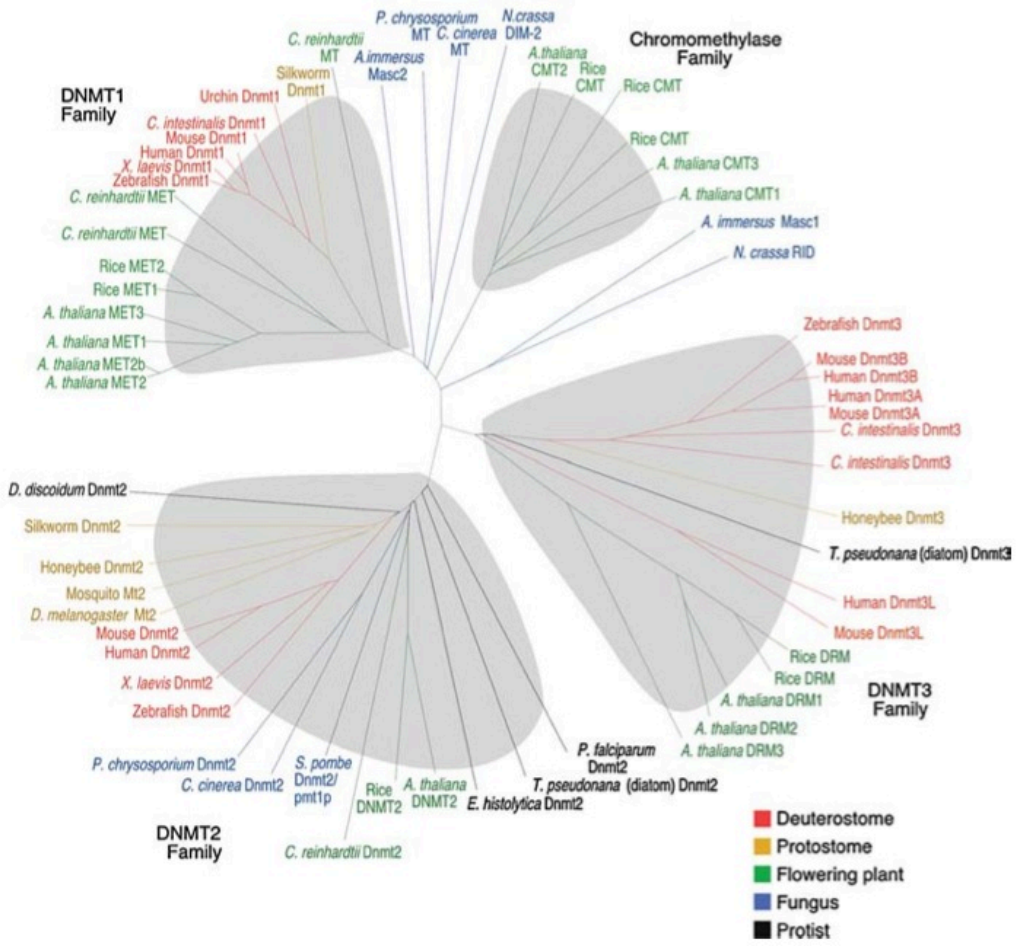

Figure 1.4 Distribution of cytosine methyltransferases and cytosine methyltransferase homologues in eukaryotes. Cytosine methyltransferases analysis revealed the existence of four distinct families of enzymes, named: Dnmt1, Dnmt2, Dnmt3 and Chromethylase. Major taxa are indicated by the color scheme shown at bottom. (Kumar *et al*., 1994).

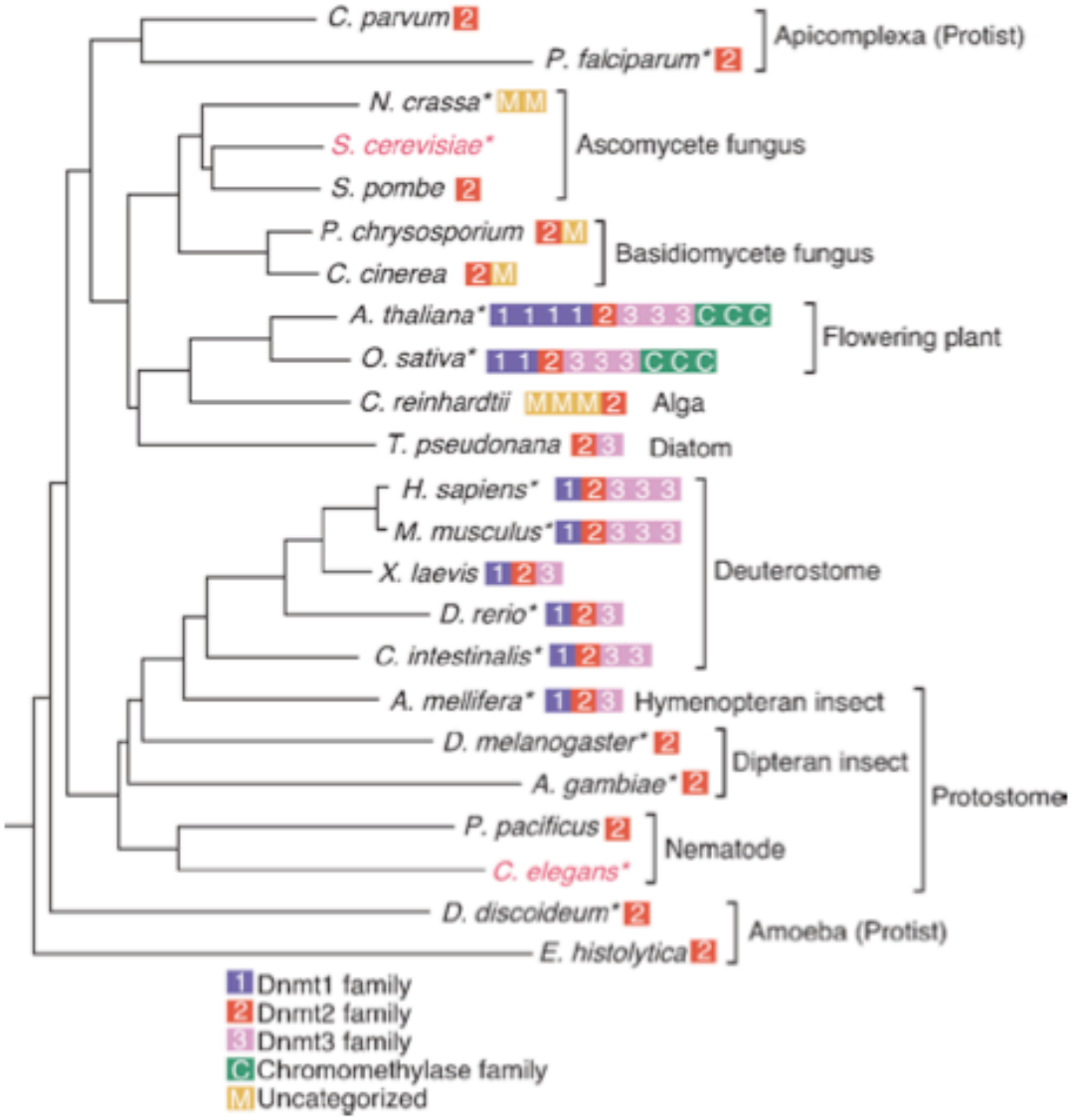

Figure 1.5 Distribution of the major cytosine methyltransferase families in eukaryotes. The color scale at bottom indicates membership in the cytosine methyltransferase families. Asterisks indicate organisms for which there is a nominally complete draft genome sequence; others are only partially sequenced, or the methyltransferase sequences were represented by expressed sequence tags. The genomes of *C. elegans* and *S. cerevisiae* lack any sequences that bear the methyltransferase motifs and are shown in red. Note that Dnmt2 is always present when the Dnmt1 and Dnmt3 families are both represented, but many organisms contain only Dnmt2 homologues. Two members of the bacterial genus Geobacter also have Dnmt2 homologues, but this is the only prokaryotic taxon known to contain members of this family. (Goll & Bestor, 2005).

Dnmt1

Dnmt1 is the most abundant DNA methyltransferase in mammalian cells, and considered to be the key maintenance methyltransferase in mammals. The first eukaryotic DNA methyltransferase to be purified and cloned was later named *Dnmt1* (Bestor *et al.*, 1988). Cedar and colleagues (1982) showed that hemimethylated DNA was methylated more rapidly than unmethylated DNA in nuclear extracts of cultured mammalian cells. Maintenance methylation provides heritability to genomic methylation patterns in a way that has no counterpart outside of DNA replication itself. The preference of Dnmt1 for hemimethylated DNA caused it to be assigned a function in maintenance methylation, although *Dnmt1* remains the only eukaryotic DNA methyltransferase to have been purified and cloned on the basis of its activity as a *de novo* cytosine methyltransferase (Bestor *et al.*, 1988). DNA substrates is greater than that of *Dnmt3A* and *Dnmt3B*, which are held to be the sole *de novo* DNA methyltransferases (Okano *et al.*, 1988). Maintenance methylation is also enforced by other factors that inhibit the de novo activity of Dnmt1 in vivo.

The sequence of mouse Dnmt1 cDNA revealed a protein of 1620 amino acids (the first 1,100 constitute the regulatory domain of the enzyme, and the remaining residues constitute the catalytic domain) that had a C-terminal domain of ~500 amino acids with clear similarities to the bacterial restriction methyltransferase M.DdeI (the only bacterial cytosine methyltransferase in the sequence databases at the time); a region of alternating glycine and lysine residues joined the C-terminal domain to a long (1100 amino acids) N-terminal domain (Steven et al., 1992).

The N-terminal domain of Dnmt1 contains a number of functional domains that have accreted over the course of evolution. Experiments have identified a sequence required for import of Dnmt1 into nuclei and a second sequence required for association with replication foci, which are micrometer-scaled structures in which DNA synthesis occurs within mammalian nuclei (Leonhardt *et al.*, 1992). Dnmt1 has a diffuse nucleoplasmic distribution in G1 phase but associates with replication foci during S phase and it is present at only very low levels in noncycling cells. Sequences very close the N terminus have been shown to interact with DMAP1 (DNA methyltransferase associated protein-1) (Rountree *et al.*, 2000). The N-terminal domain also has a role in coupling stabilization of DNA to the growth state of cells: full-length Dnmt1 is degraded in G0 cells, but when 118 N-terminal amino acids of the protein are removed by forcing translation to initiate at the second ATG codon, the protein is stabilized in G0 cells (Goll & Bestor, 2005).

In human cancer cells DNMT1 is responsible for both de novo and maintenance methylation of tumor suppressor genes (Kam-Wing *et al.*, 2006; Angela *et al.*, 2006). Dnmt1 has several isoforms: the somatic Dnmt1, a splice variant (Dnmt1b) and an oocyte-specific isoform (Dnmt1o). Dnnmt1o is synthesized and stored in the cytoplasm of the oocyte and translocated to the cell nucleus during early embryonic development, while the somatic Dnmt1 is always found in the nucleus of somatic tissue.
Dnmt1 null mutant embryonic stem cells were viable and contained a small percentage of methylated DNA and methyltransferase activity. Mouse embryos homozygous for a deletion in Dnmt1 die at 10–11 days gestation (En *et al.*, 1992).

Dnmt 3

The mammalian genome encodes two functional cytosine methyltransferases of the Dnmt3 family, Dnmt3A and Dnmt3B, which primarily methylate de novo CpG dinucleotides, and a third homologue, Dnmt3L, which lacks cytosine methyltransferase activity and functions as a regulatory factor in germ cells (Goll & Bestor, 2005).
Both recombinant proteins transfer methyl groups to hemimethylated and unmethylated substrates at equal rates and without evidence of intrinsic sequence specificity beyond the CpG dinucleotide (Okano *et al.*, 1998); in some organisms, Dnmt3A has also been reported to methylate CpA sites (Ramsahoye *et al.*, 2000). Dnmt3A and Dnmt3B are expressed in a range of adult tissues but at lower levels than Dnmt1.
Dnmt3L (DNA methyltransferase 3-like) is the sole DNA methyltrans- ferase homologue that is expressed specifically in germ cells (Aapola et al., 2000) and is related to Dnmt3A and Dnmt3B in both N- and C-terminal domains and retains the cysteine-rich domain but lacks the PWWP domain.
Similarity with Dnmt3A and Dnmt3B is seen in framework regions, but key residues within catalytic motifs have been subject to nonconservative substitutions. In addition, the protein has not been shown to possess methyltransferase activity. However, Dnmt3L is essential for establishment of a subset of methylation patterns in both male and female germ cells (Bourc'his *et al.*, 2001).

Dnmt 2

Dnmt2 was the first methyltransferase to be identified in human and represents the most conserved and shared methyltransferase among living being. For example, Dnmt2 proteins are the only methyltransferase present in *Drosophila melanogaster* (Lyko *et al.*, 2000).

Dnmt2 is also known as the “enigma methyltransferase” because even if this enzymes is present in all living being, its deletion produced non lethal mutants (on the contrary of what happen with mutations in Dnmt 1 and Dnmt3). In this context, it was not clear why this gene could have been the most conserved during evolution despite its apparent loss of function. Moreover Dnmt2 was shown not be able to methylate DNA in *in vitro* experiments (Borsatti & Mandrioli, 2004).
It is only in recent years that Goll and collegues (2006) demonstrated that Dnmt2 is not involved in DNA methylation but in the transfer of methyl groups to RNA sequences, thus being implicated in the post transcriptional regulation of gene expression. Indeed, purified recombinant human Dnmt2 methylated RNA preparations from Dnmt2 mutant mice, flies, and plants. Further experiments identified C38 in the anticodon loop of tRNAAsp as the methylation target site of Dnmt2 (Goll et al. 2006).

1.2.4 DNA methylation role

The role of DNA methylation in the organisms are different, but can be grouped in three main levels. These function are not mutually exclusive, but on the contrary, often the roles of DNA methylation in the cell are multiple.

Host genomes defence

Many repetitive elements and transposons are present in every genome and they should be regarded as highly specialized intragenomic parasites that are disseminated largely by vertical (gametic) transmission and provide no benefit to the host (Doolittle & Sapienza 1980). Indeed, the genome is an ecological niche, and it is inevitable that the resources available in it should come to lye exploited by specialized replicating entities (Orgel & Crick, 1980; Yoder *et al.*, 1997).
It is now known that the majority of cytosine methylation in plants and mammals and almost all cytosine methylation in the ascomycete fungus *Neurospora crassa* resides in repetitive elements. Much of this methylation is in transposons, which are interspersed repeated sequences that constitute more than 45% of the human genome (Smit *et al.*, 1996). It is now clear that DNA methylation represents the primary mechanism of transposon suppression in host genomes: most genomic m5C resides in transposons, and transposons are reanimated in the demethylated genomes of the mouse (Walsh *et*

al., 1999; Kato *et al*., 2003). Genome demethylation in plants (which can survive larger reductions in genomic m5C than can mammals) also causes greatly increased rates of transposon insertion (Miura *et al*., 2001; Singer *et al*., 2001). Transposons are methylated in the genomes of mammalian germ cells, and over time cytosine methylation in trans- posable elements leads to their irreversible inactivation through accumulation of C → T transition mutations arising by deamination of m5C to thymine (Hirochika *et al*., 2000).

Gene regulation during development

There are many ways that gene expression is controlled in eukaryotes, but methylation of DNA is a common epigenetic signaling tool that cells use to regulate development.

Infact, DNA methylation is an important component in several cellular processes, including embryonic development, genomic imprinting, X-chromosome inactivation, and preservation of chromosome stability. Moreover, Dnmt mutant mice which have reduced methylation levels were shown to die early in development (Davuli *et al*., 2001).

Moreover, evidence of the role of methylation in gene regulation has been found in studies that show that methylation near gene promoters varies considerably depending on cell type, with more methylation of promoters correlating with low or no transcription (Suzuki & Bird, 2008). Also, while overall methylation levels and completeness of methylation of particular promoters are similar in individual humans, there are significant differences in overall and specific methylation levels between different tissue types and between normal cells and cancer cells from the same tissue.

More in particular, methylation plays a crucial role in repressing gene expression, perhaps by blocking the promoters at which activating transcription factors should bind (Marino-Ramirez *et al*., 2004).

Imprinting

Cytosine methylation is required in both plants and mammals for the monoallelic expression of imprinted genes, which are normally expressed from only one of two identical alleles according to the sex of the parent that contributed the allele (Zilbermann & Henikoff, 2007).

Imprinted genes have allele-specific expression patterns based on parental origin of the allele in at least one tissue. DNA methylation maintains the stable expression patterns of imprinted genes in mammalian genomes (Reik & Walter, 2001). Alternation in the

expression of several imprinted genes, that can result from changes in methylation of their control elements, can lead to severe disease.

1. 2. 5 DNA methylation in animals

In mammals, DNA methylation occurs almost exclusively in the symmetric CG context and is estimated to occur at ~70–80% of CG dinucleotides throughout the genome (Ehrlich *et al.*, 1982; Law & Jacobsen, 2010).

