

1 **Epigenetic changes in eusocial insects which affect age and longevity**

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8 **Abstract**

9 Ageing, a major scientific issue, is a complex process common for all living organisms,
10 influenced by different environmental and genetic factors which are difficult to understand. It
11 is considered that epigenetic modifications, such as DNA methylation, histone post-
12 translational modification and non-coding RNA affect ageing. Eusocial insects provide an ideal
13 platform for analyzing the impact of epigenetic changes on ageing due to their phenotypic
14 plasticity. This paper summarizes most of the data published so far on epigenetic changes
15 during ageing in eusocial insects.

16 **Keywords:** histone modification, invertebrates, DNA methylation, non-coding RNA

17 **Introduction**

18 Ageing is a well-known, complex process, common to all living organisms. It is associated with
19 progressive, gradual decrease and loss of normal physiological functions and cell senescence,
20 which culminates in the onset of disease and, in the end, death of the organism^{1,2}. This process
21 depends on interaction of various environmental factors and genetics mechanisms which are
22 still not fully understood. At the molecular level, ageing is influenced by energy metabolism,
23 which has an impact on the cellular senescence, telomere shortening and autophagy, but
24 recently much progress has been made in characterising epigenetic mechanisms that underpin
25 ageing²⁻⁴. The role of epigenetics in ageing is being actively studied, and the important model

26 organisms for these studies are eusocial insects (such as honey bees, wasps, bumble bees and
27 ants), due to their phenotypic plasticity, which is an important feature of eusociality⁴⁻⁷. Social
28 insects are good example of polyphenism, unique sub-type of phenotypic plasticity, where two
29 or more distinct phenotypes arise from same genotype, and genetically identical individuals
30 express evident difference in behavior and longevity⁸. Phenotypic plasticity is also associated
31 with division of labor, which is very important for development of caste system, with lifespan
32 divergence between castes^{5,9,10}.

33 The term epigenetics comes from the Greek word *epigenesis* (*epi*: above and *genesis*:
34 generation) and it was first used by the British scientist C. H. Waddington in 1942, who defined
35 epigenetic as “the branch of biology which studies the casual interaction between genes and
36 their products which bring the phenotype into being”¹¹. Epigenetic modifications play a major
37 role in modulating genetic expression and thus establish, maintain and change the phenotype,
38 affecting behavior and longevity. Although the chromosomes in our genome contains many
39 genes, the basic physical and functional unit of heredity, the epigenome is responsible for the
40 functional use and stability of that valuable information; that is, it connects the genotype with
41 the phenotype^{2,12}.. So far, several different mechanisms of epigenetic modification of gene
42 expression have been identified, including DNA methylation, post-translational modifications
43 of histones, and the influence of regulatory non-coding RNA (ncRNA). This mini review will
44 present some of the key experiments, summed up in Table 1, that introduce us to the connection
45 between epigenetic and ageing, as well as mechanism that potentially underlie ageing and
46 longevity in eusocial insects.

47 **DNA Methylation**

48 DNA methylation is a covalent chemical modification of the 5th carbon atom of cytosine, to
49 form 5-methyl-cytosine. The donor of the methyl group is S-adenosyl methionine (SAM), and
50 the reaction is catalyzed by the enzyme DNA methyltransferase (DNMT). DNA methylation

