



LJMU Research Online

Bebane, PSA, Hunt, BJ, Pegoraro, M, Jones, ARC, Marshall, H, Rosato, E and Mallon, EB

The effects of the neonicotinoid imiacloprid on gene expression and DNA methylation in the buff-tailed bumblebee *Bombus terrestris*

<http://researchonline.ljmu.ac.uk/id/eprint/14373/>

Article

Citation (please note it is advisable to refer to the publisher's version if you intend to cite from this work)

Bebane, PSA, Hunt, BJ, Pegoraro, M, Jones, ARC, Marshall, H, Rosato, E and Mallon, EB (2019) The effects of the neonicotinoid imiacloprid on gene expression and DNA methylation in the buff-tailed bumblebee *Bombus terrestris*. *Proceedings of the Royal Society B: Biological Sciences*. 286

LJMU has developed [LJMU Research Online](#) for users to access the research output of the University more effectively. Copyright © and Moral Rights for the papers on this site are retained by the individual authors and/or other copyright owners. Users may download and/or print one copy of any article(s) in LJMU Research Online to facilitate their private study or for non-commercial research. You may not engage in further distribution of the material or use it for any profit-making activities or any commercial gain.

The version presented here may differ from the published version or from the version of the record. Please see the repository URL above for details on accessing the published version and note that access may require a subscription.

For more information please contact researchonline@ljmu.ac.uk

<http://researchonline.ljmu.ac.uk/>



LJMU Research Online

Bebane, PSA, Hunt, BJ, Pegoraro, M, Jones, ARC, Marshall, H, Rosato, E and Mallon, EB

The effects of the neonicotinoid imiacloprid on gene expression and DNA methylation in the buff-tailed bumblebee *Bombus terrestris*

<http://researchonline.ljmu.ac.uk/id/eprint/14373/>

Article

Citation (please note it is advisable to refer to the publisher's version if you intend to cite from this work)

Bebane, PSA, Hunt, BJ, Pegoraro, M, Jones, ARC, Marshall, H, Rosato, E and Mallon, EB (2019) The effects of the neonicotinoid imiacloprid on gene expression and DNA methylation in the buff-tailed bumblebee *Bombus terrestris*. *Proceedings of the Royal Society B: Biological Sciences*. 286

LJMU has developed **LJMU Research Online** for users to access the research output of the University more effectively. Copyright © and Moral Rights for the papers on this site are retained by the individual authors and/or other copyright owners. Users may download and/or print one copy of any article(s) in LJMU Research Online to facilitate their private study or for non-commercial research. You may not engage in further distribution of the material or use it for any profit-making activities or any commercial gain.

The version presented here may differ from the published version or from the version of the record. Please see the repository URL above for details on accessing the published version and note that access may require a subscription.

For more information please contact researchonline@ljmu.ac.uk

<http://researchonline.ljmu.ac.uk/>

The effects of the neonicotinoid imidacloprid on gene expression and DNA methylation in the buff-tailed bumblebee *Bombus terrestris*

P.S.A BEBANE ^{1*}, B.J. HUNT ^{1*}, M. PEGORARO* ¹, A.R.C JONES ¹, H. MARSHALL ¹, E. ROSATO ¹ & E.B. MALLON ¹⁺

1) Department of Genetics and Genome Biology

University of Leicester

University Road

Leicester, LE1 7RH

Phone: ++ (0)116 252 3488

Fax: +44 (0)116 252 3330

E-mail: ebm3@le.ac.uk

2) School of Natural Sciences and Psychology

John Moores University Liverpool

* joint first authors

+ corresponding author

Keywords: Epigenetics, Methylome, RNA-seq, BS-seq, Social insects, Pesticide

Running title: "The epigenetic effects of neonicotinoids on bumblebees"

Abstract

Neonicotinoids are effective insecticides used on many important arable and horticultural crops. They are nicotinic acetylcholine receptor agonists which disrupt the function of insect neurons and cause paralysis and death. In addition to direct mortality, there are numerous sublethal effects of low doses of neonicotinoids on bees. We hypothesize that some of these large array of effects could be a consequence of epigenetic changes in bees induced by neonicotinoids. We compared whole methylome (BS-seq) and RNA-seq libraries of the brains of buff tailed bumblebee *Bombus terrestris* workers exposed to field realistic doses of the neonicotinoid imidacloprid to libraries from control workers. We found numerous genes which show differential expression between neonicotinoid treated bees and control bees, but no differentially methylated cytosines in any context. We found CpG methylation to be focused mainly in exons and associated with highly expressed genes. We discuss the implications of our results for future legislation.

1 Introduction

2 Neonicotinoids are effective insecticides used on many important arable and horticultural crops, most
3 frequently as seed dressing. They are systemic, meaning they are absorbed by the plant and transported
4 to all tissues where they remain active for many weeks or months. This protects all parts of the plant,
5 but also means that neonicotinoids are found in the nectar and pollen of flowering crops such as oilseed
6 rape, and hence are consumed by bees (Botías *et al.*, 2015). It has also emerged that they are commonly
7 found contaminating nectar and pollen of wild flowers growing on arable farmland, providing additional
8 exposure of bees and other pollinators (Botías *et al.*, 2015; David *et al.*, 2016).

9 Neonicotinoids are nicotinic acetylcholine receptor agonists which disrupt the function of insect neu-
10 rons and cause paralysis and death. In addition to direct mortality, laboratory and field studies have
11 documented numerous sublethal effects of low doses of neonicotinoids on both honeybees and bumblebees
12 (e.g. Whitehorn *et al.* 2012; Rundlöf *et al.* 2015, reviewed in Pisa *et al.* 2015). Sublethal effects at the in-
13 dividual level include reduced fecundity of queens, reduced fertility in males, impaired immune response,
14 impaired navigation and learning, reduced pollen collection and reduced food consumption. Collectively,
15 these effects result in reduced colony growth and colony reproduction performance. The breadth of the
16 effects of neonicotinoids on bees suggests that neonicotinoids have multiple modes of action beyond their
17 designed direct impact on neurotransmission, for example their impact on immune signalling (Prisco
18 *et al.*, 2013).