Mammalian genomes, like those of all vertebrates tested so far, are globally methylated in the sense that all categories of DNA sequence (genes, transposons and intergenic DNA) are targets for CpG methylation (Eckhardt *et al*., 2006; Rabinowicz et al., 2006). Thus, unlike mosaically methylated genomes, in which methylated and unmethylated domains coexist in approximately equal proportions, mammalian genomes are dominated by methylated DNA (Rakian *et al.*, 2004). Unmethylated domains (that is, most CpG islands) account for a small fraction (1–2%) of the total (Bird, 1986; Bird *et al*., 1985) called CpG islands or simply CpGs (Bird, 2002). CpGs are largely found in promoter region of genes, having a crucial role in gene regulation. These regions are always found unmethylated (Bird, 1986). Because the vast majority of DNA is methylated to a high level, it follows that gene bodies are also methylated in vertebrates, and this has been confirmed by numerous studies (Illingworth *et al*., 2008).

1.3 Honeybee DNA methylation

The recently completed genome sequencing for the honeybee *Apis mellifera* revealed distinct homologues for Dnmt1, Dnmt2 and Dnmt3 methyltransferases as well as candidate methyl-DNA-binding protein (Schaefer & Lyko, 2007).

A closer analysis of the honeybee DNA methylation system revealed that the DNA methyltransferase orthologs Dnmt1A and Dnmt3 have enzymatic DNA methyltransferase activities (Wang *et al*., 2006). An analysis of *A. mellifera* genomic DNA by reverse phase high-performance liquid chromatography indicated the presence of 5-methylcytosine during larval and adult stages of development (Schaefer & Lyko, 2007).

Recently, bisulfite sequencing was used to delineate the methylome of the brains of honeybee queens and workers (Lyko *et al.,* 2010). This methylome analysis revealed that >75% of methylated CpGs in the honeybee are localized to exons (Figure 1.6), and that bees have negligible DNA methylation outside of CpG dinucleotides (Lyko *et al.*, 2010). Only a small fraction of data was classified as intron or promoter methylation. Indeed, the most frequent pattern in invertebrate animals is 'mosaic methylation', comprising domains of heavily methylated DNA interspersed with domains that are methylation free (Bird *et al.*, 1979; Tweedie *et al.*, 1997; Suzuki & Bird, 2010).

The exons that are targeted for methylation in honeybees lie in approximately half of the annotated genes of the genome. The remaining half of the unmethylated genes has methylation intensity below the background rate of adjacent intergenic regions, indicating that their lack of methylation is actively maintained. Unmethylated exons also have methylation intensities below the adjacent introns that are not targeted for methylation. Indeed, methylation targeting system in the honeybee is specific, to the extent that it recognizes intron–exon boundaries and that non-targeted genes and exons are not spuriously methylated (Flores & Amdam, 2011).

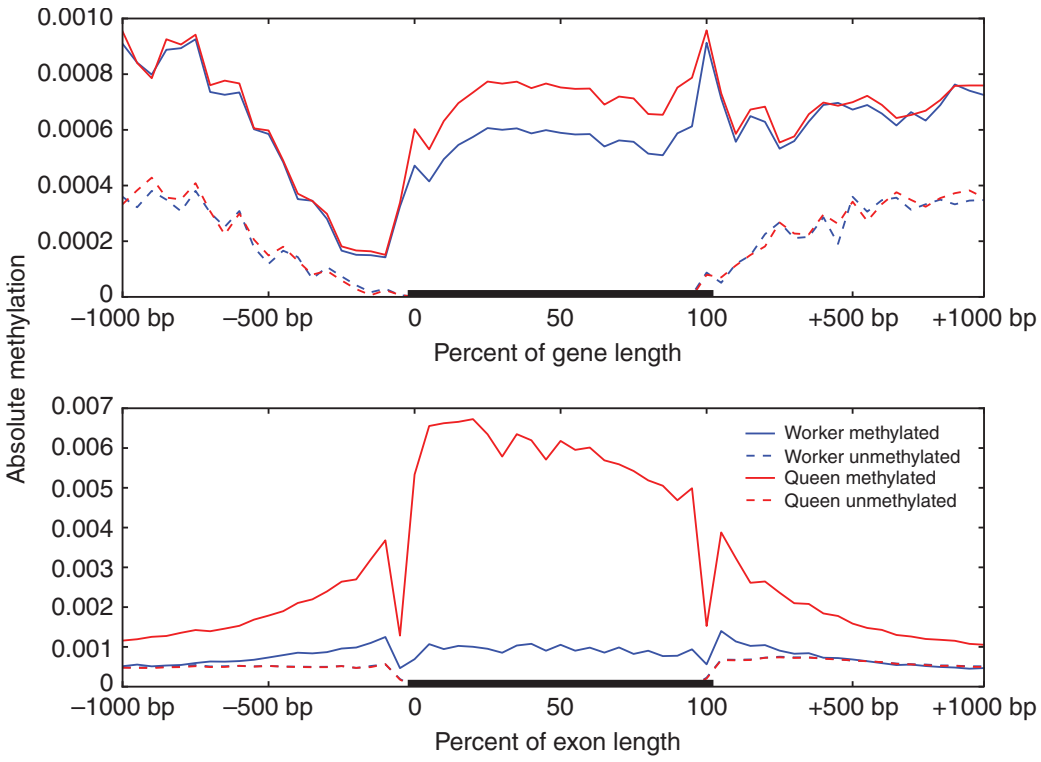

Figure 1.6 The level of absolute DNA methylation targeted to genes and exons in honeybee queens and workers. (a) Absolute DNA methylation level (total intensity of CpG methylation divided by sequence lenght). **(b)** Absolute DNA methylation levels calculated over the lenght of all exons.

Although honeybee introns are sparsely methylated compared with exon regions, the methylation intensities of the human genome increase in introns that are adjacent to methylated exons (Lister *et al.,* 2009). This difference is reflected in an opposing pattern of exon–intron CpG depletion. When further contrasting patterns of methylation in the two genomes, an obvious difference is that >70% of CpGs are targeted for methylation in humans compared with <3% in honeybees. With only a small fraction of DNA methylation detected in the promoter regions of genes, the major functional role of methylation in honeybees may be the regulation of splice variant diversity rather than to silence gene transcription (Lyko *et al.*, 2010). In contrast, promoter methylation is a widely used mechanism for transcriptional regulation in humans (Saxonov *et al.*, 2006). Humans, moreover, methylate in the CA dinucleotide context and this type of non-CpG methylation may help to maintain the pluripotency of the stem cells, as it is not observed in differentiated cells (Lister et al., 2009). Similar interspecific differences are apparent from the methylome data summarized in Table 1.1.

Species	Methylated CpGs (%)	mC context	FMR	Sample material	Genome size (Mb)	Bimodal CpG ratio	DNMT	Reference
Apis mellifera	0.51	CG	Exons	Queen and worker brains	231	Genes, exons	1, 2, 3	Lyko et al., 2010
Homo sapiens	68.40	CG, CHG, CHH	Promoters, genes	PBMC	3077	Promoters	1, 2, 3	Li et al., 2010
Homo sapiens	70–80	CG, CHG, CHH	Promoters, genes	H1, IMR90 cell lines	3077	Promoters	1, 2, 3	Lister et al., 2009
Bombyx mori	0.71	CG	Exons, introns, intragenic smRNAs	Whole larvae	431.8	None	1, 2, 3	Zemach et al., 2010
Bombyx mori	0.11	CG	Exons, introns, intragenic smRNAs	Silk gland	431.8	None	1, 2, 3	Xiang et al., 2010
Ciona intestinalis	21.60	CG	Genes	Muscle tissue	141.2	Genes	1, 2, 3	Zemach et al., 2010
Drosophila melanogaster	0.12	CG	None	Embryos 0–3 h	162.4	None	2	Zemach et al., 2010
Arabidopsis thaliana	~18	CG, CHG, CHH	Transposons, promoters, genes	Immature flower buds	115.4	None	1, 2, 3	Lister et al., 2008

Table 1. 1 Species-specific methylome attributes. Several attributes are shown that differ between species, including the percent of all cyotosine–phosphate–guanine (CpG) dinucleotides in the genome that are methylated, sequence contexts in which methylcytocine (mC) occurs, functionally methylated regions (FMRs), the type of sample material used to generate the data, approximate genome size, which genomic regions generate a bimodal distribution of CpG depletion (bimodal CpG ratio), and which classes of DNA methyltransferases (DNMTs) are contained within the species' genome. (Lyko *et al.*, 2010)

As showed in Li-Byarkay and colleagues (2013) Dnmt3 RNAi in honeybees decreased global genomic methylation level as expected and in addition caused widespread and diverse changes in alternative splicing in fat tissue. Four different types of splicing events are affected by Dnmt3 gene knockdown, and two types, exon skipping and intron

retention, was directly related to decreased methylation. These results demonstrate that one function of gene body DNA methylation in *A. mellifera* is to regulate alternative splicing. Thus demonstrating the important role of DNA methylation in affecting and regulating the phenotypic plasticity of these individual (Li-Byarkay, 2013).

One possibility that was recently uncovered by Shukla and colleagues (2011) in mammals is that exon-specific DNA methylation may affect exon-skipping by interfering with CCCTC-binding factor (CTCF) binding to the DNA. CTCF binding can promote the inclusion of nearby exons by causing RNA polymerase II pausing, and thus interference with CTCF binding has a reciprocal effect on exon inclusion (Shukla *et al*., 2011; Flores *et al*., 2012). These observations support the hypothesis that the effect of DNA methylation, i.e. either causing a repressed (heterochromatin) or open (euchromatin) chromatin state, can vary depending on the species, sequence context, DNA binding proteins, and the histone modifying enzymes that are associated with methylated DNA (Cedar *et al*., 2009; Close & Bird, 2006).

A recent study by Cingolani and colleagues (2013) confirms the role of cytosine methylation in exon in regulating alternative splicing. According to this study, skipped exons tend to be more methylated than non alternative spliced ones. Moreover, methylation driven alternative splicing of exons would have effect on the phosporilation of the protein and thus in its sensibility, subcellular localization, activities and other properties. Further research is needed to determine the mechanism by which splice junction methylation and hydroxymethylation affect mRNA splicing.

In other studies by Flores and colleagues (2012) it was found that, overall, exons included in the gene transcript contained significantly more DNA methylation than skipped exons just after the exon start site and before the exon end site. Moreover, genes that are methylated and/or alternatively spliced were longer and tent to have more exons than genes that were not methylated and not alternatively spliced. Finally, it was observed that methylated honeybee genes that are also alternatively spliced were even higher conserved across species than methylated genes that were not alternatively spliced or than alternatively spliced genes that are not methylated (Hunt *et al*., 2010). These associations suggest that gene length, DNA methylation, and alternative splicing are positively linked to gene conservation. Compared to non-methylated and non-alternatively spliced genes, methylated and/or spliced genes can result in a greater variety of transcripts (Lyko *et al*., 2010; Park *et al*., 2011). Thus, by varying the methylation and splicing pattern, genes can assume novel functions without a necessary change in their primary sequence. This may explain why the respective

genes can afford a higher conservation – they realize variation in different ways, e.g. by varation in master regulators of DNA methylation and splicing (Zeng & Yi, 2010; Takuno & Gaut, 2011).

At the individual and colony level DNA methylation is found to be widespread and associated with differential gene expression in the different castes of honeybee, thus confirming the role of DNA methylation in modulating gene expression and its importance in the biology of the honeybee (Elango *et al.*, 2009). Indeed, biological scenarios may help explain distinct implementations of transcriptional regulation by DNA methylation between taxa. In the honeybee, eusociality provides a reasonable justification for a low prevalence of DNA methylation. Dependence on a multi-caste social system necessitates phenotypic accommodation in one caste for beneficial phenotypic innovations in another.

In *A. mellifera* DNA methylation is a key component of an epigenetic network controlling a most important aspect of eusociality, the reproductive division of labor and memory(Drapeau et al., 2006; Kucharski et al., 2008).

Young larvae of the honeybee are totipotent; they can become either queens (reproductives) or workers (largely sterile helpers). DNA methylation has been shown to play a key important role in this differentiation. Different epigenetic DNA metylation pattern, related to different larvae diet is at the base of castes differentiation in honeybees (Shi *et al.*, 2011): the whole-body amount of DNA methylation in queens is lower than in workers and inhibition of de novo methylation during the development of larvae-fed workers results in queen-like individuals (Kucharski *et al.*, 2008). During the adult stage, a structure of lower genome-wide methylation in queens may accommodate a wider use of dynamic DNA methylation in worker tissues, including brain and fat body (functionally homologous to white adipose tissue), that are central to worker behavioral expression and regulation (Amdam, 2011). Honeybee worker phenotypes are plastic and diverse, but correlations between specific suites of physiological and behavioral expression are usually fitted into a predictable and temporal work schedule that may be governed, in part, by the use of DNA methylation (Flores & Amdam, 2011).

DNA methylation in *A. mellifera* is also used for storing epigenetic information, that the use of that information can be differentially altered by nutritional input, and that the flexibility of epigenetic modifications underpins, profound shifts in developmental fates,

with massive implications for reproductive and behavioral status (Kucharski *et al.*, 2008).

1.4 Honeybees and *Varroa destructor* mite

The honeybees *Apis mellifera* and *Apis cerana* are important economic insects, not only for honey production, but also for crop pollination. From an economic point of view, the value of crops created by honeybee pollination is 100 times higher than that by honey production and it has been estimated in around 14 billion dollars to worldwide agriculture annually (Morse and Calderone, 2000).

The external parasitic mite *Varroa destructor* (Anderson and Trueman, 2000) is currently the most serious threat to beekeeping around the world (De Jong *et al.*, 1982). This haemolymph-feeding mite not only weakens adult, pupal and larval honeybees, but also serves as a vector and inducer of viral infections, causing severe damage to honeybee populations world wide (Ball and Allen, 1988; Ball, 1994). Furthermore, the varroa mite has been attributed, in part, to the recent widespread Colony Collapse Disorder (CCD) as a disease vector (van Englesdorp *et al.*, 2007; Anderson *et al.*, 2008).

The varroa mite (*Varroa destructor*) switched from its natural host (*A. cerana*) to *A. mellifera*, the European honeybee, when honeybees were moved into areas where *A. cerana* is endemic. In *A. mellifera* the mite found a far less resistant host, and subsequently spread nearly worldwide (Peng *et al.*, 1987; Oldroyd, 1999). Because varroa and European honeybees have not co-evolved for a long period of time, they do not exhibit an adapted host-parasite relationship, resulting in varroa often killing its host (Le Conte *et al.*, 2010).

V. destructor is a pseudo-haplo-diploid parasite species (Martin *et al.*, 1997; Harris and Harbo, 1999) reproducing mainly through brother-sister matings, a system which largely favors the fixation of new mutations (Cornuet *et al.,* 2006). Co-evolution of the host and the parasite is driven by mutations of both the mite and the honeybee, which can lead to a more or less stable equilibrium. Heritable behavioral and physiological traits can be involved in varroa tolerance (Büchler *et al.*, 2010; Rinderer *et al.*, 2010), but the mite may counter-select those traits to increase its fitness. Mite reproduction is an important trait in varroa population dynamics and differential reproduction rates had been observed since the first infestations on honeybee were detected (Anderson, 2000).

The life cycle of the female mites (Figure 1.7) is subdivided into a phoretic phase on adult honeybees and a reproductive phase within worker or drone brood cells. For reproduction, the female mite leaves the adult honeybee and enters a brood cell with 5th instar larva shortly before the cell sealing and become stuck in the larval food at the bottom of the brood cell. Within a few hours after cell capping the larvae consume the rest of the food and set the mite free (Rosenkranz *et al.*, 2010). At that time the female mite has already started with oogenesis in the terminal oocyte (Steiner *et al.*, 1994; Garrido *et al.*, 2000; Frey *et al.*, 2013).

The female mite lays the first male egg approximately 70 h after cell capping followed by 3–5 female eggs in 30 h intervals (Martin, 1994; Rehm and Ritter, 1989). As the success of a reproductive cycle depends on the number of viable adults mated daughter mites that leave the brood cell together with the newly hatched honeybee, the duration of the postcapping period is a limiting factor and, therefore, the mother mite should start egg laying as soon as possible (Rosenkranz *et al.*, 2010).