51 most often occurs in the location where cytosine and guanine are next to each other in nucleotide
52 sequence, which is known as CpG islands¹³⁻¹⁵. DNA methylation at CpG islands is associated
53 with inhibition of gene expression, and by that, transcriptional silencing¹⁶. In most insect studies
54 methylation was predominately restricted to coding exons and absent in promotor regions¹⁶⁻¹⁸.
55 This epigenetic modification is the most studied, as it has many benefits over other
56 modifications, including the fact that DNA methylation is inherited throughout cell division,
57 which is enabled by the action of the most abundant methyltransferase, DNMT1¹³⁻¹⁵. In
58 addition to DNMT1, there are also DNMT2, 3, and 4, which are referred to as *de novo*
59 methyltransferases¹⁹⁻²¹. DNMT3 is very important as it introduces new patterns of methylation
60 and performs an important role in the development of castes in social insects²². DNMT2 was
61 previously linked to cytosine methylation, but now it is known to methylate transfer RNA
62 (tRNA)²³ and by that performs an important role in protecting insects from harmful
63 environment, as it has been shown in studies with the fruit fly *Drosophila melanogaster*^{19,20,24}.
64 In different species ageing is associated with methylation at CpG islands²⁵. Pathways that
65 control DNA methylation, unlike histone modification, are not conserved across taxa. Global
66 abundance of methylated CpG islands is various between different species^{26,27}. Insects have a
67 relatively low level of DNA methylation (0-3%), for example, CpG methylation is absent in
68 fruit flies *Drosophila melanogaster*²⁸ and beetles *Tribolium castaneum*²⁹, but it is common
69 among social insects³⁰. Humans and birds have 5% of methylated DNA, while in plants more
70 than 30% of the DNA is methylated¹³. Also, methylated CpG contents show to variate during
71 insect development, where, for instance, in honey bee, *Apis mellifera*, which was the first
72 species found to have fully functioning methylation system, embryos show to have highest level
73 of methylation in comparison with adults^{31,32}.
74 Honey bee *Apis mellifera* L. has complete DNA methylation system³³ and represents a good
75 example of phenotypic plasticity, where fertile queen develop from larvae fed with royal jelly,

76 while workers are fed with jelly that lacks in sugars and some other important ingredients³⁴.
77 Such nutritional differences may affect age and longevity in honey bees, as well as differences
78 in DNA methylation³⁵, and this is why this species is one of the most studied when it comes to
79 epigenetic modifications. Experiments on the honey bee, one of the most studied social insect,
80 have introduced the special role of DNMT3 in caste development. Kucharski et al. (2008)
81 showed that silencing of the gene for DNMT3 using simple, small interfering RNA (siRNA),
82 in newly hatched larvae, lead to development of much more queen (72%) than worker bees
83 (28%). In addition to this, comparison of heads of destined queens and ones emerged from
84 larvae with silenced DNMT3 gene, showed similarly decreased level of DNA methylation²².
85 After this, numerous studies have been conducted in order to better understand the act of DNA
86 methylation. It has been shown that methylation is important for key biological processes such
87 as development, caste determination, behavior and above all , ageing and longevity²⁴. Lyko et
88 al. (2010) performed high-resolution bisulfite sequencing on whole brain genome of queen and
89 workers honey bee, where they discovered more than 550 genes showing different patterns of
90 DNA methylation between them, which potentially affect the differences in their behavior²⁸.
91 This method analyses DNA that was treated with bisulfite, which convert unmethylated
92 cytosine to uracil, which can then be detected and compared to reference genome³⁶. Foret et al.
93 (2012) sequenced methylomes in larvae and queen heads, discovering 2399 methylated genes
94 that were significantly different in methylation between them. They showed that several highly
95 conserved signaling and metabolic pathways, such as juvenile hormone and insulin, are
96 enriched in methylated genes, which were previously shown to regulate caste determination³⁷.
97 Gene *hexamerin 110* encoding storage protein³⁸, and *dynactin p62*, conserved gene responsive
98 for feeding changes³⁹, displayed the different level in DNA methylation when comparing queen
99 bees with worker bees, as well as significantly different global level of DNA methylation in
100 bees of different age, which can be a good basis for understanding and explaining mechanism

101 of DNA methylation and how it affects ageing⁴⁰. Wang et al. (2020) compared the genomic
102 methylation of the queen and worker larvae at 3, 4, and 5 days old, using the whole-genome
103 bisulfite sequencing technique, and concluded that the fundamental traits of methylation are
104 equal among them. However, the methylation levels of queen and worker larvae showed
105 differences and varied with the age of the organism. They additionally singled out 10
106 differentially methylated genes (DMGs), as well as 13 cast-specific genes that they believe to
107 be possible molecular markers for selective breeding of this species to improve fecundity,
108 production of royal jelly, body size and foraging⁴¹.