19 We hypothesize that some of these effects could be a consequence of epigenetic changes induced by
20 neonicotinoids. Epigenetics is defined as the stable and heritable change in gene expression without any
21 change in the DNA sequence (Goldberg *et al.*, 2007). Environmental contaminants have been found to
22 affect the epigenetics of a diverse range of animal species from water fleas to polar bears (Head, 2014)
23 and include metals, endocrine disrupting compounds, air pollution, persistent organic pollutants and
24 pesticides (Vandegheuchte and Janssen, 2014), but much ecotoxicology research is centred on a direct
25 link between exposure and response (Head, 2014). Epigenetic changes have the potential to weaken that
26 link, with effects possibly manifesting much later in life or in subsequent generations. Thus if pesticide-
27 induced epigenetic changes were shown to be heritable in bees this would have implications for future
28 ecological risk assessment.

29 In social insect research the role of DNA methylation, an epigenetic marker primarily involving the
30 addition of a methyl group to a cytosine, has come under increasing scrutiny in recent years (Foret *et al.*,
31 2009; Lyko *et al.*, 2010; Glastad *et al.*, 2013; Amarasinghe *et al.*, 2014; Glastad *et al.*, 2016; Patalano *et al.*,
32 2015; Libbrecht *et al.*, 2016; Standage *et al.*, 2016; Rehan *et al.*, 2016; Glastad *et al.*, 2017; Arsenault *et al.*,
33 2018). Methylation has also been implicated in important effects on the biology of bees, including the

34 control of reproductive status (Kucharski *et al.*, 2008; Amarasinghe *et al.*, 2014) and memory (Biergans
35 *et al.*, 2012), behaviours shown to be affected by neonicotinoids (Williams *et al.*, 2015; Stanley *et al.*,
36 2015), although in the case of reproduction the link between methylation and social insect reproduction
37 is controversial (Herb *et al.*, 2018; Patalano *et al.*, 2015; Libbrecht *et al.*, 2016). DNA methylation has
38 been linked with alternative splicing in a number of insect species (Lyko *et al.*, 2010; Li-Byarlay *et al.*,
39 2013; Glastad *et al.*, 2016; Arsenault *et al.*, 2018), and with histone modifications in the ant *Camponotus*
40 *floridanus* (Glastad *et al.*, 2015). In mammals, methylation on gene promoters leads to a reduction in
41 gene expression. The effect of methylation on gene expression in insects is less well understood (Pegoraro
42 *et al.*, 2017), though high levels of methylation have been associated with highly and stably expressed
43 genes (Foret *et al.*, 2012; Bonasio *et al.*, 2012; Wang *et al.*, 2013), while in honeybees hypomethylated
44 genes are associated with caste-specific expression (Elango *et al.*, 2009; Libbrecht *et al.*, 2016; Marshall
45 *et al.*, 2019). Gene expression differences due to neonicotinoid exposure have been found in honeybee
46 larval workers, adult workers and queens (Derecka *et al.*, 2013; Aufauvre *et al.*, 2014; Christen *et al.*,
47 2016; Chaimanee *et al.*, 2016; Christen *et al.*, 2018).

48 In this study we use whole genome bisulfite sequencing (WGBS/BS-seq) and RNA-seq on brain tissue
49 of neonicotinoid exposed and control *Bombus terrestris* workers in order to elucidate the effects of the
50 neonicotinoid imidacloprid on the gene expression and methylation status of bumblebee workers.

51 **Materials and Methods**

52 *Beekeeping, experimental design and brain dissection*

53 Six colonies of *Bombus terrestris audax* were purchased from Agralan, UK. Each colony contained a
54 queen and on average ten workers and a small amount of brood. They were kept in wooden nest boxes
55 and maintained under red light at 26°C and 60% humidity on a diet of 50% v/v glucose/fructose apiary
56 solution (Meliose-Roquette, France) and pollen (Percie du set, France) (Amarasinghe *et al.*, 2014). Three
57 colonies were used for the RNA-seq experiment and the other three for the BS-seq experiment (Figure
58 S1).

59 Groups of 5 callow workers born on the same day were reared in Perspex boxes (18.5 cm x 12.5cm
60 x 6.5cm). Boxes were then randomly assign to control or treated groups. The control group was fed
61 *ad libitum* with 50% v/v apiary solution for six days whereas the treated group was fed *ad libitum*
62 with a 10ppb imidacloprid (SIGMA-ALDRICH) 50% v/v apiary solution, a field-realistic sub-lethal dose
63 (Cresswell, 2011; Blacquièrè *et al.*, 2012). After a six day chronic exposure period (Cresswell, 2011) the
64 bees were anesthetized on ice at 4°C. The brains were dissected in phosphate buffered saline (PBS) and
65 immediately frozen in liquid nitrogen and stored at -80°C. Their ovaries were checked for development to
66 ensure that only non-reproductive workers were used (Amarasinghe *et al.*, 2014; Harrison *et al.*, 2015).

67 *BS-seq*

68 **Genomic DNA extraction, sequencing and mapping**

69 Six libraries were prepared (3 colonies, control and treatment). For each colony, 10 boxes were reared
70 (5 control and 5 treatment). Each library was generated from 12 pooled brains of non-reproductive
71 workers taken at random from the relevant boxes for a total of 72 brains. Genomic DNA was extracted,
72 using QIAGEN QIAamp DNA Micro Kit following the manufacturer’s instructions. The concentration of
73 genomic DNA was measured using a Qubit® dsDNA BR Assay Kit (ThermoFisher Scientific, USA) and
74 Nanodrop. Sequencing was performed on a HiSeq 2000 machine (Illumina, Inc.) at the Beijing Genomics
75 Institute (BGI), generating 100-bp paired-end reads.

76 Poor quality reads were removed using fastQC v0.11.2 (Andrews, 2010) and adapters trimmed us-
77 ing cutadapt V1.11 (Martin, 2011) and trimmomatic V0.36 (Bolger *et al.*, 2014). Bismark v0.18.1
78 (Krueger and Andrews, 2011) was used to align the reads to the Bter_1.0 genome (Refseq accession
79 no. GCF_000214255.1 (Sadd *et al.*, 2015)), remove PCR artifacts and extract methylation calls in CpG,
80 CHH and CHG contexts (where H represents adenine, thymine or cytosine). The cytosine report files
81 from Bismark and the *B. terrestris* annotation file (GCF_000214255.1) were combined using the sqldf

82 library (Grothendieck, 2017) in R v3.4.0 (R Core Team, 2014) to generate the distribution of methylated
83 Cs over genomic features. Cytosines with less than 10X coverage were excluded. For each cytosine the 84
proportion of methylation reads over total reads was calculated.