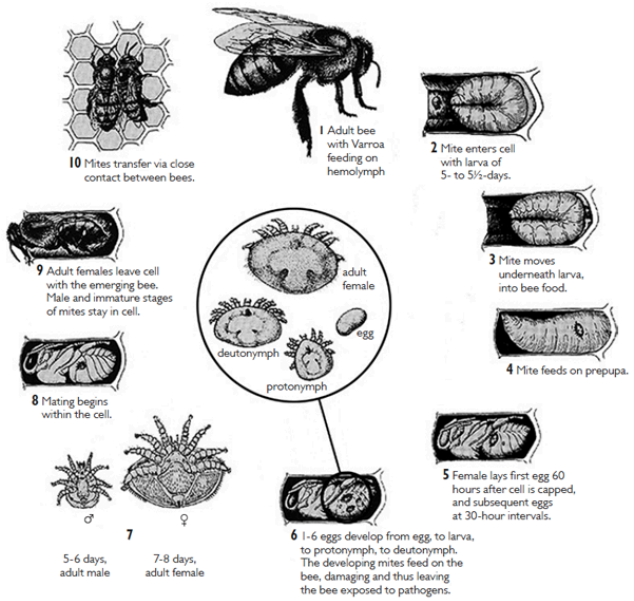

Figure 1.7 ***Varroa destructor*** **cell cycle.** The mite enters the hive on a honeybee, it will drop off when it collects the scent, juvenile pheromone, of the brood in royal jelly. Here it scurries into the cell and hides under the prepupa in the royal jelly. As soon as the cell is sealed, the fertile female will lay an egg. This first egg is that of a male. About 30 hours later, the mother mite will lay an additional egg which will be a female. The male will hatch, develop, and mature a bit faster than the female. The mother will continue laying until she has laid 2 to 6 eggs. The male mite will be mature and ready to mate with his sisters when they mature, and then he will die in the cell. The mated sisters will continue to feed on the pupa until it emerges from the cell. They will then scurry about to find another cell with brood to continue the cycle of mite rearing. The female mites are believed to live as long as 30 days to continue rearing mites (Steiner *et al.*, 1994).

Since 2006, disastrous colony losses have been reported in Europe and North America. The causes of the losses were not readily apparent and have been attributed to overwintering mortalities and to a new phenomenon called Colony Collapse Disorder (CCD). Most scientists agree that there is no single explanation for the extensive colony losses but that interactions between different stresses are likely involved. One of these is the presence of the mite, infestating the hives (Le Conte *et al*., 2010).
The phenomenon, called CCD, was identified by a set of distinctive characteristics, including the absence of dead honeybees in or near the colony and the presence of abundant brood, honey, and pollen despite vastly reduced numbers of adult workers (Cox-Foster *et al*., 2007). Losses were estimated at 23% over the winter of 2006–2007 (van Englesdorp *et al*., 2007) and at 36% over the winter of 2007–2008 (vanEngelsdorp, 2008; Johnson *et al*., 2009).

One important factor associated with honeybee mortality is the presence of the parasitic mite *Varroa destructor*. Many physical and physiological detrimental effects of the varroa mite have been described at the individual honeybee and colony levels. Repeated varroa feeding on adult honeybee and brood hemolymph injures the honeybees physically, reduces their protein content and wet and dry body weights, and interferes with organ development (Schneider & Drescher, 1987; Bowen-Walker & Gunn, 2001). The parasitic mite and the transmitted viruses contribute to morphological deformities (small body size, shortened abdomen, deformed wings), which reduce vigor and longevity, and they also influence flight duration and the homing ability of foragers (Schneider & Drescher, 1987; Koch & Ritter, 1991; Romero-Vera & Otero-Colina, 2002; Garedew *et al*., 2004; Kralj & Fuchs, 2006). The mite also weakens the honeybee's immune system, suppressing the expression of immune-related genes reducing worker survivorship and colony fitness (Yang & Cox-Foster, 2005, 2007).
Without treatment, honeybee colonies typically die within 2 years after initial varroa infestation (De Jong *et al*., 1982). Thus, several synthetic miticides are used by beekeepers for their control. Although initially effective, the continuous use of these pesticides has led to the evolution of miticide resistance within a few years (Milani, 1999). that is now widespread in Europe, USA and Canada (Elzen *et al*., 1998, 1999; Milani, 1999; Sprefacio *et al*., 2001; Elzen and Westervelt, 2002; Thompson *et al*., 2002; Skinner *et al.,* 2003). A recent study showed that most (>85%) colony fatality cases

during winter in Canada were significantly associated with varroa mite infestation despite colonies being treated with synthetic miticides (Guzman-Novoa *et al.*, 2010). This suggests that mite populations are becoming more difficult to control in recent years. In addition, the use of synthetic miticides in honeybee hives raises the risk of contamination of honey and other hive products (Ruijter, 1995; Wallner, 1999; Hamidduzzaman *et al.*, 2012).

1.5 Honeybees and varroa transmitted parasites

The role of varroa mites as a vector in transmitting viruses from infected individuals to healthy honeybees has been demonstrated (Cox-Foster *et al.* 2007). Varroa feeds on brood and adult honeybees and moves quickly from one honeybee to another (Le Conte & Arnold, 1987). The varroa mite can facilitate the horizontal transmission of viruses from nurse honeybees to larvae through larval food and via brood to adults (Ball, 1985; Chen *et al.*, 2004). It can also be transmitted vertically by drones via semen and by queens via virus infected eggs (Yue *et al.*, 2006, 2007). In addition, there is evidence for horizontal mite-to-mite transmission of viruses (Bowen-Walker *et al.*, 1999; Chen *et al.*, 2004, 2005). A few of those virus can replicate in the varroa mite and are present in mite saliva, which suggests that varroa is likely an active biological vector for honeybee viruses (Ongus *et al.*, 2004; Shen *et al.*, 2005; Chen *et al.*, 2006).

Unfortunately, for honeybee viruses neither cell culture models nor satisfactory cellular and molecular data exist. Therefore, the more descriptive terms overt and covert infections have to be used.

Typically, overt infections are those in which the virus-infected host develops obvious disease symptoms and covert infections are asymptomatic. These two broad categories cover a spectrum of infection strategies, the boundaries of which may not always be clearly defined. Even the distinction between overt and covert is not always clear cut if, like it is the case for insect viruses, the definition and observation of symptoms are difficult, because clinical or laboratory diagnosis in its classical (vertebrate) sense are in most of the cases not available. In the absence of physiological and serological parameters defining disease symptoms the only usable symptoms for insects are obvious morphological or behavioral changes or ultimately, death (Shen *et al.*, 2005).

However, honeybee viruses rarely cause easily detectable (i.e. visual) symptoms. This might suggest that they should be classified as covert infections. Occasionally, these viruses do cause symptoms (trembling, inability to flight, crippledness, death) in their hosts often accompanied by highly elevated virus titres easily detectable even by antibody-based detection methods (Ball, 1985).

Of those viruses infecting honeybees worldwide, acute bee paralysis virus (ABPV), black queen cell virus (BQCV), Kashmir bee virus (KBV), and sacbrood virus (SBV) are the most common infections in the United States an Europe (Cox-Foster *et al*. 2007). Most honeybee viruses are single stranded RNA viruses; they are isometrical, about 20–30 nm in diameter and non occluded possessing a buoyant density in CsCl ranging from 1.33 to 1.42 g/ml, and a 100-190S sedimentation coefficient (Bailey, 1976). To date, the complete genome sequences of six honeybee viruses, ABPV (Govan *et al*., 2000), BQCV (Leat *et al*., 2000), DWV (Fujiyuki *et al*., 2004), KBV and SBV (Ghosh *et al*., 1999) have been reported. The genomes of ABPV, BQCV, and KBV are monopartite bicistronic with non-structural genes at the 50 end and structural genes at the 30 end, while the genome of KV, SBV and DWV are monopartite monocistronic genomes with structural genes at the 50 end and non-structural genes at the 30 end. Based largely on phylogenetic analyses using these sequences, a new virus family, Dicistroviridae, has been proposed to include several of the honeybee viruses (Mayo, 2002).
In nature, BQCV, DWV, KBV, and SBV infect larvae and pupae as well as adult honeybees, while ABPV affects only adult honeybees. Diagnosis of honeybee virus infections is difficult because honeybee viruses usually persist as inapparent infections and cause no overt signs of disease (Bailey, 1967). More overmixed virus infections in honeybees are quite widespread in nature, as were detected mixed infections of BQCV, DWV, KBV, and SBV in adult worker honeybees and brood.

Black Queen Cell Virus (BQCV)

Black queen-cell virus (BQCV) is one of 18 viruses isolated from honeybees (Allen & Ball, 1996; Ball & Bailey, 1991). It was first isolated from queen pre-pupae and pupae, found dead in their cells (Bailey & Woods, 1977). The name of the virus was derived from darkened areas on the walls of cells containing infected pupae. Pupae were found to contain large numbers of isometric virus particles, 30 nm in diameter. Particles contained a single genomic RNA (8,550 nucleotides) and four capsid proteins, with molecular masses of 34, 32, 29 and 6 kDa. BQCV multiplied readily when injected into

pupae, but could not be similarly propagated in caged adult honeybees. However, it did multiply in adult honeybees if ingested with spores of the microsporidian parasite *Nosema apis* (Bailey *et al.*, 1983). A correlation was also observed between the incidence of BQCV in dead field honeybees from colonies in the UK; both showed peak infections during spring and early summer (Allen & Ball, 1996).

Deformed Wing Virus (DWV)

For DWV, the situation is somewhat different. DWV sequences could be detected in all stages of development from egg to adult workers, queens and drones, all of them without any obvious signs of disease (Chen *et al.*, 2005). DWV positive sperm could be collected from healthy appearing drones and artificial insemination using this virus-positive sperm did not result in any clinical symptoms in the offspring (Yue *et al.*, 2006). These results suggest vertical transmission through sperm and eggs to larvae, pupae, and adult honeybees. Additionally, viral sequences could be detected in brood food indicating horizontal transmission through feeding, again resulting in an asymptomatic infection (Yue and Genersch, 2005). Therefore, DWV causes true covert infections in all life stages of the honeybee and these viruses are transmitted vertically as well as horizontally. Overt DWV infections are inevitably associated with varroa destructor. Only when the virus is transmitted by *V. destructor* during pupal development, the characteristic clinical symptoms (malformed appendages, shortened and bloated abdomens, miscolouring) are present in the hatching honeybee (Ball & Allen, 1988; Bowen-Walker *et al.*, 1999; Martin, 2001; Martin *et al.*, 1998; Yue & Genersch, 2005). Hence, the infection strategy of DWV depends upon the mode of transmission. Horizontal (feeding) and vertical transmission of DWV in the absence of *V. destructor* causes asymptomatic, covert infections. Vectorial transmission of DWV by *V. destructor*, i. e., transmission by “injecting” the virus into pupae leads to overt infections. Early experiments already suggested this connection between mode of transmission and occurrence of clinical symptoms: injection bioassay with DWV using young pupal honeybees demonstrated the causal relationship between crippled wings of honeybees originating from injected pupae and DWV (Bailey & Ball, 1991).

Kashmire Bee Virus (KBV)

KBV is known to cause covert infections. For instance, individuals can carry virus loads up to 106 KBV particles without affecting longevity (Bailey & Gibbs, 1964) and pupae were shown to carry KBV without any signs of disease (Anderson & Gibbs, 1988; Dall, 1985). As typical for covert infections, persisting KBV remains fully competent and can reemerge to cause an overt infection (death): this is observed by injecting potassium buffer or insect ringer into covertly infected individuals (Anderson & Gibbs, 1988). KBV cause honeybee mortality and colony collapse in association with *V. destructor* (Allen & Ball, 1996; Ball & Allen, 1988). However, for both viruses it is not yet definitely demonstrated whether they need to be transmitted through the mite to cause an overt infection (like it is the case for DWV) or if mite infestation is the trigger for reactivation of the virus. Early experiments which demonstrated that both viruses can cause fatal infections when injected (Bailey & Ball, 1991; Bailey & Gibbs, 1964) do not help here since injection bioassays bypass the natural transmission routes and for many invertebrate pathogens bypassing the integument almost assures infection. Accordingly, it has been shown recently that *V. destructor* was able to transmit KBV but no clinical symptoms or honeybee mortality was recorded as a result of the experimental vector-borne KBV infection (Chen *et al*., 2004). Therefore, we still need to show that transmission of KBV through *V. destructor* (Ball, 1989) involves infectious viruses which cause overt infection before we can decide whether KBV also use mixed infection strategies or only cause covert infections which may be reactivated through mite infestation.

Sacbrood Virus (SBV)

SBV causes fatal acute and, therefore, overt infections in honeybee larvae (Bailey, 1975). SBV could also be demonstrated in pupae (Dall, 1985) and adult honeybees without obvious signs of disease (Anderson & Gibbs, 1989; Bailey, 1969; Bailey & Fernando, 1972) and in eggs (Chen *et al*., 2006) indicating vertical transmission. Extracts from apparently healthy whereas SBV positive honeybees gave rise to overt SBV infection in the injected honeybees (Bailey, 1976) demonstrating that SBV causing covert infections remained fully able to cause well characterized overt infections. Absence of clinical symptoms, vertical transmission and the covert virus' ability to still cause an overt infection fit with our definition of covert infection. Hence, SBV has a mixed infection strategy and the type of infection (overt versus covert) is depending on the life stage of the infected honeybee.

2. AIMS OF THE STUDY

2.1 Symbiosis

Symbiosis (i. e. “living together”) is close and often long-term interaction between two or more different biological species.

In 1877, Albert Bernhard Frank used the word symbiosis (which previously had been used to depict people living together in community) to describe the mutualistic relationship in lichens. In 1879, the German mycologist Heinrich Anton de Bary defined it as "the living together of unlike organisms” (Douglas & Angela, 2010).

Even if common sense, but many scientists too, believed the term symbiosis should only refer to persistent mutualisms, symbiosis should be applied to any types of persistent biological interactions (i.e. mutualistic, commensalistic, or parasitic - Table 2.1). After more than a hundred years of debate (Martin *et al.*, 2012), currently the latter "de Bary" definition or an even broader definition (i.e. symbiosis as all species interactions), with absence of the restrictive definition (i.e. symbiosis = mutualism) is used (Martin *et al.*, 2013).

Interaction	Species A	Species B
Commensalism	Receives benefit	Not affected
Mutualism	Receives benefit	Receives benefit
Parasitism	Receives benefit	Harmed

Table 2.1 Classification of symbioses. Classification of symbioses is based on the effect of the interaction on the species involved.

Some symbiotic relationships are obligate, meaning that both symbionts entirely depend on each other for survival. For example, many lichens consist of fungal and photosynthetic symbionts that cannot live on their own (Douglas & Angela, 2010). Others are facultative, meaning that they can, but do not have to live with the other organism.

Symbiotic relationships include those associations in which one organism lives on another (ectosymbiosis), or where one partner lives inside the other (endosymbiosis).
An important category of symbioses are the intestinal symbioses, in which host delegate to their consortium of microbes living within it (called “microbiome”) the entire digestion ,thus linking their nutrition, an essential function for host life, to their symbiotic microbiome.

Even if symbioses have alway been studied at the individual level (Douglas & Angela, 2010), recently it has became more and more clear that symbioses also entails a communication at a genomic level (Medina & Sacks, 2010). Indeed, many are the known cases of gene transfer between hosts and symbionts. An illustrative example is the relationship between aphids and their obligate mutualists, the bacteria *Buchnera aphidicola*: after millions years of symbioses (the estimations ranges between 150-200 my), bacterial genes are now found in aphids genome (Nikoh *et al.*, 2010).
Recently the term of holobiome and holobiont were introduced in scientific literature (Rosenberg, 2007). In this context, the hologenome is defıned as the sum of the genetic information of the host and its symbiotic microorganisms (Zilber-Rosenberg & Rosenberg, 2007), including an animal or plant host and its symbiotic microorganisms (Knight *et al.*, 2012). The idea of holobiont is not new: this term was coined originally by Margulis and Foster in 1991, and later adopted into various parts of evolutionary biology.
The holobiont was appropriated for use particularly in reference to the large symbiotic communities residing in corals (Rowan, 1998). In general, a holobiont is any organism and all of its associated symbiotic microbes, including parasites, mutualists, synergists, and amensalists (Rosenburg & Zilber-Rosenburg, 2011). This is the unit of selection in the hologenome theory (as opposed to the traditional evolutionary theory, which states that individuals are the units acted upon by natural selection). The hologenome theory, while a new concept in name, has actually been considered in some form or another with the recognition of symbionts as important to host health (Slonczewski & Foster, 2011).

2.2 DNA methylation and symbioses

Among scientific literature, many are the cases in which epigenetic and symbioses are intimately connected.

Much of the most exemplificative cases are from the bacterial symbioses field. Many bacteria are known for they pathogenic role in different organisms. For instance, in the case of *Salmonella* bacteria DNA methylation was proved to play a crucial role in bacterial virulence (Douglas & Heithoff, 1999). Although the molecular mechanism through which this occur has not already been elucidated, it is known that the methylation of adenine residues of DNA is required for *Salmonella* pathogenesis (Douglas & Heithoff, 1999).

In plants symbiotic bacteria perform nitrogen fixation in the host roots, an important source of nitrogen in agricultural production. For example 90% of nitrogen is biologically fixed in well-nodulated soybean plants.
In these bacteria, selectively methylated bases (C and A) are best known as important agents for restriction-modification systems, which distinguish self and non self DNA to protect bacteria from invaders. In this system, the host DNA is methylated and only unmethylated DNA is digested by cognate restriction endonucleases. Thus in plant-symbiotic bacteria DNA methylation contributes to the establishment and maintenance of symbiotic plant-bacteria relationships (Ichida *et al.*, 2006).