109 Similar mechanisms have been observed in other social insects such as ants, wasps and bumble
110 bees, showing significant changes in DNA methylation during ageing, as well as caste-specific
111 methylation patterns. In bumble bee *Bombus terrestris* workers, significant age-related increase
112 in DNMT3 expressed in fat body was observed, suggesting a novel association between ageing
113 and methylation⁶. In experiment with primitively social paper wasp *Polistes dominula*, Weiner
114 et al. (2013) showed surprisingly increased overall DNA methylation and caste-related
115 differences in site-specific methylation, suggesting the role of DNA methylation in
116 physiological and behavioral regulations⁴². Recent studies in jewel wasp *Nasonia vitripennis*
117 show that knocking-out DNMT1 gene leads to failure in cellularization and gastrulation of the
118 embryo, which demonstrates that reduction in DNA methylation is associated with decreased
119 gene expression⁴³. Evolutionarily conserved DNA methyltransferase has an important role in
120 the reproductive caste development in social insects, like ants²³. It regulates phenotypic
121 plasticity of size in carpenter ants, and it is correlated with age-related and caste-specific gene
122 expression. However, patterns of DNA methylation also change during development and caste
123 specific gene expression in ants^{44,45}.

124 DNA methylation is reversible and this can be done in 2 ways: (1) passive demethylation- by
125 blocking DNMT1 maintain process during DNA replication; (2) active demethylation- by

126 enzyme family called TET (Ten-eleven translocation) dioxygenases, which oxidize methyl
127 group on cytosine to form 5-hydroxymethyl-cytosine, which can, in mammals, be oxidized to
128 5-formyl-cytosine and 5-carboxyl-cytosine, which is finally converted into cytosine. 5-
129 hydroxymethyl-cytosine is present in insect, but there is no knowledge about the presence of
130 other two forms in insects^{36,46,47}. Studies of honey bee *A. mellifera* have proven that this species,
131 like many different insects including bumble bee *Bombus terrestris* and wasp *Nasonia*
132 *vitripennis*²³, has only one type of TET dioxygenase, while in vertebrates 3 types of this enzyme
133 have been discovered^{47,48}. These enzymes were found in different tissues and developmental
134 stages in honey bee^{49,50}. In addition to this TET dioxygenases are most expressed in the brain
135 of honey bee workers, however, the level of their expression does not correspond to the level
136 of 5-hydroxymethyl-cytosine, which implies that these enzymes possibly have other roles as
137 well¹⁵.

138 **Post-translational histone modifications**

139 Post-translational modification of histone is a type of epigenetic modification that involves
140 structural changes in chromatin, which is responsible for the packaging and organization of
141 DNA in the nucleus, and affect various important biological processes^{4,24}. DNA is wrapped
142 around small, positively charged proteins, called histones (H1, H2a, H2b, H3, and H4), with
143 amino acid tails subjected to various changes. Chromatin is the manager of DNA availability
144 for processes such as transcription, replication and DNA reparation, so it plays a major role in
145 the developmental trajectories of phenotype construction²⁴. Post-translational modifications of
146 histone tails operate together with DNA methylation in regulation of gene expression³. These
147 modifications include methylation, acetylation, phosphorylation, ubiquitination, ADP-
148 ribosylation, etc. which aim to activate or repress transcription in order to regulate gene
149 expression. The configuration of chromatin, which can be condensed or relaxed, depends on

150 these modifications. More than 160 histone modifications have been detected in insects, but
151 most of the mechanisms are still not fully understood¹³.

152 During histone methylation, methyl groups are relocated to lysine or arginine side chains,
153 altering the DNA packaging, which is proven to regulate insect development and longevity^{24,51}.

154 Two enzymes, histone methyltransferase (HMT) and histone demethylase (HDM), play
155 important roles in these modifications^{24,52}. Methylation of H3K9, H3K27 and H4K20 is said to
156 be associated with transcription repression, while methylation of H3K4, H3K36 and H3K79 is
157 associated with active chromatin³. Studies conducted in honey bee worker and queen larvae 96
158 h after hatching, showed a well-preserved methylation pattern of H3K4 and H3K36, indicating
159 an important role in caste development⁵³. Bonasio et al. (2012) identified 27 proteins containing
160 the conserved SET histone methyltransferase domain (proteins that methylate histone on lysine)
161 in ants *Camponotus floridanus* and 22 in *Harpegnathos saltator*, but there are still missing
162 crucial results on how this affect ageing⁵⁴. Nevertheless, there is a little work done in this field
163 of study, so additional research is required in order to better understand the mechanism of action
164 behind histone methylation in other social insect, such as ants and wasps.