85 **Methylation differences between treatments**

86 Differential methylation analysis was performed using methylKit (Akalin *et al.*, 2012). Bismark cytosine 87
reports were filtered to exclude loci with extreme low or high coverage (< 10 or > 500 reads) and those 88 not
covered in all samples. A mixture of binomial model (Cheng and Zhu, 2014) was used to make per-
89 loci methylation status calls and only loci identified as methylated in at least one sample were tested. A 90
logistic regression test was applied using overdispersion correction, controlling for colony as a covariate, 91
and adjusting p-values for multiple testing using the SLIM method. A minimum change in methylation
92 between treatments of 10% was used to filter results.

93 *RNA-seq*

94 **RNA extraction and Illumina sequencing**

95 Eighteen libraries were prepared (three colonies, three replicates per colony, two conditions). For each
96 colony, 6 boxes were reared (3 control and 3 treatment). Each library was generated from 3 pooled
97 brains of non-reproductive workers taken from the relevant boxes, for a total of 54 brains. Total RNA
98 was isolated utilizing the GenElute Mammalian Total RNA Miniprep Kit. DNA and RNAase activity
99 was eliminated using (Sigma-Aldrich DNase I treatment kit) following the manufacturer's instruction.
100 RNA concentration and integrity were determined by Bioanalyzer using the RNA Nano Kit (Agilent
101 Technologies). From each sample we isolated an average of 0.8 mg of RNA. Two samples appeared
102 degraded and were not used. Nine control and seven treated samples were prepared and sequenced
103 on HiSeq 200 (Illumina, Inc.) at Beijing Genomics Institute (BGI) and 100-bp paired-end reads were
104 generated.

105 BGI removed adaptor sequences, contamination and low-quality reads from raw data. Base calling
106 and quality scoring of the raw reads were visualized using fastQC v 0.11.2 (Andrews, 2010). The clean
107 reads for each sample were aligned to the reference genome Bter_1.0 genome (Refseq accession no.
108 GCF_000214255.1 (Sadd *et al.*, 2015)) using Hisat2 v2.0.4 (Kim *et al.*, 2015) with default parameters.
109 The output sam file was sorted and converted to a bam file using samtools (Li *et al.*, 2009). Aligned
110 reads were assembled and quantified using the assembler stringtie v1.3.3b (Pertea *et al.*, 2015).

111 **Differential gene expression analysis**

112 A table of raw counts was generated using a Python script
113 (<https://github.com/gpertea/stringtie/blob/master/prepDE>) and analysed using DESeq2 (Love

114 *et al.*, 2014) in R v3.4.0 (R Core Team, 2014) to estimate differentially expressed genes using an
115 FDR-adjusted p-value threshold of 0.05 and controlling for colony effects. Genes with less than 10
116 reads were discarded from analysis. The normalized read counts were \log_2 transformed. The quality
117 of replicates was assessed by plotting read counts of samples against one another and assessing the
118 dispersion and presence of any artefacts between samples (Rich *et al.*, 2018). A principal-component
119 analysis was performed to visualize diversity between samples within treatment and between condition.

120 *GO term enrichment and KEGG analysis*

121 A list of GO terms for the bumblebee were made by annotating the transcriptome using trinotate (default
122 settings) (Hébert *et al.*, 2016) and blast2GO (against RefSeq) (Conesa *et al.*, 2005). These lists were
123 combined, using the pipeline implemented in Amar *et al.* 2014 with a K value of 1. A hypergeometric test
124 was applied and significant GO terms identified after BH correction ($p_{\text{corrected}} < 0.05$) (Benjamini and
125 Hochberg, 1995) using GOstats (Falcon and Gentleman, 2007), with all RNA features in the bumblebee
126 genome used as a background (GCF_000214255.1). We filtered these to only those terms present in
127 three or more DEGs and used REVIGO (Supek *et al.*, 2011) to cluster and visualise enriched GO terms,
128 selecting the whole UniProt database and SimRel semantic similarity measure.

129 The clusterprofiler R package (version 3.8.1) (Yu *et al.*, 2012) identified differentially expressed genes
130 associated with KEGG pathways using the whole UniProt database. A hypergeometric test was applied
131 and significant KEGG pathways were identified after BH correction ($q_{\text{value}} < 0.05$) (Benjamini and
132 Hochberg, 1995).

133 Results

134 *Methylation analysis*

135 The overall sequence alignment rate was 67.21% ± 1.53% (mean ± standard deviation). The proportion of
136 methylated cytosine reads calculated by Bismark were 0.53% ± 0.05% for CpGs, 0.37% ± 0.05% for CHGs,
137 0.38% ± 0.07% for CHHs and 0.4% ± 0.06% for CNs or CHNs ((H = A, C, or T). While insect methylation
138 levels are often low (Glastad *et al.*, 2017) these methylation levels are lower even than in the honey bee,
139 *Apis mellifera*, estimated at ~1% at the genome level using similar metrics (Feng *et al.*, 2010; Bewick
140 *et al.*, 2017). In a CpG context, across all samples, 0.15% ± 0.03 % of loci with a minimum coverage
141 of 10 reads were considered methylated by the mixture of binomial model. The distribution of CpG
142 methylation shows a mild bimodal distribution with the vast majority of sites being not or only modestly
143 methylated and a few fully methylated (Figure S2 A). Methylated CpGs are more abundant in coding
144 regions (seven fold) and exons (five fold) than introns (Figure 1 A). Non-CpG per-loci methylation levels
145 were reported as less than 0.001% by the mixture of binomial model. This, in conjunction with the
146 uniformity of non-CpG methylation across genomic features (Figure 1 B,C), led to the conclusion that
147 such levels were indistinguishable from error and as such were excluded from subsequent analysis.

148 **Methylation differences between control and neonicotinoid treated samples**

149 In total 4,424,986 loci were analysed using the mixture of binomial model, which subsequently identified
150 6,080 sites to test. No differentially methylated loci were identified using logistic regression at a q-value of
151 0.05 or 0.1. MethylKit includes an option to pool replicates into single control/treatment samples and use
152 Fisher's exact test; using this approach we identified a small number of differentially methylated CpGs
153 at q-value < 0.1, including loci within *histone-lysine N-methyltransferase 2C*, *histone acetyltransferase*
154 *p300*, *CXXC1* (a transcriptional activator that binds to unmethylated CpGs), and genes involved with
155 axon formation (supplementary data, diff_meth_fisher).