Moreover, *Wolbachia pipientis* is very “popular” bacteria in scientific literature. Wolbachia is particularly know for its double role in the symbiosis, depending on the animal it infects. Indeed, wolbachia is able to infect both arthropods (such as insects) and nematodes. The relationship established is consequently different: wolbachia can be generally considered a mutualist when interacts with nematodes, and a parasite in the interaction with arthropods (even if many exceptions to this generalization are known).
In the parasitic relationships with arthropods wolbachia exhibits several phenotypes (i.e. cytoplasmic incompatibility; parthenogenesis, male killing; feminization; true parasitism; true mutualism). In one of them wolbachia is able to feminize males infected by the bacteria in order to maximize its transmission (via eggs). In a recent study (Negri *et al.*,

2009) it was demonstrated that in the symbiosis between wolbachia and insect, the feminization was correlated to a different DNA methylation pattern in the male: parasitized feminized males with ovaries possess a female imprinting DNA methylation pattern, those with testes maintain the same methylation pattern of males, indicating that the wolbachia infection is able to modulate host genomic imprinting through the modification of the DNA methylation pattern in the host (Negri *et al.*, 2009).

Parasites are a great example of the importance of epigenetics in symbioses (Wyse et al., 2013). Indeed, parasites employ sophisticated mechanisms to escape host defenses, mainly based on the evasion from the immune system of the host by periodically and rapidly changing the coat of proteins displayed on its surface through a process called antigenic variation. In many cases, epigenetic mechanisms are at the base of antigenic variation in the host.
For example, *Trypanosoma brucei* is a unicellular parasite that, in its mammalian host, lives in the bloodstream and interstitial spaces, causing the fatal disease African sleeping sickness in humans. *T. brucei* evades its host's immune system by several chromatin modifications and active DNA methylation (Figueired *et al.*, 2009)

2.3 Aims of the study

In this study the possibility that epigenetics and in particular DNA methylation could be involved in symbioses was investigated.
This study, among the different epigenetic mechanism presents in the cells, focused in particular on DNA methylation due its properties:

- Its is a simple and energetically low cost chemical modification in the cell.
- It is a very fast modification. Rapid modification can account for very fast and low variations in the environment. This make DNA methylation a very receptive and responsive tool for the cell.
- It has a great magnitudo effect: small changes in the DNA methylation pattern of a cell are able to produce great effects in the phenotype of the cell itself and, moreover on the phenotype of the individuals.

These properties make DNA methylation a suitable mechanism through which two species, come in contact during a symbiosis event, and can (genomically) communicate.

In this context, a symbiosis between arthropods was used a case study make a light on the possible role of epigenetics in the context of symbioses. In particular, symbiosis between European honeybee (*Apis mellifera*) and its host, the parasitic mite *Varroa destructor* was studied. As mentioned in the previous section, this system is a three level symbiosis. Infact, the mite infesting honeybee is also a vector for several viruses which can infect *Apis mellifera* as a consequence of the mite infestation (Figure 2.1). More in general, the main aim of this study is to verify if there is a correlation between the variation of the DNA methylation pattern and the presence of parasites (mite and virus).

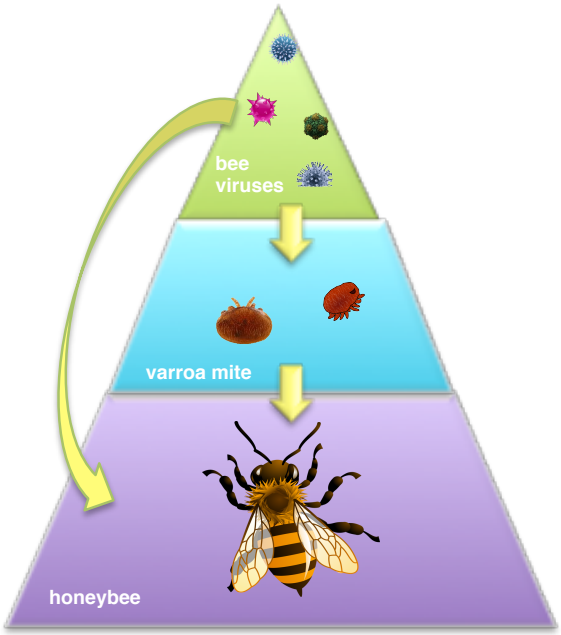

Figure 2.1 Representation of three level bee-mite-virus symbiosis. Mite infesting honeybee can be infected by bee viruses which can be transmitted to the bee during the mite interaction.

3. MATERIALS AND METHODS

3.1 Samples collection

60 samples were collected in 12 different beehives in Lombardy region (see Appendix for sample details). In every case blue-eyes stage (14-15 day old) bee larvae (Figure 3.1) were collected from worker cells in the hives and stored in absolute ethanol at -20°C. This stage is called blue-eyes stage (indicated by red arrow) because of the early development of the honeybee eyes pigmentation.

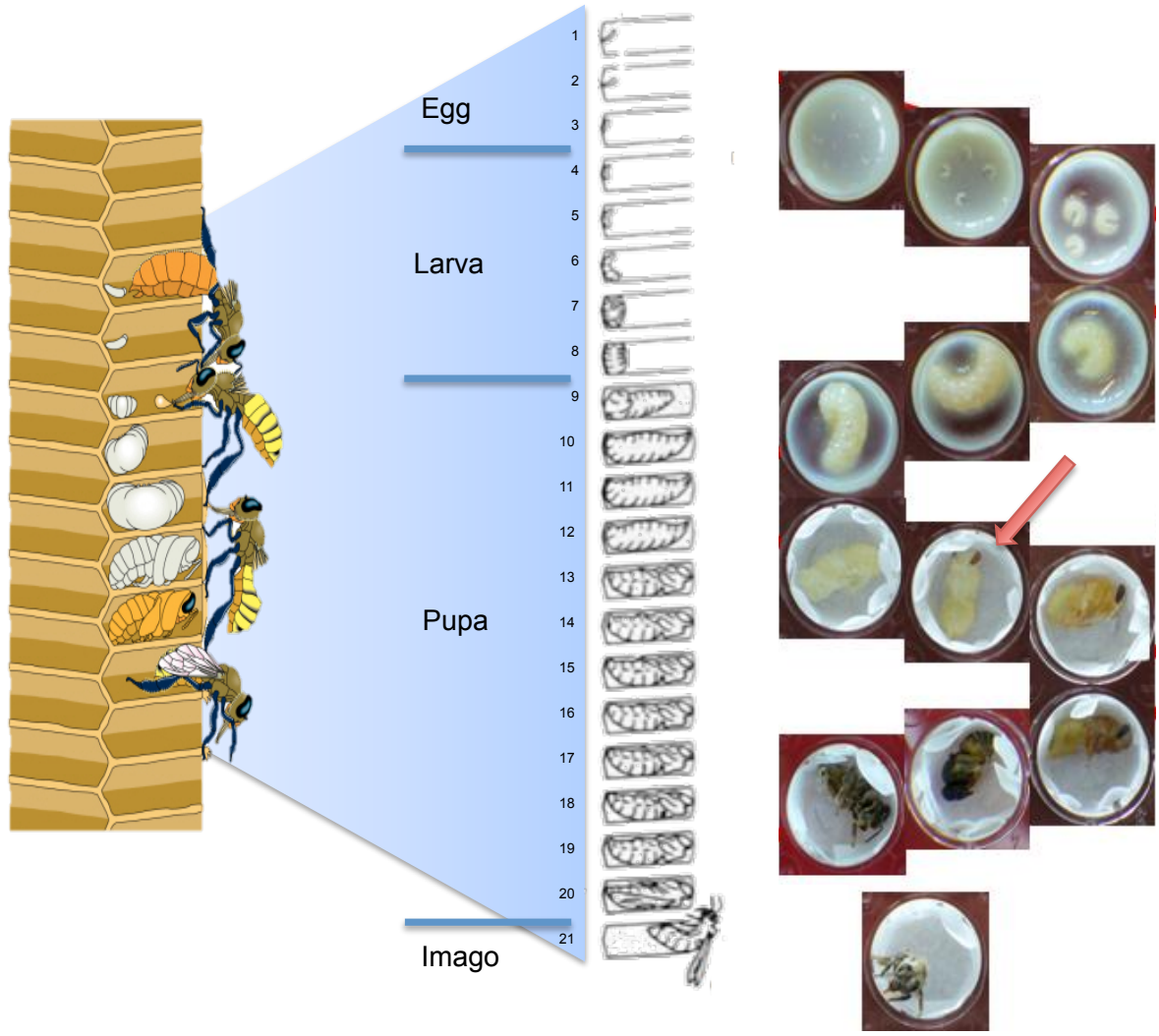

Figure 3.1 Worker honeybee development. Left image represent the development of worker bee in the hive. Right images represent details of the development from the egg to the imago (adult). Sample were collected at 13-15 day of development. (Image modified from Gätschenberger *et al.*, 2013)

Once stored, samples were vouchered following the protocol specified by the Biorepositories initiative (http://www.biorepositories.org). The aim of this on-line registry is the creation of a system that links the records in a databases (such as DNA sequences database) to the specimens from which the data (e.g. the DNA sequences) derived. In particular, each voucher name is composed by the combination of three parts, for example MIB:ZPL:05335:

- the universally-recognized acronym for the institution that holds the voucher specimen (in this case the acronym “MIB“ – University of Milano - Bicocca);
- the institution’s code to indicate the collection in which the voucher specimen is kept (in our case we used the code “ZPL”, deriving from the name of our lab: ZooPlantLab);
- the unique catalog number (or other identifier) in the catalog of specimens stored in that collection (e.g. 05335).

3.2 Genomic analyses

3.2.1 Genomic level DNA methylation analyses

To asses differences in the global methylation level between parasitized and non parasitized bees, Fourier Transformed Infrared Spectroscopy (FTIR) was used.

Fourier transform infrared spectroscopy (FTIR) is a technique used to obtain an infrared spectrum of absorption, emission, photoconductivity or Raman scattering of a solid, liquid or gas. A FTIR spectrometer simultaneously collects spectral data in a wide spectral range. This confers a significant advantage over a dispersive spectrometer which measures intensity over a narrow range of wavelengths at a time. FTIR has made dispersive infrared spectrometers all but obsolete (except sometimes in the near infrared), opening up new applications of infrared spectroscopy.

FTIR analysis has a great usage also in molecular biology. Infact, FTIR has the advantage of generating structural information of the entire DNA molecule in a single spectrum as a sort of “snapshot” of all data, including the conformational states or possible contaminants present in the sample.

The technique is widely used for systematic studies of nucleic acids (e. g. sequence variations, covalent modifications) since it is fast and non-destructive so that it can be

used in *in vivo* experiments (Banyay & Gräslund, 2002). The FTIR region of interest when studying nucleic acids is 1800-800 cm^{-1}.

FTIR analysis can also show the level of methylation in a DNA sample. For this reason, in this study, FTIR approach was used to asses a possible difference in the global DNA methylation level between parasitized and non-parasitized samples.
As demonstrated by Banyay and Gräslund (2002) the presence of DNA methylation on a DNA is reflected in the FTIR spectrum in three different regions, due to the changes in the DNA conformation caused by the methylation presence :
- The sugar conformation region (900 - 790 cm^{-1})
- The glycosidic bon rotation region (1440 - 1330 cm^{-1})
- The base pairing/base sacking interaction region (1760 - 1480 cm^{-1})

DNA methylation is shown to modify, proportionally to the intensity of the DNA methylation level itself, the spectrum of these three regions. Thus, by measuring the intensity and conformation of the spectrum in these regions it possible to asses the global level of DNA methylation.

To perform FTIR analyses, a great amount of pure DNA was required (500 ng). Classical DNA extraction kits, commonly used in molecular biology, do not allow suitable DNA yields for FTIR analysis. Infact, the amount of DNA required for molecular biology analysis (PCR and bisulfite sequencing) is much lower than the amount necessary for FTIR performing. Moreover commercial DNA extraction kits do not provide totally chemically pure DNA: in every kit tested with FTIR analysis the residual presence of buffer in the final eluted DNA was revealed. Finally, the composition of buffer used in commercial kits is so complex that its spectrum overlap the range of the DNA spectrum. In this case, it is impossible, in a FTIR analysis, to discriminate between pure DNA (in which a possible DNA methylation effect can be measured) and a buffer-contaminated sample (in which both positive and negative errors may be measured).
For these reasons, a special protocol for bee DNA extraction was set up. For the DNA extraction only non-interfering buffers were used.
DNA extraction was performed from the samples by phenol-chloroform extraction. Indeed, bot phenol and chloroform spectra do not overlap with the DNA spectrum.

For every samples studied, genomic DNA was extracted from 200-400 mg of larvae tissue.

Preparation of samples consisted of one or more washes with cold (i.e. about 4°C) NaCl solution 0.9% to remove as well as possible ethanol from tissues. Samples were then cut into small pieces or powdered after freezing with liquid nitrogen.

The extraction of nucleic acids involves adding an equal volume of phenol, chloroform and isoamil alcohol solution (25:24:1) to an aqueous solution of lysed cells or homogenized tissue. Subsequent mixing allows two the phases to separate. Centrifugation of the mixture yields two phases: the lower organic phase and the upper aqueous phase. DNA contained in the aqueous phase is collected and purified with several ethanol washes. Finally DNA is eluted in 100 µl sterile water.

Purified DNA concentration of each sample was estimated fluorometrically. Samples with at least 500 ng/µl were used for FTIR analysis.

The FTIR experiments of each sample were repeated three times to obtain reproducible data. 1 µl of the extracted DNA was deposited onto a BaF_2 window and dried at room temperature for about 30 min.

FTIR absorption spectra from 4000 to 600 cm^{-1} were acquired in the transmission mode by coupling the UMA 500 infrared microscope, equipped with a nitrogen cooled MCT detector (narrow band, 250 µm), to a FTS 40A spectrometer (Digilab-USA) at 2 cm^{-1} resolution, 20 kHz speed, 256 scan co-additions, and triangular apodization. Absorption spectra with a low noise level were obtained by setting the microscope aperture at about 100 µm x 100 µm. The background spectrum was collected before each measurement and no baseline correction was required on the spectra. Spectra were only corrected for possible residual water vapor.

A second derivative analysis of the DNA spectra region was performed after a 15 point smoothing by the Savitzky–Golay method (3rd polynomial, 13 smoothing points), using the GRAMS/32 software (Galactic Industries, USA). The second derivative spectra were always normalized at the phosphate band around 1100 cm^{-1}.

3.2.2 Gene level methylation analyses

Selection of the study regions

According to Navayas *et al* (2010) the honeybee-mite interaction involves three main components: the cellular damage, the embryonic development, the cognitive-behavioral

one (figure 3.2). In this study seven genes involved in this pathway were selected (Table 3.1).

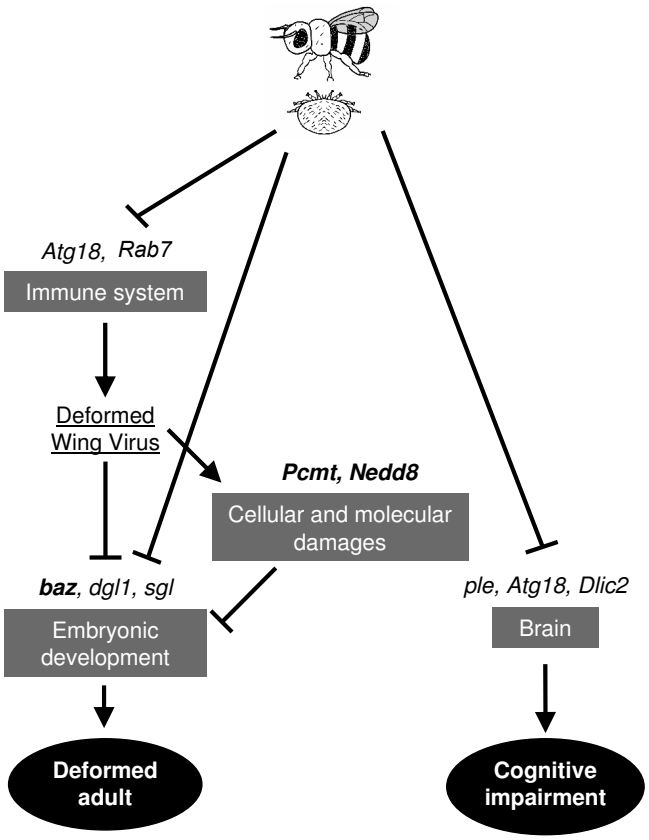

Figure 3.2 Hypothetical pathways and models of honeybee responses to varroa-parasitism. Arrows and dashes indicate positive and negative regulation, respectively. One of the consequences of the varroa parasitism is a decline in immune capacity which induces the proliferation in honeybees of viruses. The boost of viruses multiplication might cause cellular and molecular damage, inducing the production of protein repair and the labelling of proteins for degradation. Mites might decrease the production of dopamine (*Ple*) and inhibit genes known for indirectly preventing neural degeneration in aged adults, which could explain the cognitive impairment often observed in adults parasitized by varroa. (Navajas *et al.*, 2010)

Gene	Role	Functions
Atg18	✦ IMMUNE SYSTEM ✦BRAIN	- Early component of autophagy machinery - Involved in the formation of preautophagosomal structures and development of mature phagosomes
Dlic2	✦ BRAIN	- Idrolase involved in the axogenesis, axon cargo transport and dendrite morphogenesis

Gene	Role	Functions
Nedd8	✦ CELLULAR AND MOLECULAR DAMAGES	- Neddilation process - Cellular proliferation - Regulation of protein stability - Virus-induced effect
Pcmt	✦ CELLULAR AND MOLECULAR DAMAGES	- Protein repair - Protein modification process
Ple	✦ BRAIN	- Tyrosine hydroxilase - Involved in courtship behavior - Involved in the thermotaxis
Rab7	✦ IMMUNE SYSTEM	- Involved in endosome to lisosome transport - Involved in autophagic cell death - Involved in the negative regulation of symbiont in the host
Sgl5	✦ EMBRYO DEVELOPMENT	- Involved in olfactory behavior - Segment polarity determination - virus-induced effect

Table 3.1 Gene studied. For every gene considered in this work, role and functions in the honeybee are listed.