165 Histone acetylation, the first discovered modification, is based on the addition of an acetyl
166 group from acetyl-CoA to the amino group of lysine, by the action of the enzyme histone
167 acetyltransferase (HAT). Histone tails undergo rapid acetylation and deacetylation by histone
168 deacetylase (HDAC), and the half-life of acetylated histones is only a few minutes. HAT and
169 HDAC are often part of larger enzyme complexes that have different activities, and each has a
170 coordinated function, recognizes specific regions of chromatin and makes the necessary
171 modifications. Histone acetylation can activate transcription by reducing histone-DNA
172 interactions, recruiting transcription factors, and remodeling chromatin structure^{52,55,56}.

173 Dickman et al. (2013) identified 23 post-translational modifications in 96 h old honey bee
174 larvae, an important time point for key genetic modification triggered by consumption of royal

175 jelly, but also two acetylation, in particular H3K9 and H3K14, which showed to be mutually
176 depended⁵⁷. Simola et al. (2013) conducted similar experiment with ant *C. floridanus*, and they
177 found that gene changes in histone modifications, especially H3K27 acetylation could be
178 powerful predictor of caste identity⁵⁸.

179 The latest studies of Choppin et al. (2021) on *Temnothorax rugatulus* ants, showed that feeding
180 workers with chemical inhibitor of histone acetylation (C646) and deacetylation, trichostatin A
181 (TSA), affect gene expression, especially by downregulating genes with immunity, ageing and
182 longevity functions. They concluded the importance of histone acetylation in phenotypic
183 plasticity of this species⁵⁹. In contrast, previous work done by Hu et al. (2017) showed that
184 treatment of honey bees with Na-butyrate, well known HDAC inhibitor, led to increased histone
185 acetylation, but also prolonged their life span compared to untreated bees⁶⁰. HDAC inhibitors
186 commonly present in royal jelly are necessary for development of queen bees, which may
187 indicate that royal jelly has direct influence on histone acetylation level and thereby caste
188 determination⁶¹. As of today, numerous studies of histone modification in insect were
189 conducted, but it is still unclear how they affect ageing and longevity, therefore additional
190 experiments are required.

191 **Non-coding RNA (ncRNA)**

192 Non-coding RNAs (ncRNAs) are DNA transcripts, but rather than being translated into
193 proteins, they play an essential role in gene expression regulation at the post-translational stage,
194 where they affect gene expression and chromatin remodeling by binding to their targets⁵². There
195 are several distinct ncRNAs: micro RNA (miRNA), small interfering RNA (siRNA), PiWi
196 interacting RNA (piRNA), long non-coding RNA (lncRNA), and circular RNA (circRNA)^{9,62–}
197 ⁶⁵. Along with DNA methylation, they play a crucial role in paramutations, which occur when
198 one allele affects another from the same locus and causes hereditary modifications in that allele,
199 but are also involved in protecting insects from viral infections^{12,13}. These ncRNAs play an

200 important role in epigenetic modifications, by affecting age and longevity, as it has been shown
201 in experiment with *C.elegans* where total miRNA and piRNA are decreasing during ageing⁶⁶.
202 Genetic modulations of certain ncRNAs affect the life span of insects, in addition to the
203 modulation of protein factors that alter ageing^{52,66} CircRNA increases in age-dependent way,
204 while miRNA, in general, affect age and longevity by interaction with different genes and, by
205 that, alter gene expression, but also target 3'-UTR of mRNA affecting transcriptional
206 repression⁵². Several experiments on social insect such as honey bee *A. mellifera*, and two ant
207 species *C. floridanus* and *H. saltator*, showed the differences in expression of miRNA,
208 suggesting its role in development of different phenotypes. Experiments conducted on honey
209 bees have shown that royal jelly, the primary food for larvae from which queen develop,
210 contains numerous ncRNAs, including piRNA, siRNA⁶⁷, and miRNA⁶⁸, that play a role in post-
211 translational silencing of genes, providing, therefore, an additional level of epigenetic
212 regulation^{12,69}. miRNAs are much more common in jelly-feeding larvae, while reproductive
213 queens have significantly higher levels of piRNA, so these RNAs are thought to play a role in
214 the development of specific phenotypes in the caste⁶⁹⁻⁷¹. Honey bee is the most popular model
215 organism for investigating different biological processes, but there are still missing information
216 of how ncRNA affect ageing in honey bee, as well as in other social organisms such as ants,
217 wasps and bumble bee.