156 *Expression analysis*

157 Alignment rate to the genome was 93.6% (92.1 to 94.1) and after filtering a total of 10,772 genes were
158 analysed. All libraries from the same treatment showed low variation in their gene expression patterns
159 (Figure S3, S4).

160 **Differential expression**

161 A total of 405 genes were differentially expressed: 192 genes upregulated and 213 downregulated in
162 neonicotinoid samples compared to controls (see supplementary data: differentially_expressed_genes).

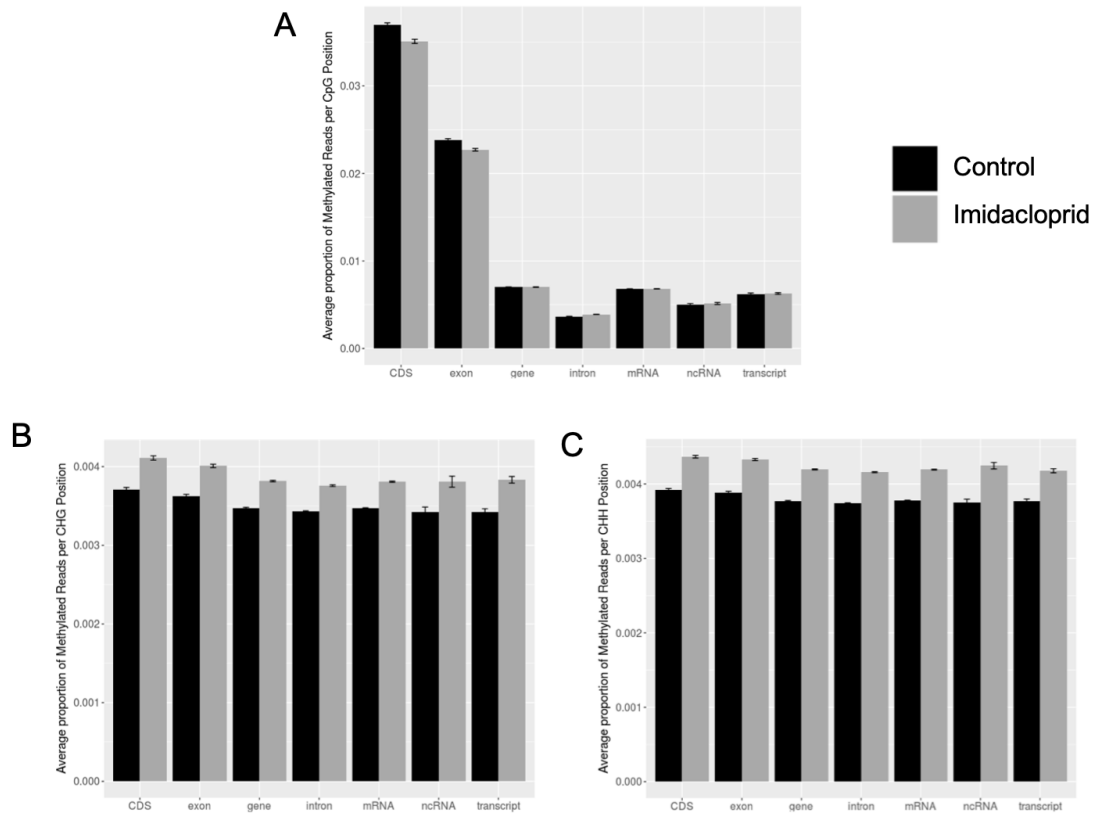


Figure 1: Methylated Cs distribution. Average proportion of methylation reads SD per CpG (A), CHG (B) and CHH (C) positions over genomic features. Control samples in black and Neo treated samples in grey.

163 Four cytochrome P450 (CYP) genes were differentially expressed, two upregulated and two downreg-
 164 ulated. Upregulated genes in neonicotinoid treated bees also include *apyrase* that hydrolyzes ATP to
 165 AMP, the neuropeptide receptor *pyrokinin-1 receptor* and *ionotropic receptor 25a* that is involved in
 166 circadian clock resetting in *Drosophila* (Chen *et al.*, 2015). Downregulated genes include *neurexin*, in-
 167 volved in synaptic formation and maintenance, *peptide methionine sulfoxide reductase*, involved in repair
 168 of oxidation-damaged proteins, and a number of genes related to photoreceptor function. Three genes
 169 belonging to the homeotic box gene (Hox) family were downregulated in neonicotinoid treated bees.
 170 *lethal(2)essential for life (Efl21)* displayed the highest down regulation. We found 105 enriched biologi-
 171 cal process GO terms (BH corrected $p < 0.05$) associated with differential gene expression (supplementary
 172 data: expression_GO), subsequently clustered using REVIGO to 58 terms (Figure S5). Many of the most
 173 significantly enriched terms were associated with energy reserve metabolism. Also enriched were terms
 174 associated with apoptotic processes, apoptotic cell clearance, immune effector processes, cell death and
 175 response to chemical stimulus. No KEGG pathways were over represented for differentially expressed
 176 genes ($q < 0.05$).

177 *DNA methylation - Expression correlation*

178 We calculated the average percentage of methylated reads per gene for the most differentially expressed
179 genes (\log_2 fold-change > 0.5 or < -0.5) and non-differentially expressed genes (Figure 2), fitting a
180 generalized linear model (GLM) with a quasi binomial error distribution with treatment (control vs
181 neonicotinoid) and expression state (DEG vs. non-DEG) as independent variables. There was no signif-
182 icant interactions between the independent variables (interaction model versus main effects only model:
183 $\chi^2 = -0.014$, d.f. = 1, $p = 0.82$). For CpGs, non-differentially expressed genes had more methylation
184 than differentially expressed genes ($z_{1,19673}=4.641$, $p<0.001$). There was no significant treatment effect
185 on methylation levels ($z_{1,19673}=-0.772$, $p=0.692$).