Despite the essential role of DNA methylation in splicing regulation and honeybee biology (e.g. caste differentiation), according to scientific literature (Flores *et al.*, 2011), honeybee genomes are poorly methylated (0.1 - 2%) and, moreover, DNA cytosine methylation in honeybees is restricted to gene bodies and, in particular, to exons. For this reason, the selection of study regions focused only on the codifying sequences of the gene considered.

Seven selected genes were analyzed with the online bioinformatic tool EMBOSS Newcpgreport (Rice *et al.*, 2000 - http://www.ebi.ac.uk/Tools/seqstats/emboss_newcpgreport/) and CpG site were predicted in every sequence. For every gene, exons containing the greater number of CpG dinucleotides (which could be methylated) were selected for further analyses as the most representative (Figure 3.3).

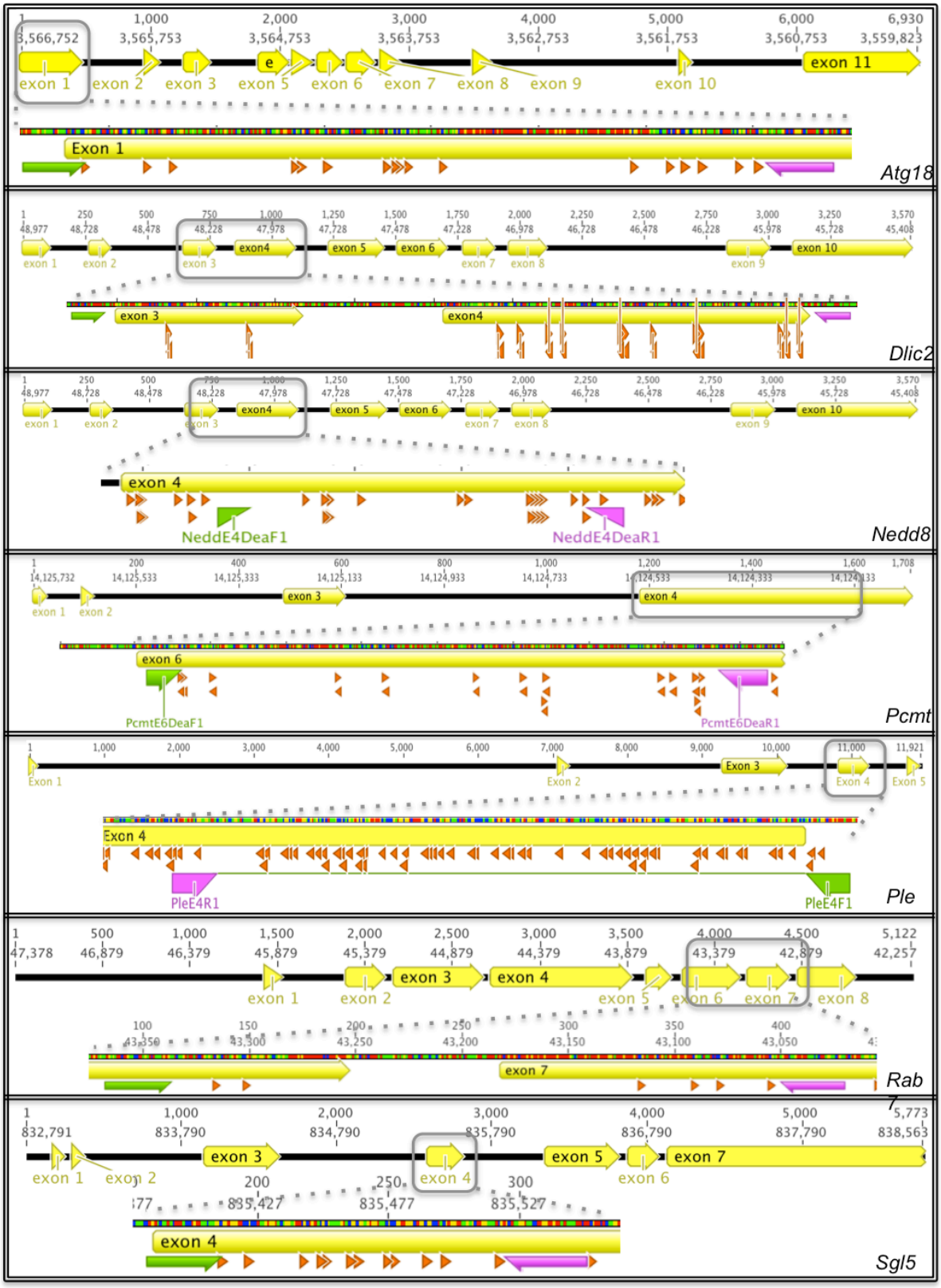

Figure 3.3 Representation of the studied regions. For every gene studied, exons (in yellow), primer binds forward and reverse (green and purple respectively) and CpG site (orange) are shown.

Bisulfite treatment

To analyse exon level methylation bisulfite treatment was performed. Sodium bisulfite is a selective mutagenic molecule: treating DNA with bisulphite converts cytosine residues to uracil, but leaves 5-methylcytosine residues unaffected. Thus, bisulfite treatment introduces specific changes in the DNA sequence that depend on the methylation status of individual cytosine residues, yielding single-nucleotide resolution information about the methylation status of a segment of DNA (Figure 3.4).

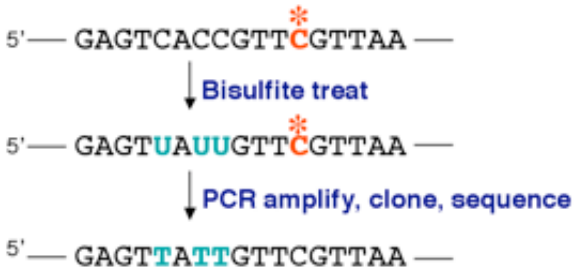

Figure 3.4 Sodium bisulfite reaction. Socium bisulfite converts unmethylated Cs in Uracile. Then subsequent PCR convert U in T. Red asterisks shows methylated Cs.

The DNA methylation pattern can then be inferred from the comparison between the reference genomic sequence (pre bisulfite treatment) and the sequence treated with sodium bisulfite (Figure 3.5).

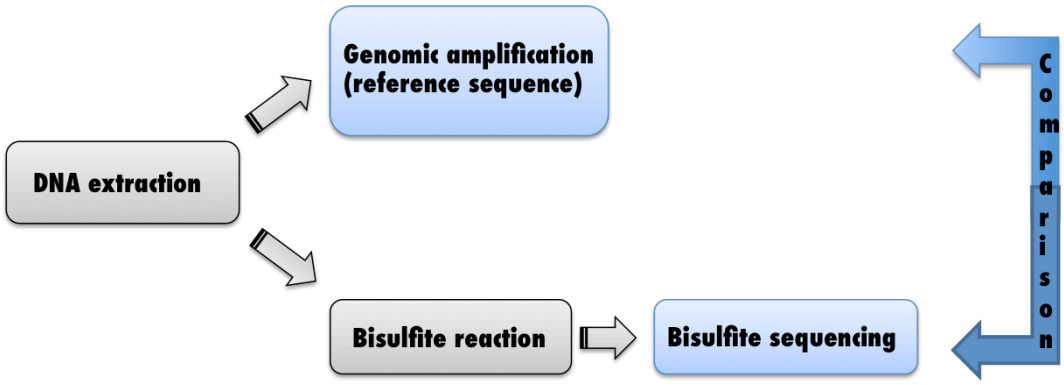

Figure 3.5 Scheme of the laboratory procedures. DNA is extracted from every sample. Then the reference sequence database was obtained by genomic amplification. Extracted DNA is then treated with sodium bisulfite and the bisulfite sequencing was performed. Finally, DNA methylation pattern was obtained comparing reference sequences with treated ones.

DNA extraction

For every samples studied, genomic DNA was extracted from 50-100 mg of larvae tissue.

Preparation of samples consisted of one or more washes with cold (i.e. about 4°C) NaCl solution 0.9% to remove as well as possible ethanol from tissues. Samples were then cut into small pieces or powdered after freezing with liquid nitrogen.

DNA was then extracted using DNeasy Blood & Tissue Kit by QIAGEN and following manufacturer instructions to obtain high-quality DNA, free of polysaccharides or other metabolites that might interfere with successive amplification processes. DNA was then eluted into 50 ul sterile water and used for subsequent reactions (genomic amplification and bisulfite treatment).

Purified DNA concentration of each sample was estimated both fluorometrically and by comparison of ethidium bromide-stained band intensities with λ DNA standard.

Genomic amplification

In order to set up the reference sequences dataset, genomic DNA was amplified for every gene studied. For each selected sample, at least 10 ng of the extracted DNA were used as a template in a PCR reaction to amplify the selected regions. The reactions were carried out in a final volume of 20 µl with the following final concentrations:

1X buffer including 1.5 – 2.5 mM $MgCl_2$
0.2 mM of each dNTP
1 µM of each forward and reverse
0.5 U/µl taq (MasterTaq kit, Eppendorf™)

with the following PCR program 94°C 1'; (94°C 45"; T°m 45"; 72°C 90') x 39 cycles; 72°C 10'; 60°C 5'. Primer and relative melting temperatures (T°m) used for each gene amplification are listed below in table 3.2

Target region	Primer name	Primer sequence 5'-3'	Amplicon length (bps)	T°m (°C)
Atg18	**AtgE1F1**	TTTCAGTTTCATCATTCCGTG	391	56
	AtgE1R1	TTTGCGAGGTTCATTAGTCC		
Dlic2	**DlicE4F1**	TAACTAACTAATAAAAAAT	600	54
	DlicE4R1	GTCATATTTTTAAATTATTT		
Nedd8	**NeddE4F1**	GGCGCAGGATTATAAAGTTCA		

Target region	Primer name	Primer sequence 5'-3'	Amplicon length (bps)	T°m (°C)
	NeddE4R1	CGGGTCTCGAGAAAAGAAAAT	488	56
Pcmt	**PcmtE6F1**	GATTGGGATATGCTGCTGAC	535	56
	PcmtE6R1	TGTTGAATCATTTTACGTGCG		
Ple	**PleE4F1**	TATTTCTCTTGTCCGATTCGC	347	56
	PleE4R1	ACGAAGTAGATCGGTTGGTAA		
Rab7	**Rab7E7F1**	GACAGATTAGAATTGAGTGAT	480	56
	Rab7E7R1	TACCAAAAGACAGCGTTATTA		
Sgl5	**SglE4F1**	TTCTTTCTTTCACAACTAACCG	285	56
	SglE4R1	AATCCAATGCTCGTAAACCTT		

Table 3.2 Genomic primers. Detailed information about the primer used for the genomic DNA amplification.

Amplicons size was assessed by electrophoresis in 1.5% agarose gels stained with ethidium bromide. PCR products were gel purified (using the Perfectprep Gel Cleanup, Eppendorf™) and the heavy DNA strands were bidirectionally sequenced using an ABI 155 3730XL automated sequencer at Macrogen Inc., Korea. Manual editing of raw traces and subsequent alignments of forward and reverse sequences were conducted with Geneious sequence alignment editor version 4.5.1 created by Biomatters. Available from http://www.geneious.com/ .

Bisulfite reaction

For the gene level methylation analyses, sodium bisulfite reaction was performed using Epitect Bisulfit Kit, QIAGEN. Since manufacturer instructions were set up on plant and vertebrate DNA, where methylation levels are higher than in invertebrates (two order of magnitude higher), manufacturer protocol for the reaction was changed as following. Buffer were used at the same volume and concentration, reaction time was halved to reduce DNA damaging and loss caused by the extensive use of a mutagen, sodium bisulfite, and by the high temperature of the reaction (94°C). Modified DNA was the eluted in 20 µl of sterile water and stored at -20°C.

For each selected sample, at least 15-20 ng of the modified DNA were used as a template in a PCR reaction to amplify the selected regions. The reactions were carried out in a final volume of 20 µl with the following final concentrations:

1X buffer including 1.5 – 2.5 mM $MgCl_2$
0.2 mM of each dNTP
1 µM of each forward and reverse
0.5 U/µl taq (MasterTaq kit, Eppendorf™)

with the following PCR program: 94°C 1'; (94°C 45"; T°m 45"; 72°C 90') x 39 cycles; 72°C 10'; 60°C 5'.

To amplify modified sequences, primer were designed using a specific tool for DNA methylation analysis: MethPrimer (Li & Dahiya, 2002). This software takes in consideration the possibility of a mutation in the primer sequence, thus choosing as primer sequence for modified DNAs, a trait of nucleotide with low CpG. Primer used for bisulfite modified DNA are listed below in table 3.3:

Target region	Primer name	Primer sequence 5'-3'	Amplicon length (bps)	T°m (°C)
Atg18	**AtgE1Dea_F1**	GAATTTGATGTTTTAGTTTTATTATTT	376	50
	AtgE1Dea_R1	TACTATATTTTAATCCCTATATCAATACAT		
Dlic2	**DlicE4Dea_F1**	TAACTAACTAATAAAAAAT	490	50
	DlicE4Dea_R1	GACATTCTTCCAAAGTAGTT		
Nedd8	**NeddE4Dea_F**	TATTTTTTGTTTTGGAATGTATGTGT	515	54
	NeddE4Dea_R	ACAAAATAAAATTTAACAACCCATAACA		
Pcmt	**PcmtE6Dea_F**	GATTAATTGATTTTTGGTGGA	400	50
	PcmtE6Dea_R	TATCAATAATTTATAAATAAATATTCTATT		
Ple	**PleE6_F1**	TATTTCTCTTGTCCGATTCGC	350	50
	PleE6_R1	ACGAAGTAGATCGGTTGGTAA		
Rab7	**RabE7Dea_F1**	AGTGTAATAAGTTTTAATGTATATTTAGA(	347	50
	RabE7Dea_R1	ACTAAACCATTAAAAAATTCTTCAAATTC		
Sgl5	**SglE4Dea_F1**	AGATTTTATTAAATTTAGAATTTTTGG	162	50
	SglE4Dea_R1	ATTAAAATATTCTTCCTTAAAATCCAATAC		

Table 3.3 Bisulfite primers. Detailed information about the primer used for the modified DNA amplification

Amplicons size was assessed by electrophoresis in 1.5% agarose gels stained with ethidium bromide and PCRs products were gel purified (using the Perfectprep Gel Cleanup, Eppendorf™).

Since DNA methylation is tissue, cell and time specific, amplicons from the modified-PCR reaction need to be cloned.

Cloning was then performed to separate single DNA methylated sequences using the pGEM®-T Easy plasmid vector (Promega, Madison, Wisconsin) for the ligation reaction. Ligation was carried out using T4 DNA Ligase and incubated overnight at 4°C. Recombinant plasmids were transformed into competent *E. coli* DH5a Competent Cells (Promega, Madison, Wisconsin) and, after overnight growth on LB medium plates at 37°C, colonies containing plasmids with inserts were screened by X-gal-mediated blue/ white selection.

For every gene studied 10 clones were picked up with a pipette tip and put into tubes containing 40 µl of sterile water. After denaturation at 95°C for 10 minutes, 2 µl of this solution was amplified for each clone by PCR using bisulfite primers.

DNA purification and sequencing were then performed as described previously.

Amplicons size was assessed by electrophoresis in 1.5% agarose gels stained with ethidium bromide. PCR products were gel purified (using the Perfectprep Gel Cleanup, Eppendorf™) and the DNA was sequenced using an ABI 155 3730XL automated sequencer at Macrogen Inc., Korea.

Bioinformatic analyses

Clone sequences were aligned and checked for their quality using BiqAnalyzer (Bock *et al.*, 2005). Biq Analyzer software aligns clone sequences with the respective reference sequences, then check sequences for their quality and finally calculate the methylated Cs percentage present in the sequence.

Output of BiqAnalyzer sequences were then analyzed using BISMA Calculation and Computation (Rhode *et al.*, 2008) that allows to compare the level of DNA methylation in different genes.

3.2.3 Virus screening

Every sample was checked for the presence of virus in the larvae. For the virus screening RNA was extracted from every samples using TriPure reagent (Sigma-Aldrich).