218 **Conclusion**

219 Epigenetics and ageing are extremely popular areas of research, particularly as they are
220 interrelated. Social insects holds a great promise in epigenetic studies. They play a crucial role
221 as model organisms, due to their phenotypical plasticity and flexible ageing and longevity. It is
222 fascinating how the division in the castes works and how much individuals differ significantly
223 in morphology and behavior as well as in life expectancy, regardless of a very similar genotype.
224 In addition to this, social insects are simple to grow in laboratory conditions. Therefore,

225 research of epigenetic changes and ageing in social insects is essential for understanding the
226 mechanisms behind these processes, as well as how they can be influenced. Even though a lot
227 of studies, especially in DNA methylation, has been done, there is still lack of knowledge that
228 may explain epigenetic changes in more detail. Further analysis on histone posttranslational
229 modifications is particularly required, to better understand which genes are affected by
230 modifications and the way these changes affect ageing or caste formation. In recent years,
231 RNA research has become very popular, especially since it had been determined that ncRNAs
232 have an impact on the epigenetics and ageing of the organism. Additional research is also
233 needed to better explain the mechanisms by which certain ncRNAs affect the genome and cause
234 epigenetic changes. Understanding the link between ageing and epigenetics is challenging in
235 any model organism. Social insect provide more manipulable system, but difficulties still arise.
236 To understand these link, we may need to dig deeper, at cellular level, to understand what is
237 happening in different cell types and how does that affect phenotype. Understanding all of this
238 may lead us to some new studies, which can potentially lead to identification of therapeutics
239 that may be used to treat ageing-related disorders as well as slow the ageing processes of the
240 organism.

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- 411

412 **Table 1.** Summary of processes in which epigenetic regulations play important role in eusocial
 413 insects.

Family	Species	Epigenetic modification	Process regulation
Apidae	<i>Apis mellifera</i>	DNM	Caste development ²²
Apidae	<i>Apis mellifera</i>	DNM	Not yet defined ^{38,39}
Apidae	<i>Apis mellifera</i>	DNM	Caste behavior ²⁸
Apidae	<i>Apis mellifera</i>	DNM	Caste determination ³⁷
Apidae	<i>Apis mellifera</i>	DNM	Caste specific gene expression ⁴¹
Apidae	<i>Bombus terrestris</i>	DNM	Ageing ⁶
Vespidae	<i>Polistes dominula</i>	DNM	Physiology and behaviour ⁴²
Formicidae	<i>Linepithema humile</i>	DNM	Reproductive development ⁵⁴
Formicidae	<i>Camponotus floridanus</i>	DNM	Caste-specific gene expression ⁴⁴
Pteromilidae	<i>Nasonia vitripennis</i>	DNM	Genes for cellularization and gastrulation of embryo ⁴³
Apidae	<i>Apis mellifera</i>	HPTM	Caste development ⁵³
Formicidae	<i>Camponotus floridanus</i> <i>Harpegnathos venator</i>	HPTM	Potentially ageing ⁵⁴
Apidae	<i>Apis mellifera</i>	HPTM	Caste development ⁵⁷
Formicidae	<i>Camponotus floridanus</i>	HPTM	Caste identity ⁵⁸
Formicidae	<i>Temnothorax rugatulus</i>	HPTM	Downregulate genes with immunity, ageing and longevity functions ⁵⁹
Apidae	<i>Apis mellifera</i>	ncRNA	Ageing and longevity ⁶⁰
Formicidae	<i>Camponotus floridanus</i>	ncRNA	Development of different phenotypes ⁶⁹⁻⁷¹
Formicidae	<i>Harpegnathos saltator</i>		

414 DNM, DNA methylation; HPTM, histone post-translation modification; ncRNA, non-coding RNA.