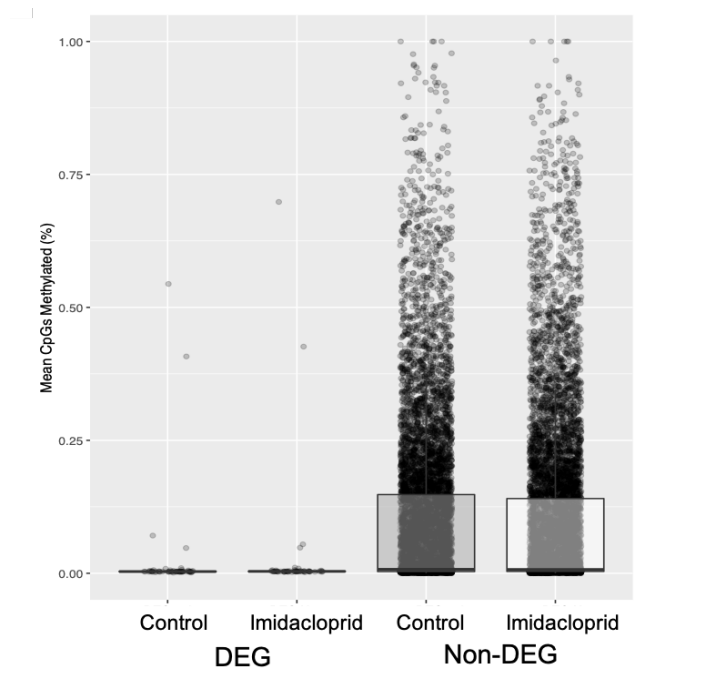


Figure 2: Average percentage of methylated CpG per gene. Differentially expressed genes (DEG) and non differentially expressed genes (nonDEG) are plotted separately. Dots represent genes.

186 To have a more fine scale understanding of the correlation between methylation and expression, we
187 plotted mean proportion of methylation per gene against ranked expression level (\log_{10} fpkm per gene)
188 in 100 bins (from low to high) (Figure 3) fitting a linear model with treatment and expression level as
189 independent variables. There was no significant interaction between expression's and treatment's effects
190 on methylation (interaction model versus main effects only model: $F_{1,189} = 1.0347$, $p = 0.3104$). We
191 found a significant association between expression and methylation ($F_{1,189} = 281.654$, $p = < 2 \times 10^{-16}$).
192 Neonicotinoid treated bees had comparable levels of CpG methylation to control bees ($F_{1,189} = 1.8125$,
193 $p = 0.1798$).

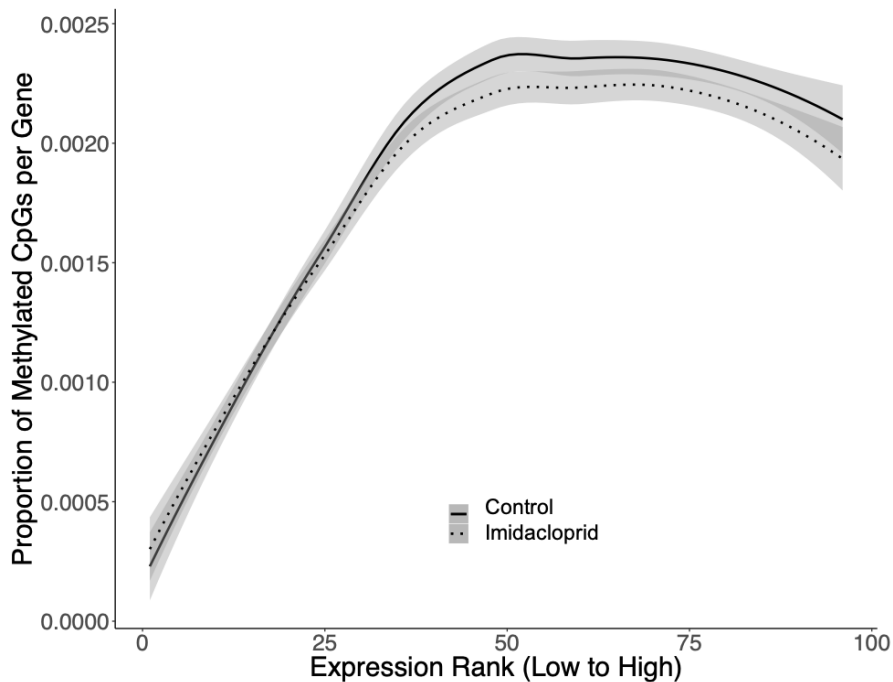


Figure 3: The proportion of methylated CpGs is plotted against gene expression rank. One hundred "bins" of progressively increasing level of expression were generated and genes with similar level of expression have been grouped in the same bin. Solid lines represent control samples and dotted lines neonicotinoid treated samples. The grey shading represents 95% confidence intervals.

194 Discussion

195 We found numerous genes which show differential expression between bees treated with field realistic doses
 196 of the neonicotinoid imidacloprid and control bees. We found CpG methylation to be focused in exons,
 197 and high CpG methylation was associated with highly expressed genes, but no differentially methylated
 198 loci were detected between treatments. Non-differentially expressed genes had higher methylation levels
 199 than differentially expressed genes.

200 Four cytochrome P450 (CYP) genes were identified as differentially expressed, in line with other stud-
 201 ies assessing the impact of insecticides on honeybees (Shi *et al.*, 2017; Li *et al.*, 2017; Derecka *et al.*, 2013;
 202 Wu *et al.*, 2017; Christen *et al.*, 2018). Two were upregulated (CYP6k1 and 4c3) and two downregulated
 203 (28d1 and 9e2). CYP6, 9 and 28 genes are linked to xenobiotic metabolism and resistance to insecticides
 204 (Feyereisen, 2006) and CYP6 genes specifically have been found to be upregulated in honeybees after
 205 treatment with sublethal doses of the neonicotinoid Thiamethoxam (Shi *et al.*, 2017), as has CYP4C1
 206 after treatment with the neonicotinoid Clothianidin (Christen *et al.*, 2018). The CYP9Q subfamily were
 207 recently shown to be responsible for bee sensitivity to neonicotinoids (Manjon *et al.*, 2018).