For every samples 100 mg of tissue were fragmented and collected in a tube; 1ml of TriReagent and 0.2 ml chloroform were added to each samples, then incubated for 10 minutes at room temperature. Room temperature incubation allows nucleoprotein complexes to completely dissociate from RNA: after centrifugation RNA-containing phase was then collected in a new tube. RNA was then washed with 0.5 ml of Isopropilic Alchol and Ethanol 70% (twice). Finally, samples were dried and pellet was eluted in 50 µl distilled water.

In order to remove any DNA trace, RNA was treated with DNAse I, using DNAse I Amplification Grade kit by Sigma-Aldrich, according to manufacturer protocol.

Pure RNA was then reverse-transcripted to cDNA to use as template for the subsequent PCR screening of bee viruses. In this context, iScript cDNA synthesis kit by Biorad was used according to manufacturer protocol.

Finally, PCR reactions for bee virus screening were set up in a 20 µl final volume using 10-15 ng cDNA as template and reagents at the following final concentrations:

1X buffer including 1.5 – 2.5 mM $MgCl_2$
0.2 mM of each dNTP
1 µM of each forward and reverse
0.5 U/µl taq (MasterTaq kit, Eppendorf™)

For the screening reactions, the following primers were used:

virus	primer name	Primer sequence (5'-3')	fragment lenght (bps)	T °m	reference
BQCV	BQCV_F1	GGACGAAAGGAAGCCTAA AC	424	60	Nielsen *et al.*, 2008
	BQCV_R1	ACTAGGAAGAGACTTGCACC			
DWV	DWV_F1	CTTACTCTGCCGTCGCCCA	194	56	Chen *et al.*, 2004
	DWV_R1	CCGTTAGGAACTCATTATCGCG			
KBV	KBV_F1	GATGAACGTCGACCTATTGA	393	56	Nielsen *et al.*, 2008
	KBV_R1	TGTGGGTTGGCTATGAGTCA			

virus	primer name	Primer sequence (5'-3')	fragment lenght (bps)	T °m	reference
SBV	SBV_F1	ACCACCCGATTCCTCAGTAG	487	56	Nielsen *et al.*, 2008
	SBV_R1	CCTTGGAACTCTGCTGTGTA			

Table 3.3 Virus screening primers. Detailed information about the primer used for the screening of viruses in the samples collected.

Virus were amplified using the following PCR program: 50° 30''; 94°C 5'; (94°C 30''; T°m 30''; 72°C 60'') x 35 cycles; 72°C 7'. Then their presence was checked by electrophoresis in 1.5% agarose gels stained with ethidium bromide. In every amplification random samples were gel purified (using the Perfectprep Gel Cleanup, Eppendorf™) and then sequenced, using an ABI 155 3730XL automated sequencer at Macrogen Inc., Korea, to check for correct primer targeting.

Bioinformatic analyses

The analyses of virus presence data consisted of two steps: firstly, data were collected and a matrix was created; secondly, model to test hypotheses were created.
The first model was created to test the possibility that virus presence in the samples was correlated to varroa mite infestation. For this reason a linear model was used considering as dependent variable the virus presence in the sample, and, as independent variable, the presence of varroa, and assuming a Poisson error distribution.
To test the possibility that the number of viruses and the DNA methylation were correlated, a general model was used assuming as dependent variable the number of virus infesting the samples, and, as independent variable, the methylation percentage in the samples.

Models were fitted with the lmer procedure (Baer & Maechler, 2009) in the lme4 package in R 2.8.1 R Development Core Team, 2011 http://spatial-analyst.net/book/node/330).

4. RESULTS AND DISCUSSION

During the present study two group of samples are considered: the non parasitized, or control group (V-) and the parasitized one (V+). Parasitized group comprises all the samples in which a mite was found on the body of pupae or on the bottom of the cell in the apiary, during the sampling.

Detailed list of the results is shown in Table 4.1

sample	**methylation %**							**virus presence/ absence**				**virus tot**
	Atg11	*Rab7*	*Sgl5*	*Dlic2*	*Ple*	*Pcmt*	*Nedd8*	DWV	SBV	KBV	BQCV	
MIB:ZPL:00397	0	50	0	0	13.1	6.7	0	+	+	+	+	4
MIB:ZPL:00398	0	50	0	6	13.1	6.7	0	+	+	+	-	3
MIB:ZPL:00608	2.3	50	15.4	12.5	12	8.3	7.7	+	+	-	-	2
MIB:ZPL:00609	2.3	50	4.6	12.5	7.6	8.3	7.7	+	-	-	-	1
MIB:ZPL:00716	23.5	83.3	36.9	50	14.9	33.3	92	-	-	-	-	0
MIB:ZPL:00717	23.5	83.3	36.9	50	14.9	33.3	92	-	-	-	-	0
MIB:ZPL:01147	7.8	53.3	15.4	6	9.7	8.3	15	+	-	-	-	1
MIB:ZPL:01146	2.3	50	4.6	12.5	7.6	6.7	15	+	+	-	-	2
MIB:ZPL:03907	7.8	53.3	15.4	12.5	9.7	8.3	15	+	-	-	-	1
MIB:ZPL:03910	16.5	62.5	21.2	25	7.6	16.7	30	-	-	-	-	0
MIB:ZPL:03913	2.3	50	4.6	6	7.6	6.7	7.7	+	+	-	-	2
MIB:ZPL:03919	16.5	62.5	21.1	25	7.6	16.7	30	-	-	-	-	0
MIB:ZPL:03920	28	70	36.9	50	10.5	20.8	61	+	-	-	-	1
MIB:ZPL:03921	28	70	36.9	50	16.6	25	76	-	-	-	-	0
MIB:ZPL:03923	11.8	73.3	30.8	25	19.3	20.8	61	+	-	-	-	1
MIB:ZPL:03924	11.8	73.3	30.8	25	19.3	20.8	61	-	-	-	-	0
MIB:ZPL:03930	14.1	62.5	16.9	25	12	16.7	30	-	-	-	-	0
MIB:ZPL:03931	14.1	56.7	4.6	14	10	12.5	30	+	-	-	-	1
MIB:ZPL:03933	2.9	50	4.6	12.5	7.6	6.7	7.7	+	-	+	-	2
MIB:ZPL:03935	7.8	53.3	16.9	12.5	9.7	8.3	15	+	-	-	-	1
MIB:ZPL:03937	28	90	36.9	50	19.3	33.3	92	-	-	-	-	0
MIB:ZPL:03938	28	90	36.9	50	19.3	33.3	92	-	-	-	-	0
MIB:ZPL:03939	23.5	83.3	36.9	37.5	14.9	25	76	-	-	-	-	0
MIB:ZPL:03941	7.8	53.3	15.4	12.5	9.7	8.3	15	+	-	-	-	1
MIB:ZPL:03943	14.1	62.5	15.4	12.5	9.7	6.7	23	+	-	-	-	1
MIB:ZPL:03953	0	50	0	6	7.6	6.7	0	+	+	+	+	4
MIB:ZPL:03955	7.8	53.3	15.4	14	9.7	8.3	7.7	+	-	-	-	1
MIB:ZPL:03957	14.1	56.7	21.2	6	13.1	12.5	23	-	-	-	-	0
MIB:ZPL:03959	14.1	56.7	21.2	12.5	13.1	12.5	30	-	-	-	-	0
MIB:ZPL:03961	7.8	53.3	15.4	25	9.7	8.3	15	+	-	-	-	1
MIB:ZPL:03975	2.9	62.5	4.6	12.5	7.6	6.7	7.7	+	+	-	-	2
MIB:ZPL:03977	7.8	56.7	15.4	25	10	6.7	15	+	-	-	-	1
MIB:ZPL:05314	24.7	86.7	33.8	37.5	16.6	33.3	76	-	-	-	-	0

sample	methylation %							virus presence/ absence				virus tot
MIB:ZPL:05315	28	70	36.9	50	19.3	33.3	76	-	-	-	-	0
MIB:ZPL:05316	17.6	70	15.4	37.5	10.5	20.8	46	+	-	-	-	0
MIB:ZPL:05317	11.8	86.7	33.8	37.5	14.9	25	46	-	-	-	-	0
MIB:ZPL:05318	17.6	86.7	33.8	37.5	14.9	25	61	-	-	-	-	0
MIB:ZPL:05319	28	70	36.9	50	19.3	33.3	76	-	-	-	-	0
MIB:ZPL:05320	24.7	86.7	33.8	37.5	16.6	33.3	76	-	-	-	-	0
MIB:ZPL:05321	17.6	70	15.4	37.5	14.9	20.8	61	-	-	-	-	0
MIB:ZPL:05322	16.5	62.5	16.9	14	13.1	16.7	30	-	-	-	-	0
MIB:ZPL:05323	16.5	62.5	16.9	14	13.1	16.7	30	-	-	-	-	0
MIB:ZPL:05324	11.8	62.5	21.2	12.5	12	8.3	23	-	-	-	-	0
MIB:ZPL:05327	24.7	66.7	12.7	37.5	16.6	25	76	-	-	-	-	0
MIB:ZPL:05328	28	90	36.9	50	19.3	33.3	92	-	-	-	-	0
MIB:ZPL:05329	23.5	90	36.9	37.5	16.6	25	61	-	-	-	-	0
MIB:ZPL:05334	24.7	86.7	33.8	50	16.6	25	92	-	-	-	-	0
MIB:ZPL:05335	17.6	83.3	33.8	25	10.5	20.8	61	-	-	-	-	0

Table 4.1 Detailed results. For every sample, DNA methylation percentage in every gene, virus presence and the number of virus infecting the sample is shown.

4.1 Genomic level DNA methylation

For the analysis of DNA methylation at a genomic (global) level FTIR was used. In particular, three regions of the DNA spectrum were studied.
A total of 24 samples were analyzed with FTIR, 10 parasitized individuals and 10 controls.

FTIR analysis revealed no difference in the DNA spectra. In particular, no differences were detected in the three regions in study. Such pattern indicates that samples parasitized by varroa have, at a global level, the same level of DNA methylation than control samples. In other word, no differences in the global DNA methylation pattern were detectable.
A possible explanation of this pattern lies in the honeybee genome architecture. As previously mentioned, honeybee genome is very AT rich, so there are few C which can be methylated. Moreover DNA methylation is far than recurrent and interspersed in the genome. It is, infact, confined to the codifying regions and in particular to exons. The low level of methylable Cs in the genome and the disposition of DNA methylation in the genome can be seen as constraints to any possible changes in the global DNA

methylation pattern, resulting in no measurable difference in the DNA methylation at a global level.

Being the methylation confined to particular regions, any possible effect of parasites on the epigenetics of the honeybee would, more likely, posses two characteristics:

- it must be short: little but precise (at a sequence) differences in the epigenome (i. e. the sum of all epigenetic informations in genome). Indeed, it is known, in scientific literature, that DNA methylation as a great magnitudo effect on phenotypes.
- it is rare (i.e. low frequent), due to the scarce C methylable in the honeybee genome.

4.2 Gene level DNA methylation

4.2.1 Sodium bisulfite reaction

In the of study gene level methylation sodium bisulfite sequencing was used. A total of 1610 sequences were analyzed: five sequences from 46 samples in every genes studied (7 genes).

Bisulfite sequencing analysis, which consists of treatment of DNA with the mutagen sodium bisulfite, was set up for honeybee genome study. Indeed this genome is very AT-rich, thus low quantity of Cs is available for methylation, thus resulting in a great loss of DNA during the reaction with sodium bisulfite.

Yields obtained using manufacturer protocols were too low for the subsequent PCR reaction (less than 20% of reaction product could be used in PCR).

Using the modified protocols greater quantity of DNA was obtained with a good DNA quality. These products were used for subsequent reaction.

4.2.2 Analysis of DNA methylation between genes.

A total of 151.340 CpG sits were studied. DNA methylation profiles were almost the same among the five replicates of each sample with a few minor differences.

Bisulfite sequencing shown very different DNA methylation levels in the genes analyzed in this study. Infact, overall DNA methylation ranges from 80% in *Rab7* gene (V-) to 16.16% in *Ple* Figure 4.1).

Moreover, this difference in the methylation percentages in different genes are reflected also in the two sample groups considered: in both parasitized and non parasitized sample, genes show very different DNA methylation levels (Figure 4.2).
This difference is though to be due to two main factors. One important point to consider is that different genes have different role in the cells and, moreover, are expressed according to different regulation. Secondly, bee genomes are very AT rich. This implies a relative depletion of CpG which can be methylated. According to this, different DNA methylation levels can presumably reflect the level of CpG which can be methylated in the sequence.

In addition, these differences were present independently from the presence of the mite on the honeybee: control samples (V-) were also methylated. This pattern reflects what is also reported in scientific literature: honeybee genomes are methylated. Infact, in honeybees DNA methylation was proved to have a key role in the biology of the cell and gene regulation (for example in the caste differentiation), even if no clear information about the exact role has already been published (Flores & Amdam, 2010).

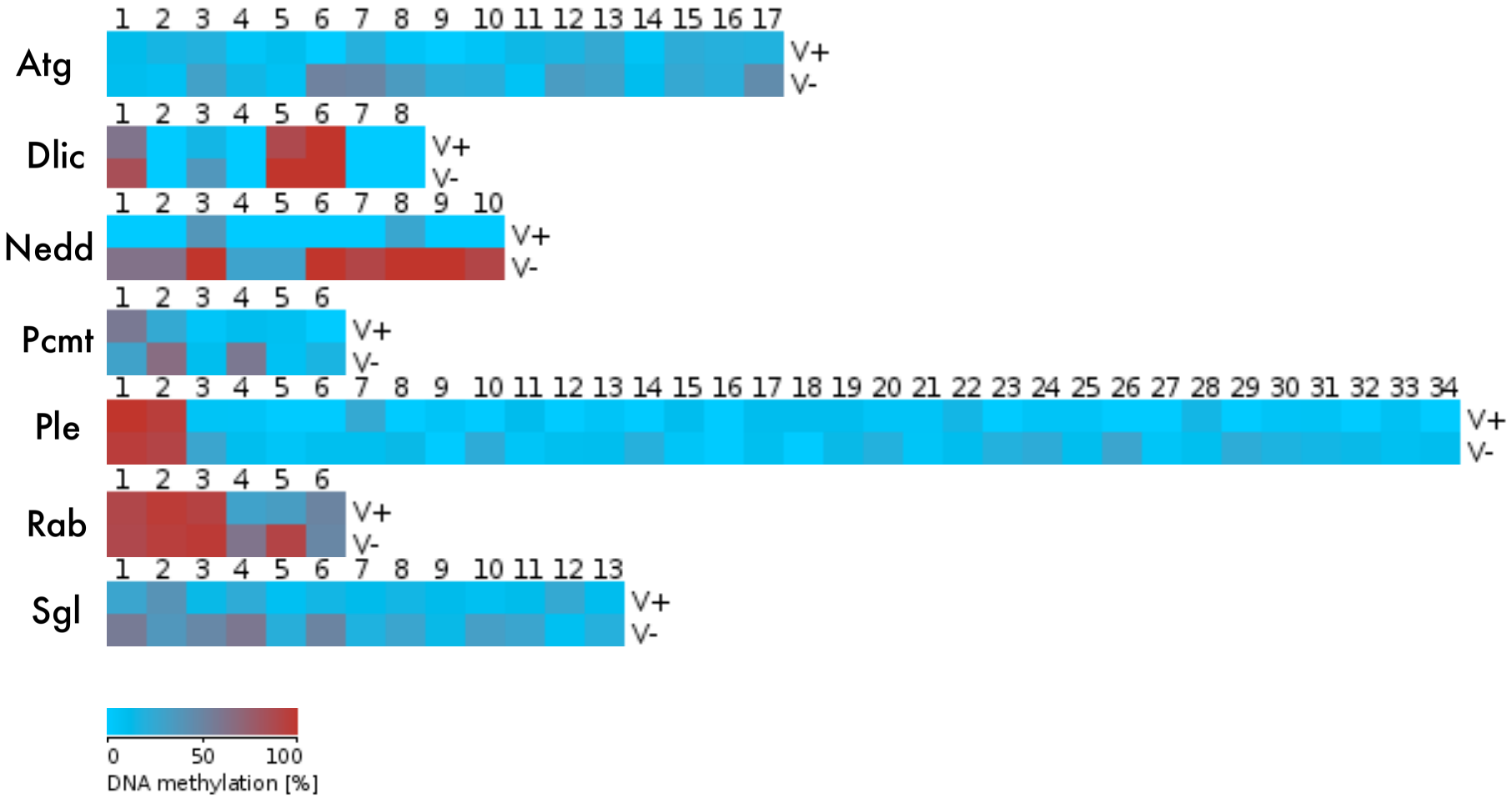

Figure 4.1: Bisulfite sequencing results. In this graph, the CpGs present in every gene are numbered and represented in color ranging from blue to red according to the DNA methylation level: high methylated Cs are shown in red and low methylated ones in blue. In every gene the two groups V+ and V- are considered.

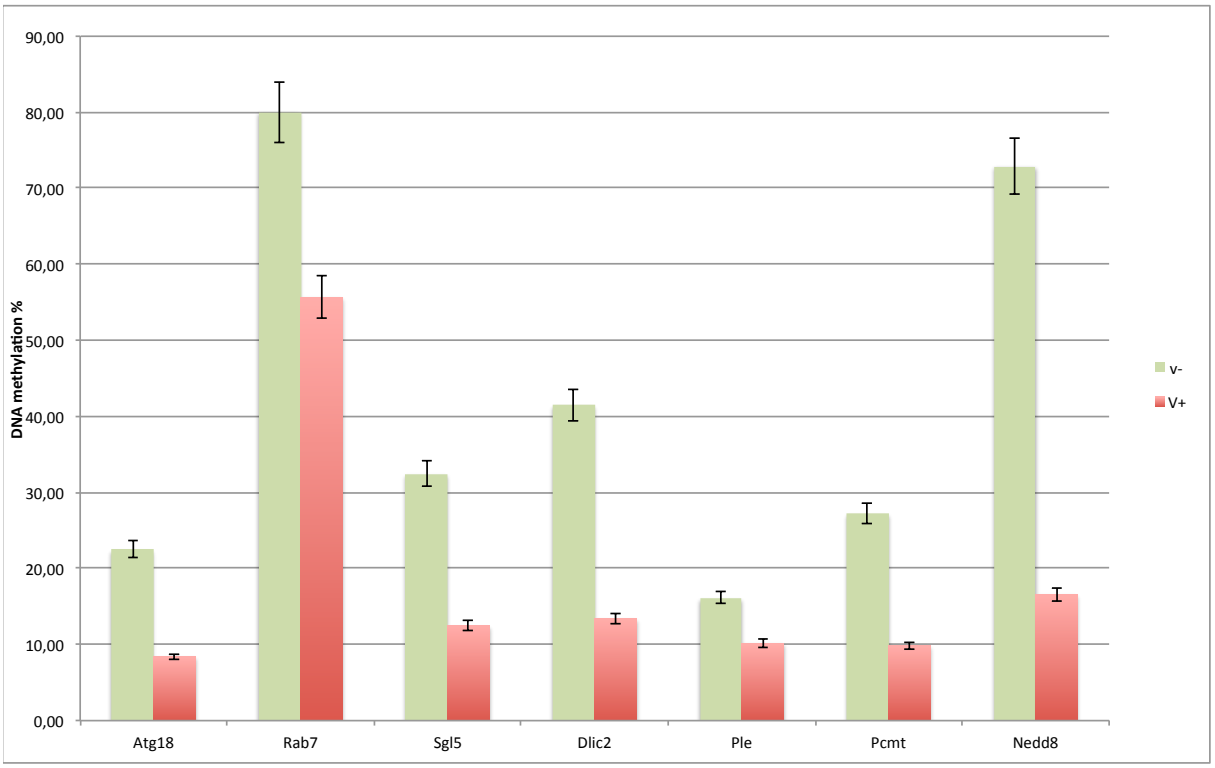

Figure 4.2 DNA methylation levels of control and parasitized samples. The level of methylation in control (green) groups is always higher than the methylation level in parasitized honeybee (red).

These result also highlight an important consideration. Despite the differences in the DNA methylation levels of the gene studied, the two genes which function was directly related to the presence of parasites are also the most methylated genes: in both parasitized and non parasitized samples *Rab7* and *Nedd8* show the highest levels of DNA methylation (80% and 72.86% respectively in control samples; 55.64% and 16.55% respectively in parasitized samples).

A similar pattern is probably linked to the importance of the activity of these two genes in the context of honeybee development. It is already known that genes involved in the immune system are over-expressed during the larval and pupal stage, probably as mechanism preventing the infection by pathogens (Navajas *et al.*, 2010).

4.2.3. Parasitized *vs.* control DNA methylation

The key point of bisulfite sequencing analysis consisted of the comparison of average methylation levels in control and parasitized group for every gene.

A very important result came out from this analysis: for every gene considered in this study, the level of DNA methylation decreases in the parasitized samples (Table 4.2 ; figure 4.3).

Indeed, in every case samples infested from *Varroa destructor* were always less methylated the their correspondent control sample.

Gene	V- methylation %	V+ methylation %	$\Delta_{V-/V+}$ (%)
Atg18	22.48	8.38	14.1*
Dlic2	41.48	13.40	28.08*
Nedd8	72.86	16.55	56.31*
Pcmt	27.25	9.85	17.39*
Ple	16.16	10.13	6.03*
Rab7	80.00	55.64	24.36*
Sgl5	32.45	12.47	19.98*

Table 4.2 Bisulfite sequencing results. DNA methylation percentages in control and parasitized samples are shown. In the last columns, differences among control and varroa samples are listed. * marked values are statistically significative ($p< 0.005$).

Despite no informations are currently known about the physiological mechanisms, such a repetitive and consistent correlation between methylation and varroa presence allows to think to DNA methylation as a possible mechanism involved in honeybee and mite symbioses.

As shown in Lyko and collegues (2010) study, DNA methylation in honeybee is linked to the regulation of alternative splicing. In particular a decreased level of methylation spanning the conditional splicing event (insertion or skipping of the cassette-exon) may impede the inclusion frequency of this exon into the mature transcript. According to these results, varroa infested samples, which show a decrease in the methylation level are affected during the splice events.

According to the pattern shown by this results, two hypothesis are possible to explain the correlation between varroa presence and DNA methylation decreasing.

According to the first hypothesis (called the “varroa hypothesis”) the presence of varroa in the honeybee sample allow DNA methylation to decrease, thus the widespread of

aberrant splice variants of the gene studied. This may result in a loss of protein in the cells (and of course, in the individuals), compromising the immune system, cognitive function and development of the honeybee and making the honeybee more susceptible to varroa infestation.

According to a second hypothesis (called the “honeybee hypothesis”) differences in DNA methylation can be present even without varroa presence on the honeybees. Samples with reduced DNA methylation also have reduced levels of functional proteins in the three compartments, thus increasing the possibility of a subsequent varroa infestation.

My thesis work cannot discriminate between the “varroa” and the “honeybee hypotheses”. Indeed, to shed greater light further studies are needed, possibly with a laboratory control of varroa infestation of the hives.

However, it can be concluded that the “honeybee hypothesis” to explain the correlation between methylation decreasing and parasite infestation is less probable than the “varroa hypothesis”: indeed, any of the control samples showed DNA methylation levels similar to those present in parasitized honeybees.

4.3 Virus screening

All the samples used in this study were checked for the five most common honeybee virus presence according to the methodic previously described.

Results show that viruses are strongly present both in non parasitized and parasitized samples (figure 4.3). In particular the percentage of sample infected by virus is 9 % in control samples and 69% in parasitized honeybees. The presence of mite-transmitted viruses in non infested honeybee is probably due to past infestation of the mite in the hive or, less probably, are due to local cross contamination between hives in the same region. Scarce and rare cases of horizontal contamination between nearly hives are documented in scientific literature (Le Conte *et al.*, 2010), but are still under discussion.

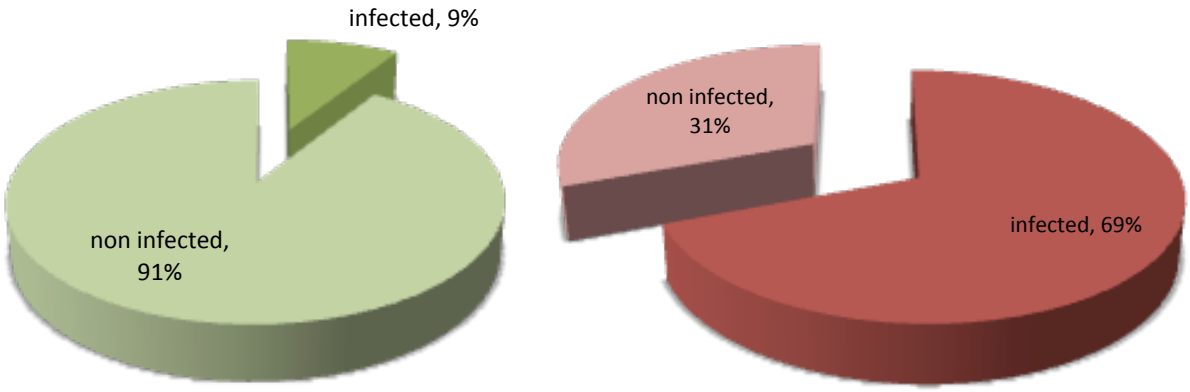

Figure 4.3 Virus infection. Virus infection in control (green) and varroa parasitized (red) group are shown. Infected and non-infected samples are shown in dark and light colors respectively.

In this study the correlation between viruses infection and mite infestation in the honeybee was tested by a linear model and considering as dependent variable the virus presence in the sample, and, as independent variable, the presence of varroa, and assuming a Poisson error distribution:

mv <- lm(virus_tot~factor(varroa)

This analysis shown a correlation between the presence of viruses and the infestation of honeybees by *Varroa destructor*, indicating varroa as a good vector for virus pathogens in the hives (Table 4.3).

Coefficients	Estimated	Std Error	t value	Pr(>ltl)
(Intercept)	0.09091	0.18823	483	631*
factor(varroa)V+	1.10140	0.25576	4.306	8.62e-05*

Table 4.3 Virus infection and varroa correlation. Values form the model used to test the hypothisis of varroa as a vector of viruses in the hives are shown. * marks significative results.

One important result from the virus screening analysis is about the distribution of viral pathogens in the samples (figure 4.4). Infact, virus infections can be divided in four types, according to the number of viruses infecting the honeybee: honeybees can be infected by one (single infection), two (double infection), 3 (triple infection) or four viruses (quadruple infection).

Notably all the single infection are due to Deformed Wing Virus (DWV) only, making this the most widespread virus in the hives sampled in this study. This is in accordance with current scientific literature report (Chen *et al.*, 2004).
Double infection are due to DWV and sacbrood (SBV) or Kashmire bee viruses (KBV). SBV is thus the second most widespread in the samples.

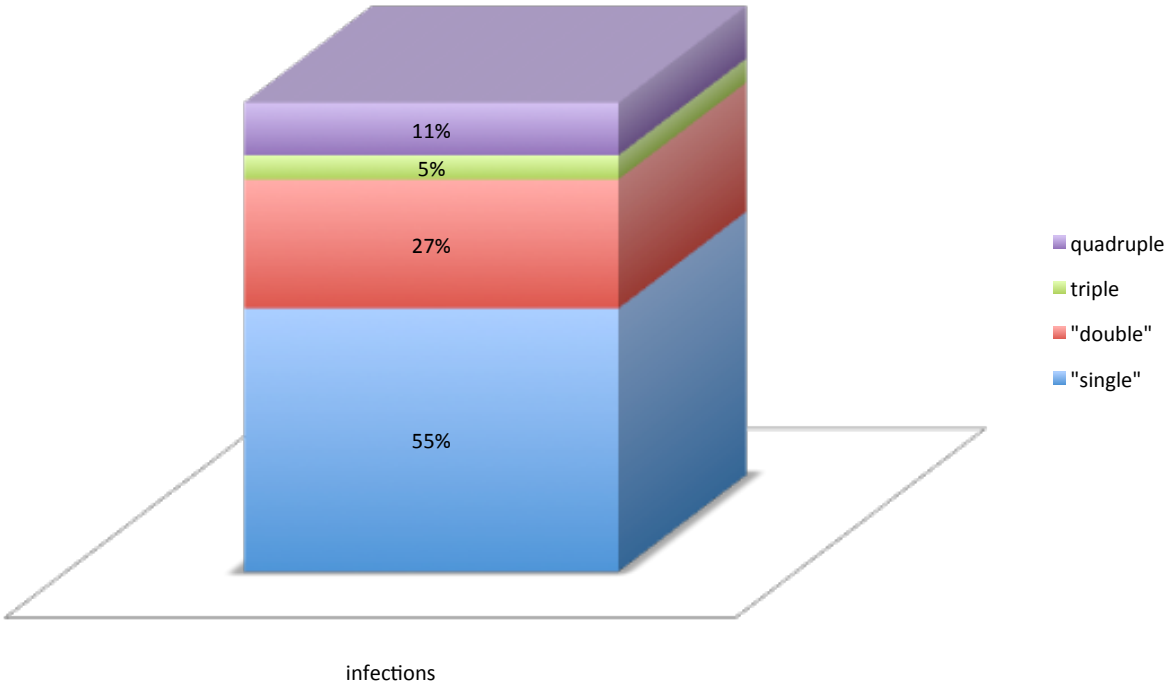

Figure 4.4 virus distribution. Virus distribution can be a single infection by DWV (blue), double infection (red), triple infection (green) or quadruple infection (purple).

To test the possibility that the number of viruses and the DNA methylation were correlated, a general model was used assuming as dependent variable the number of viruses infesting the samples, and, as independent variable, the methylation percentage in the samples:

model <- lm(Vj ~ factor(Vtot))

where Vj are the gene considered in this study and the factor are the number of viral parasite in each sample (1, 2, 3 or 4 virus).
Detailed result from the model are listed in table 4.4.

Gene	Coefficient	Estimate	Std error	t value	Pr>(ltl)
Atg11	intercept	0.20532	0.01057	19.433	<2e-16 *
	factor(virus_tot)1	-0.10124	0.01929	-5.248	4.47e-06*
	factor(virus_tot)2	-0.17992	0.02714	-6.628	4.45e-08*
	factor(virus_tot)3	-0.20532	0.05690	-3.609	0.000798*
	factor(virus_tot)4	-0.20532	0.04092	-5.018	9.56e-06*
Dlic2	intercept	0.34696	0.02387	14.534	< 2e-16 *
	factor(virus_tot)1	-0.16238	0.04359	-3.726	0.000563*
	factor(virus_tot)2	-0.23496	0.06133	-3.831	0.000410*
	factor(virus_tot)3	-0.28696	0.12856	-2.232	0.030866*
	factor(virus_tot)4	-0.31696	0.09246	-3.428	0.001351*
Nedd8	intercept	0.60964	0.04104	14.856	< 2e-16*
	factor(virus_tot)1	-0.37598	0.07492	-5.018	9.54e-06*
	factor(virus_tot)2	-0.51804	0.10542	-4.914	1.34e-05*
	factor(virus_tot)3	-0.60964	0.22099	-2.759	0.008489*
	factor(virus_tot)4	-0.60964	0.15893	-3.836	0.000404*
Pcmt	intercept	0.24096	0.01251	19.260	< 2e-16*
	factor(virus_tot)1	-0.13630	0.02284	-5.967	4.08e-07*
	factor(virus_tot)2	-0.17076	0.03214	-5.313	3.61e-06*
	factor(virus_tot)3	-0.17396	0.06737	-2.582	0.013310*
	factor(virus_tot)4	-0.17396	0.04845	-3.590	0.000842*
Ple	intercept	0.149071	0.005976	24.945	< 2e-16*
	factor(virus_tot)1	-0.044655	0.010911	-4.093	0.000184*
	factor(virus_tot)2	-0.064271	0.015353	-4.186	0.000138*
	factor(virus_tot)3	-0.018071	0.032182	-0,562	0.577343
	factor(virus_tot)4	-0.045571	0.023145	-1.969	0.055425
Rab7	intercept	0.75182	0.01908	39.407	< 2e-16*
	factor(virus_tot)1	-0.17765	0.03483	-5.100	7.28e-06*
	factor(virus_tot)2	-0.22682	0.0490	-4.628	3.39e-05*
	factor(virus_tot)3	-0.25182	0.10274	-2.451	0.01838*

Gene	Coefficient	Estimate	Std error	t value	Pr>(ltl)
	factor(virus_tot)4	-0.25182	0.07389	-3.408	0.00143*
Sgl5	intercept	0.28671	0.01617	17.736	< 2e-16*
	factor(virus_tot)1	-0.11871	0.02951	-4.022	0.000229*
	factor(virus_tot)2	-0.21911	0.04153	-5.276	4.08e-06*
	factor(virus_tot)3	-0.28671	0.08705	-3.294	0.001985*
	factor(virus_tot)4	-0.28671	0.06261	-4.579	3.96e-05*

Table 4.4 Virus infection and DNA methylation correlation. Values form the model used to test the hypothesis of a correlation between viral infection and DNA methylation are listed. Residual standard error: 0.03162 on 43 degrees of freedom. Multiple R-squared: 0.4078, Adjusted R-squared: 0.3527. F-statistic: 7.402 on 4 and 43 DF, p-value: 0.0001254. * indicates significative estimate.

Results shown that among the honeybees that are infested by *Varroa destructor*, the samples infected by viruses have the lower methylation levels. In particular, the DNA methylation level decreases with the number of viruses in the sample: single infected samples are more methylated than double infested samples, which are lower methylated than triple infested and so on (Figure 4.5).