208 The identification of differentially expressed genes associated with synaptic transmission (supplemen-
 209 tary data: expression_GO) is to be expected, given that we used brain tissue and given the known target

210 effects of neonicotinoids. The identification of a downregulated *neurexin* gene aligns with the results of
211 Shi *et al.* (2017). The effect seen here on metabolic pathways has also been found in honeybees, with GO
212 term enrichment for catabolic carbohydrate and lipid metabolism (Christen *et al.*, 2018). These authors
213 suggested that due to the intensive energy demands of the brain, negative effects on metabolic pathways
214 could affect brain function and therefore behaviour. During the review period a further study was pub-
215 lished examining gene expression changes in *B. terrestris* after exposure to neonicotinoids, again showing
216 changes in carbohydrate and lipid metabolism (Colgan *et al.*, 2019). *Eft21*, the most downregulated
217 gene identified, has been found to be involved in foraging behaviour in bees (Hernández *et al.*, 2012), a
218 potential genetic link to the findings of Mommaerts *et al.* (2009). Impaired foraging has implications for
219 pollination, reproduction and overall colony survival. Downregulation of carbohydrate metabolism path-
220 ways has also been shown in honeybee larvae (Derecka *et al.*, 2013; Wu *et al.*, 2017). Also downregulated
221 were three hox genes. This may be indicative of an impaired immune system, as hox genes have been
222 found to play a role in invertebrate innate immune responses (Uvell and Engström, 2007; Irazoqui *et al.*,
223 2008). Hox genes have been found to be downregulated in response to insecticide treatment in honeybees
224 (Aufauvre *et al.*, 2014). The bumblebee visual system may also be impacted by imidacloprid treatment,
225 given the downregulation of genes such as protein scarlet, protein glass and ninaC.

226 No differentially methylated loci between control and treatment were identified using a logistic re-
227 gression model, and we suggest that if acute neonicotinoid exposure does alter methylation status in *B.*
228 *terrestris* it is subtle and the data reported here may be underpowered to detect it due to low per-sample
229 coverage. A small number of differentially methylated loci were identified by pooling replicates and using
230 Fisher’s exact test (supplementary data: diff_meth_fisher), but unlike logistic regression this approach
231 cannot control for covariates and the results should be treated with caution. Using this approach a CpG
232 loci in *CXXC-type zinc finger protein 1* was identified as hypermethylated in neonicotinoid-treated bees;
233 this gene also was upregulated in that group. In mammals, CXXC1 is a transcriptional activator that
234 binds to unmethylated CpGs to regulate gene expression (Shin Voo *et al.*, 2000). Other loci identified
235 by pooling were located within *histone acetyltransferase p300* and *histone-lysine N-methyltransferase 2C*.
236 These findings raise the possibility that neonicotinoids may have a more detectable effect over a longer
237 period through a cascade of epigenetic processes. A study on the effects of imidacloprid on bumblebees
238 found no effect on mortality or reproduction over 11 weeks using 10 ppb when workers were not required
239 to forage for food, while 20 ppb affected mortality and foraging was impaired at both doses (Mommaerts
240 *et al.*, 2009). It may therefore be that a higher dose or longer exposure time might have a detectable
241 impact on CpG methylation, and further work investigating chronic rather than acute exposure to im-
242 idacloprid at different doses would be valuable. Also worthy of investigation is the potential effect on
243 epigenetic processes other than DNA methylation, such as histone modification, which has been found

244 to have a similar, but non-redundant, association with gene expression in the ant *Camponotus floridanus*
245 (Glastad *et al.*, 2015).

246 We found patterns of CpG methylation to be in line with other insect species. It is mainly focused
247 in exons (Glastad *et al.*, 2017), and high CpG methylation was associated with highly expressed genes
248 (Figure 3) (Arsenault *et al.*, 2018; Bonasio *et al.*, 2012; Glastad *et al.*, 2013; Libbrecht *et al.*, 2016;
249 Patalano *et al.*, 2015; Wang *et al.*, 2013), and non-differentially expressed genes showed higher levels of
250 methylation (Glastad *et al.*, 2013, 2016; Libbrecht *et al.*, 2016; Sarda *et al.*, 2012). As well as inducing no
251 changes in methylation at individual loci, neonicotinoids appear to have no effect on overall levels of CpG
252 methylation (see Figures 2 and 3). This failure to identify methylation differences between experimental
253 groups is consistent with findings of robust methylation between castes in various insects (Hunt *et al.*,
254 2010) but contrasts with studies finding differences resulting from removal of maternal care (Arsenault
255 *et al.*, 2018), or within castes with differing reproductive status (Marshall *et al.*, 2019).

256 Non-CpG methylation plays a role in gene silencing in flowering plants (Stroud *et al.*, 2014) and to
257 a lesser extent, in mammals (Dyachenko *et al.*, 2010). In this study, while we identified a very small
258 number of loci showing methylation in CHG/CHH contexts we could not exclude the possibility that
259 much of it was noise, as bisulfite sequencing is prone to false positives from sources such as incomplete
260 bisulfite conversion, miscalled bases and SNPs. Overall, we conclude that there is no notable methylation
261 of non-CpG cytosines in *B. terrestris*, as with the honeybee (Lyko *et al.*, 2010) and *Nasonia vitripennis*
262 (Wang *et al.*, 2013). In contrast to the preponderance of CpG methylation in exons, we found that
263 CHH and CHG methylation was uniformly spread throughout genes (Figure 1) a pattern which would
264 be consistent with the idea that there is no significant methylation in these contexts.

265 Recently, it has become clear that epigenetics can play a role in the interplay between man-made
266 chemicals and natural ecosystems, and their constituent species (Vandegheuchte and Janssen, 2014).
267 Hymenopteran insects (ants, bees and wasps) are ideal models to study this. They are both strongly
268 affected by man-made chemicals and are important emerging models for epigenetics, with a number of
269 species with relatively small genomes showing a confirmed role for methylation in their biology (Glastad
270 *et al.*, 2011; Weiner and Toth, 2012; Welch and Lister, 2014; Yan *et al.*, 2014).

271 However, on the evidence of this study, imidacloprid does not appear to have epigenetic effects, at
272 least through DNA methylation. This finding is important in the context of future legislation for pesticide
273 control, as it is evidence suggesting a potential lack of transgenerational effects on *B. terrestris* with the
274 use of imidacloprid.

275 **Acknowledgements**

276 Thanks to Dr. Swidbert Ott and Prof. Dave Goulson for discussions. EBM, BJH and MP were funded
277 by NERC grant NE/N010019/1. PSAB was supported by a scholarship from the Human Capacity De-
278 velopment Program (Koya University - Iraq). HM was supported by a NERC CENTA DTP studentship.
279 ARCJ was supported by a BBSRC MIBTP DTP studentship. This research used the ALICE High
280 Performance Computing Facility at the University of Leicester.

281 **Data accessibility**

282 All sequencing data related to this project can be found under NCBI BioProject PRJNA524132.

283 **Authors' contributions**

284 EBM, ER and PSAB designed the study. PSAB carried out the experiments. PSAB, BJH, MP, ARCJ
285 and HM analysed the data. MP, PSAB and EBM wrote the initial draft. All authors were involved in
286 redrafting.