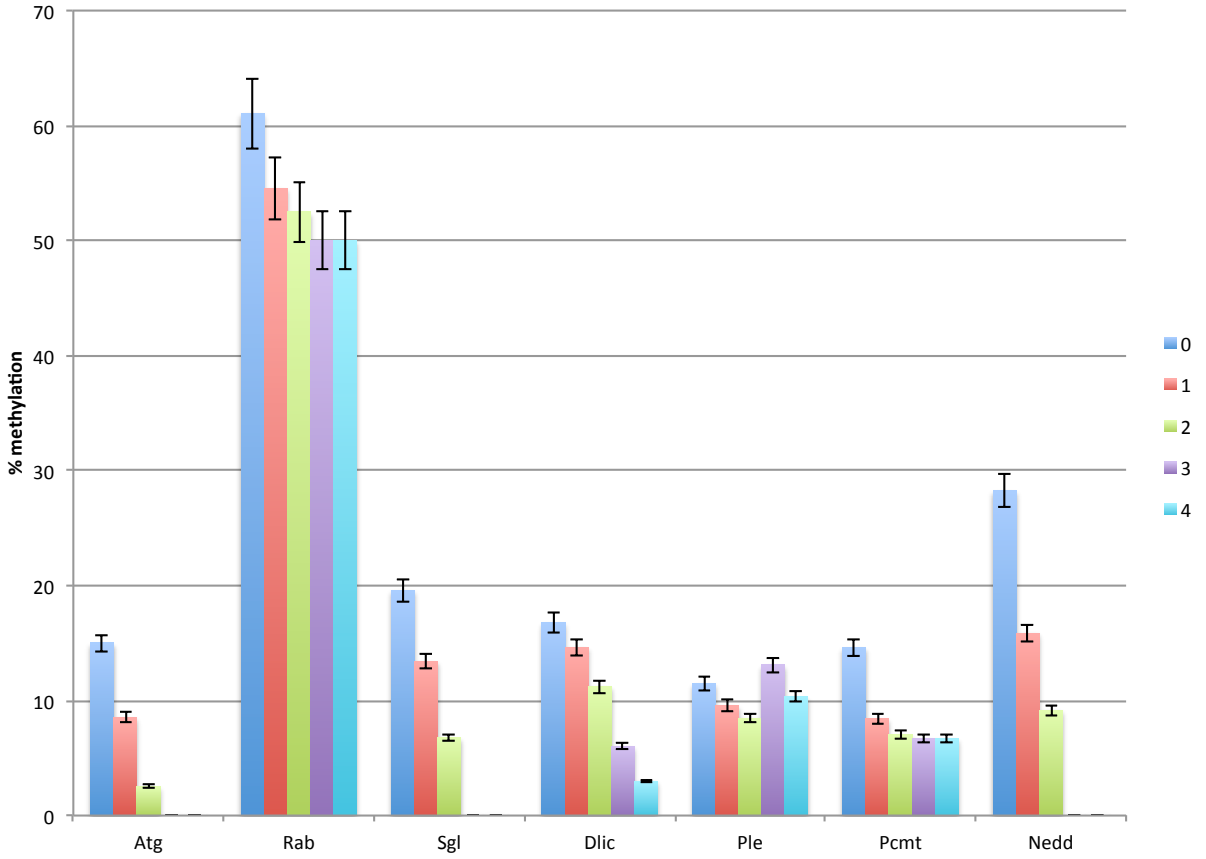

Figure 4.5 Virus infection and DNA methylation correlation. For every genes the methylation levels in the different infestation group is shown

Taken together results show a strong correlation between the presence of symbionts (in this case parasites) in the honeybee and the level of DNA methylation and in particular with a decreasing in methylation.

Results from the virus analyses confirm the hypothesis according to which the DNA methylation and symbioses are strongly linked. Despite no hypothesis about the mechanism have been formulated in this work, a strong correlation was demonstrated, suggesting an important link between these widespread and fundamental processes: epigenetics and symbioses.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

The main objective of this study was to investigate the DNA methylation patterns in the context of a symbiosis between two arthropod species: the honeybee *Apis mellifera* and its parasitic mite *Varroa destructor*.
This study concerns two main field of biology: epigenetics (i.e the study of changes in phenotypes which do not alter genotypes) and symbioses (i.e. the relationship between two different species). As mentioned in previous chapters, when a symbiosis occur, two entities (species) interact and thus two different genomes came in contact. It is clear that in this context molecular communications occur between two species, even if, in most of the cases, we currently do not know the “language” of these communications.

Because of its properties, DNA methylation is always more tough as a good candidate to embody (or take part) the “language” in the interaction among species. In this work, this hypothesis was considered and studied for the first time in the context of a well known symbiosis between arthropods.
In particular, DNA methylation was investigated at two levels: a global, genomic, level and a detailed, genetic level.

As shown by results, discussed in the previous section, no differences in the global level of DNA methylation was found between honeybees infested by mites and non parasitized individuals. These results are in accordance with scientific literature data, according to which DNA methylation in insects, and in particular in the model organism *Apis mellifera*, is restricted to particular regions of the genome and is present at very low levels, making the discrimination at a global level very difficult.

Detailed analysis were conducted at a lower scale, focusing on the DNA methylation of honeybee genes involved in the symbiosis with the parasitic mite *Varroa destructor*. Results shown a correlation between a decreasing in the methylation levels and the

presence of varroa on honeybee. These results allow us to raise the hypothesis that DNA methylation may be involved in the symbiosis between honeybees and mites.
Moreover, since *Varroa destructor* is a vector for several honeybee virus, the possible relationships between virus presence in the honeybee infested by varroa and DNA methylation was studied. Results show a correlation between the number of viruses in the honeybee, that is a measure of the level of infection, and the decreasing in honeybee DNA methylation.

Taken together these results support the idea of DNA methylation as a good candidate mechanism which may have a possible role in the symbiosis regulation.
In this study no hypothesis about any possible mechanism of interaction between DNA methylation and symbiosis was studied. It can therefore been assumed that since DNA methylation in honeybee is involved in the regulation of alternative splicing, alteration in the correct DNA methylation pattern caused by environmental factors affect protein expression: alternative splice variant may not have the same protein function or could be non functional at all.

Even if further studies, such as the artificial manipulation of the honeybee and mite symbiosis, are needed to clarify how DNA methylation of honeybee and the presence of symbionts are linked, results from this work support the connection between epigenetics and symbiosis.
Moreover, further studies can be conducted in different models organisms to clarify the role of epigenetics in the context of symbioses and considering that the knowledge of the mechanism (the “language”) is the first step towards its possible manipulation. In other words, the understanding of how epiegenetics can modulate symbioses, may became a key point in control of some important and relevant parasitic relationships with great impact on medical and agroeconomic fields.

From an ecological point of view, epigenetics processes are involved in a complex matrix of interaction (Figure 5.1) concerning both gene expression and ecological interactions: epigenetic mechanisms used in the developmental program of an organism can be sensitive to the environment. Indeed, epigenetic mechanism, such as DNA methylation, provide a means of extending the flexibility of the genome by affecting changes to the transcriptome and thus to increase phenotypic plasticity.

Phenotypic plasticity is ubiquitous property in plants and animals that enables a population to achieve phenotypic variability with respect to environmental changes despite genetic uniformity. More generally, epigenetic processes may increase phenotypic plasticity and may alter the evolutionary potential of organism in response to the abiotic (environmental) and biotic (symbionts) stresses.

Assuming that symbiotic events, such parasitosis, are part of environmental changes, phenotypic plasticity would play a fundamental role in the response of host to their symbionts.

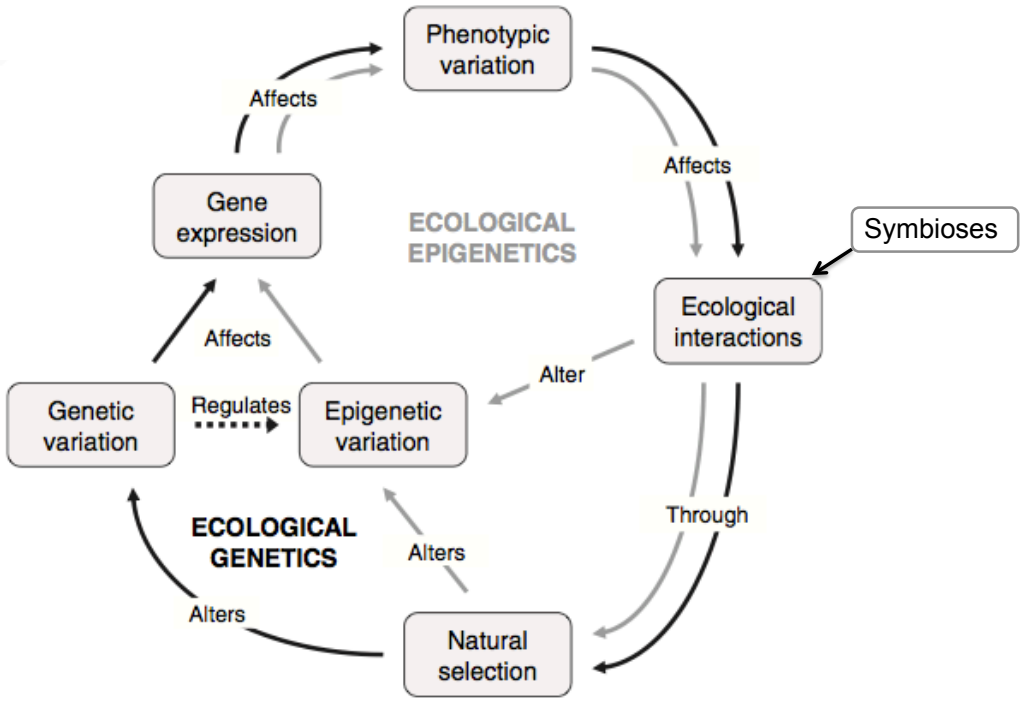

Figure 5.1 Ecological genetics and ecological epigenetics. On one hand, epigenetic processes may provide a second inheritance system, that allows evolution by natural selection. On the other hand, epigenetic variation, unlike genetic variation, may be altered directly by ecological (and thus by symbiotic) interactions. Modified from Bossdorf *et al.*, 2008.

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APPENDIX

VOUCHER NAME	SAMPLE DETAIL		LOCALITY			COLONY	NOTE
	SPECIES	CASTE AND STAGE					
MIB:ZPL: 00397	*Apis mellifera*	worker larvae	Italia	LO	Lodi		parasitized by *Varroa destructor*
MIB:ZPL: 00398	*Apis mellifera*	worker larvae	Italia	LO	Lodi		parasitized by *Varroa destructor*
MIB:ZPL: 00608	*Apis mellifera*	worker larvae	Italia	LC	Monticello brianza		parasitized by *Varroa destructor*
MIB:ZPL: 00609	*Apis mellifera*	worker larvae	Italia	LC	Monticello brianza		parasitized by *Varroa destructor*
MIB:ZPL: 00716	*Apis mellifera*	worker larvae	Italia	LC	Monticello brianza		_
MIB:ZPL: 00717	*Apis mellifera*	worker larvae	Italia	LC	Monticello brianza		_
MIB:ZPL: 01143	*Apis mellifera*	worker larvae	Italia	LC	Monticello		parasitized by *Varroa destructor*
MIB:ZPL: 01144	*Apis mellifera*	worker larvae	Italia	LC	Monticello		parasitized by *Varroa destructor*
MIB:ZPL: 01145	*Apis mellifera*	worker larvae	Italia	LC	Monticello		parasitized by *Varroa destructor*
MIB:ZPL: 01146	*Apis mellifera*	worker larvae	Italia	LC	Monticello		parasitized by *Varroa destructor*
MIB:ZPL: 01147	*Apis mellifera*	worker larvae	Italia	LC	Monticello		parasitized by *Varroa destructor*
MIB:ZPL: 03907	*Apis mellifera*	worker larvae	Italia	LC	Monticello brianza		Parasitized by *Varroa destructor*
MIB:ZPL: 03910	*Apis mellifera*	worker larvae	Italia	LC	Monticello brianza		Parasitized by *Varroa destructor*
MIB:ZPL: 03913	*Apis mellifera*	worker larvae	Italia	LC	Monticello brianza		Parasitized by *Varroa destructor*
MIB:ZPL: 03919	*Apis mellifera*	worker larvae	Italia	LC	Monticello brianza		Parasitized by *Varroa destructor*
MIB:ZPL: 03920	*Apis mellifera*	worker larvae	Italia	MB	Besana brianza		_
MIB:ZPL: 03921	*Apis mellifera*	worker larvae	Italia	MB	Besana brianza		_

MIB:ZPL: 03922	*Apis mellifera*	worker larvae	Italia	MB	Besana brianza		_
MIB:ZPL: 03923	*Apis mellifera*	worker larvae	Italia	MB	Besana brianza		_
MIB:ZPL: 03924	*Apis mellifera*	worker larvae	Italia	MB	Besana brianza		_
MIB:ZPL: 03925	*Apis mellifera*	worker larvae	Italia	LC	Monticello brianza		_
MIB:ZPL: 03930	*Apis mellifera*	worker larvae	Italia	BG	Nembro		Parasitized by *Varroa destructor*
MIB:ZPL: 03931	*Apis mellifera*	worker larvae	Italia	BG	Nembro		Parasitized by *Varroa destructor*
MIB:ZPL: 03933	*Apis mellifera*	worker larvae	Italia	BG	Nembro		Parasitized by *Varroa destructor*
MIB:ZPL: 03935	*Apis mellifera*	worker larvae	Italia	BG	Nembro		Parasitized by *Varroa destructor*
MIB:ZPL: 03937	*Apis mellifera*	worker larvae	Italia	BG	Nembro		_
MIB:ZPL: 03938	*Apis mellifera*	worker larvae	Italia	BG	Nembro		_
MIB:ZPL: 03939	*Apis mellifera*	worker larvae	Italia	BG	Nembro		_
MIB:ZPL: 03941	*Apis mellifera*	worker larvae	Italia	LC	Barzanò		Parasitized by *Varroa destructor*
MIB:ZPL: 03943	*Apis mellifera*	worker larvae	Italia	MB	Carnate		Parasitized by *Varroa destructor*
MIB:ZPL: 03953	*Apis mellifera*	worker larvae	Italia	MB	Carnate		Parasitized by *Varroa destructor*
MIB:ZPL: 03955	*Apis mellifera*	worker larvae	Italia	MB	Carnate		Parasitized by *Varroa destructor*
MIB:ZPL: 03957	*Apis mellifera*	worker larvae	Italia	MB	Carnate		Parasitized by *Varroa destructor*
MIB:ZPL: 03959	*Apis mellifera*	worker larvae	Italia	MB	Carnate		Parasitized by *Varroa destructor*
MIB:ZPL: 03961	*Apis mellifera*	worker larvae	Italia	MB	Carnate		Parasitized by *Varroa destructor*

MIB:ZPL: 03963	*Apis mellifera*	worker larvae	Italia	LC	S. Girolamo		Parasitized by *Varroa destructor*
MIB:ZPL: 03965	*Apis mellifera*	worker larvae	Italia	LC	S. Girolamo		Parasitized by *Varroa destructor*
MIB:ZPL: 03969	*Apis mellifera*	worker larvae	Italia	BG	Val Seriana		Parasitized by *Varroa destructor*
MIB:ZPL: 03972	*Apis mellifera*	worker larvae	Italia	BG	Val Seriana		Parasitized by *Varroa destructor*
MIB:ZPL: 03975	*Apis mellifera*	worker larvae	Italia	BG	Val Seriana		Parasitized by *Varroa destructor*
MIB:ZPL: 03977	*Apis mellifera*	worker larvae	Italia	BG	Val Seriana		Parasitized by *Varroa destructor*
MIB:ZPL: 03978	*Apis mellifera*	worker larvae	Italia	BG	Val Seriana		_
MIB:ZPL: 05314	*Apis mellifera*	worker larvae		LC	Cremella		_
MIB:ZPL: 05315	*Apis mellifera*	worker larvae		LC	Cremella		_
MIB:ZPL: 05316	*Apis mellifera*	worker larvae		LC	Cremella		_
MIB:ZPL: 05317	*Apis mellifera*	worker larvae		LC	Cremella		_
MIB:ZPL: 05318	*Apis mellifera*	worker larvae		LC	Cremella		_
MIB:ZPL: 05319	*Apis mellifera*	worker larvae		LC	Cremella		_
MIB:ZPL: 05320	*Apis mellifera*	worker larvae		LC	Cremella		_
MIB:ZPL: 05321	*Apis mellifera*	worker larvae		LC	Cremella		_
MIB:ZPL: 05322	*Apis mellifera*	worker larvae		LC	Cremella		Parasitized by *Varroa destructor*
MIB:ZPL: 05323	*Apis mellifera*	worker larvae		LC	Cremella		Parasitized by *Varroa destructor*

MIB:ZPL: 05324	*Apis mellifera*	worker larvae		LC	Cremella		Parasitized by *Varroa destructor*
MIB:ZPL: 05325	*Apis mellifera*	worker larvae		LC	Cremella		Regina (ultimo stadio pupale)
MIB:ZPL: 05327	*Apis mellifera*	worker larvae		LC	Cremella		_
MIB:ZPL: 05328	*Apis mellifera*	worker larvae		LC	Cremella		_
MIB:ZPL: 05329	*Apis mellifera*	worker larvae		LC	Cremella		_
MIB:ZPL: 05333	*Apis mellifera*	worker larvae		MI	Varese		_
MIB:ZPL: 05334	*Apis mellifera*	worker larvae		MI	Varese		_
MIB:ZPL: 05335	*Apis mellifera*	worker larvae		LC	Cremella		_

Appendix: samples details. For every sample collected, voucher number, species, stage and locality of collection are shown. Bold samples were analyzed in this study.