287 **Supplementary material**

288 Supplementary figures are available in the supplementary figures file. Supplementary data is available at
289 <https://doi.org/10.6084/m9.figshare.6796802>.

290 **Figure legends**

291 Figure 1: Methylated Cs distribution. Average proportion of methylation reads SD per CpG (**A**), CHG
292 (**B**) and CHH (**C**) positions over genomic features. Control samples in black and Neo treated samples in
293 grey.

294 Figure 2: Average percentage of methylated CpG per gene. Differentially expressed genes (DEG) and
295 non differentially expressed genes (nonDEG) are plotted separately. Dots represent genes.

296 Figure 3: The proportion of methylated CpGs is plotted against gene expression rank. One hundred
297 "bins" of progressively increasing level of expression were generated and genes with similar level of
298 expression have been grouped in the same bin. Solid lines represent control samples and dotted lines
299 neonicotinoid treated samples. The grey shading represents 95% confidence intervals.

References

- 300
301 Akalin, A., Kormaksson, M., Li, S., Garrett-Bakelman, F. E., Figueroa, M. E., Melnick, A., and Mason,
302 C. E. 2012. methylKit: a comprehensive R package for the analysis of genome-wide DNA methylation
303 profiles. *Genome Biology*, 13: R87.
- 304 Amar, D., Frades, I., Danek, A., Goldberg, T., Sharma, S. K., Hedley, P. E., Proux-Wera, E., Andreasson,
305 E., Shamir, R., Tzfadia, O., and Alexandersson, E. 2014. Evaluation and integration of functional
306 annotation pipelines for newly sequenced organisms: the potato genome as a test case. *BMC Plant*
307 *Biology*, 14(1): 329.
- 308 Amarasinghe, H. E., Clayton, C. I., and Mallon, E. B. 2014. Methylation and worker reproduction in the
309 bumble-bee (*Bombus terrestris*). *Proceedings of the Royal Society B: Biological Sciences*, 281(1780):
310 20132502.
- 311 Andrews, S. 2010. FastQC A Quality Control tool for High Throughput Sequence Data.
- 312 Arsenault, S. V., Hunt, B. G., and Rehan, S. M. 2018. The effect of maternal care on gene expression
313 and DNA methylation in a subsocial bee. *Nature Communications*, 9(1).
- 314 Aufaivre, J., Misme-Aucouturier, B., Viguès, B., Texier, C., Delbac, F., and Blot, N. 2014. Transcriptome
315 Analyses of the Honeybee Response to *Nosema ceranae* and Insecticides. *PLOS ONE*, 9(3): e91686.
- 316 Benjamini, Y. and Hochberg, Y. 1995. Controlling the False Discovery Rate: A Practical and Powerful
317 Approach to Multiple Testing. *Journal of the Royal Statistical Society. Series B (Methodological)*,
318 57(1): 289–300.
- 319 Bewick, A. J., Vogel, K. J., Moore, A. J., and Schmitz, R. J. 2017. Evolution of DNA Methylation across
320 Insects. *Molecular Biology and Evolution*, 34(3): 654–665.
- 321 Biergans, S. D., Jones, J. C., Treiber, N., Galizia, C. G., and Szyszka, P. 2012. DNA Methylation Mediates
322 the Discriminatory Power of Associative Long-Term Memory in Honeybees. *PLoS ONE*, 7(6): e39349.
- 323 Blacquièrre, T., Smagghe, G., van Gestel, C. A. M., and Mommaerts, V. 2012. Neonicotinoids in bees: a
324 review on concentrations, side-effects and risk assessment. *Ecotoxicology*, 21(4): 973–992.
- 325 Bolger, A. M., Lohse, M., and Usadel, B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence
326 data. *Bioinformatics*, 30(15): 2114–2120.
- 327 Bonasio, R., Li, Q., Lian, J., Mutti, N. S., Jin, L., Zhao, H., Zhang, P., Wen, P., Xiang, H., Ding, Y.,
328 Jin, Z., Shen, S. S., Wang, Z., Wang, W., Wang, J., Berger, S. L., Liebig, J., Zhang, G., and Reinberg,
329 D. 2012. Genome-wide and Caste-Specific DNA Methylomes of the Ants *Camponotus floridanus* and
330 *Harpegnathos saltator*. *Current Biology*, 22(19): 1755–1764.
- 331 Botías, C., David, A., Horwood, J., Abdul-Sada, A., Nicholls, E., Hill, E., and Goulson, D. 2015. Neon-
332 icotinoid Residues in Wildflowers, a Potential Route of Chronic Exposure for Bees. *Environmental*
333 *Science & Technology*, 49(21): 12731–12740.
- 334 Chaimanee, V., Evans, J. D., Chen, Y., Jackson, C., and Pettis, J. S. 2016. Sperm viability and gene
335 expression in honey bee queens (*Apis mellifera*) following exposure to the neonicotinoid insecticide
336 imidacloprid and the organophosphate acaricide coumaphos. *Journal of Insect Physiology*, 89: 1–8.
- 337 Chen, C., Buhl, E., Xu, M., Croset, V., Rees, J. S., Lilley, K. S., Benton, R., Hodge, J. J. L., and
338 Stanewsky, R. 2015. *Drosophila* Ionotropic Receptor 25a mediates circadian clock resetting by tem-
339 perature. *Nature*, 527(7579): 516–520.
- 340 Cheng, L. and Zhu, Y. 2014. A classification approach for DNA methylation profiling with bisulfite
341 next-generation sequencing data. *Bioinformatics (Oxford, England)*, 30(2): 172–179.
- 342 Christen, V., Mittner, F., and Fent, K. 2016. Molecular Effects of Neonicotinoids in Honey Bees (*Apis*
343 *mellifera*). *Environmental Science & Technology*, 50(7): 4071–4081.

- 344 Christen, V., Schirrmann, M., Frey, J. E., and Fent, K. 2018. Global Transcriptomic Effects of En-
345 vironmentally Relevant Concentrations of the Neonicotinoids Clothianidin, Imidacloprid, and Thi-
346 amethoxam in the Brain of Honey Bees (*Apis mellifera*). *Environmental Science & Technology*,
347 52(13): 7534–7544.
- 348 Colgan, T. J., Fletcher, I. K., Arce, A. N., Gill, R. J., Rodrigues, A. R., Stolle, E., Chittka, L., and Wurm,
349 Y. 2019. Caste- and pesticide-specific effects of neonicotinoid pesticide exposure on gene expression in
350 bumblebees. *Molecular Ecology*, 28(8): 1964–1974.
- 351 Conesa, A., Götze, S., García-Gómez, J. M., Terol, J., Talón, M., and Robles, M. 2005. Blast2go: A uni-
352 versal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics*,
353 21(18): 3674–3676.
- 354 Cresswell, J. E. 2011. A meta-analysis of experiments testing the effects of a neonicotinoid insecticide
355 (imidacloprid) on honey bees. *Ecotoxicology*, 20(1): 149–157.
- 356 David, A., Botías, C., Abdul-Sada, A., Nicholls, E., Rotheray, E. L., Hill, E. M., and Goulson, D.
357 2016. Widespread contamination of wildflower and bee-collected pollen with complex mixtures of
358 neonicotinoids and fungicides commonly applied to crops. *Environment International*, 88: 169–178.
- 359 Derecka, K., Blythe, M. J., Malla, S., Genereux, D. P., Guffanti, A., Pavan, P., Moles, A., Snart, C.,
360 Ryder, T., Ortori, C. A., Barrett, D. A., Schuster, E., and Stöger, R. 2013. Transient Exposure to Low
361 Levels of Insecticide Affects Metabolic Networks of Honeybee Larvae. *PLOS ONE*, 8(7): e68191.
- 362 Dyachenko, O. V., Schevchuk, T. V., Kretzner, L., Buryanov, Y. I., and Smith, S. S. 2010. Human
363 non-CG methylation. *Epigenetics*, 5(7): 569–572.
- 364 Elango, N., Hunt, B. G., Goodisman, M. A. D., and Yi, S. V. 2009. DNA methylation is widespread and
365 associated with differential gene expression in castes of the honeybee, *Apis mellifera*. *Proceedings of*
366 *the National Academy of Sciences*, 106(27): 11206–11211.
- 367 Falcon, S. and Gentleman, R. 2007. Using GStats to test gene lists for GO term association. *Bioinform-*
368 *atics*, 23(2): 257–258.
- 369 Feng, S., Cokus, S. J., Zhang, X., Chen, P.-Y., Bostick, M., Goll, M. G., Hetzel, J., Jain, J., Strauss,
370 S. H., Halpern, M. E., Ukomadu, C., Sadler, K. C., Pradhan, S., Pellegrini, M., and Jacobsen, S. E.
371 2010. Conservation and divergence of methylation patterning in plants and animals. *Proceedings of the*
372 *National Academy of Sciences*, 107(19): 8689–8694.
- 373 Feyereisen, R. 2006. Evolution of insect P450. *Biochemical Society Transactions*, 34(6): 1252–1255.
- 374 Foret, S., Kucharski, R., Pittelkow, Y., Lockett, G. A., and Maleszka, R. 2009. Epigenetic regulation of
375 the honey bee transcriptome: unravelling the nature of methylated genes. *BMC Genomics*, 10(1): 472.
- 376 Foret, S., Kucharski, R., Pellegrini, M., Feng, S., Jacobsen, S. E., Robinson, G. E., and Maleszka, R.
377 2012. DNA methylation dynamics, metabolic fluxes, gene splicing, and alternative phenotypes in honey
378 bees. *Proceedings of the National Academy of Sciences*, 109(13): 4968–4973.
- 379 Glastad, K. M., Hunt, B. G., Yi, S. V., and Goodisman, M. a. D. 2011. DNA methylation in insects: on
380 the brink of the epigenomic era. *Insect Molecular Biology*, 20(5): 553–565.
- 381 Glastad, K. M., Hunt, B. G., and Goodisman, M. a. D. 2013. Evidence of a conserved functional role for
382 DNA methylation in termites. *Insect Molecular Biology*, 22(2): 143–154.
- 383 Glastad, K. M., Hunt, B. G., and Goodisman, M. A. D. 2015. DNA Methylation and Chromatin Organi-
384 zation in Insects: Insights from the Ant *Camponotus floridanus*. *Genome Biology and Evolution*, 7(4):
385 931–942.
- 386 Glastad, K. M., Gokhale, K., Liebig, J., and Goodisman, M. A. D. 2016. The caste- and sex-specific
387 DNA methylome of the termite *Zootermopsis nevadensis*. *Scientific Reports*, 6: 37110.

- 388 Glastad, K. M., Arsenault, S. V., Vertacnik, K. L., Geib, S. M., Kay, S., Danforth, B. N., Rehan, S. M.,
389 Linnen, C. R., Kocher, S. D., and Hunt, B. G. 2017. Variation in DNA Methylation Is Not Consistently
390 Reflected by Sociality in Hymenoptera. *Genome Biology and Evolution*, 9(6): 1687–1698.
- 391 Goldberg, A. D., Allis, C. D., and Bernstein, E. 2007. Epigenetics: A Landscape Takes Shape. *Cell*,
392 128(4): 635–638.
- 393 Grothendieck, G. 2017. *squidf: Manipulate R Data Frames Using SQL*. R package version 0.4-11.
- 394 Harrison, M. C., Hammond, R. L., and Mallon, E. B. 2015. Reproductive workers show queenlike gene
395 expression in an intermediately eusocial insect, the buff-tailed bumble bee *Bombus terrestris*. *Molecular*
396 *Ecology*, 24(12): 3043–3063.
- 397 Head, J. A. 2014. Patterns of DNA Methylation in Animals: An Ecotoxicological Perspective. *Integrative*
398 *and Comparative Biology*, 54(1): 77–86.
- 399 Herb, B. R., Shook, M. S., Fields, C. J., and Robinson, G. E. 2018. Defense against territorial intrusion
400 is associated with DNA methylation changes in the honey bee brain. *BMC Genomics*, 19(1): 216.
- 401 Hernández, L. G., Lu, B., da Cruz, G. C. N., Calábria, L. K., Martins, N. F., Togawa, R., Espindola,
402 F. S., Yates, J. R., Cunha, R. B., and de Sousa, M. V. 2012. Worker Honeybee Brain Proteome.
403 *Journal of Proteome Research*, 11(3): 1485–1493.
- 404 Hunt, B. G., Brisson, J. A., Yi, S. V., and Goodisman, M. A. D. 2010. Functional Conservation of DNA
405 Methylation in the Pea Aphid and the Honeybee. *Genome Biology and Evolution*, 2: 719–728.
- 406 Hébert, F. O., Grambauer, S., Barber, I., Landry, C. R., and Aubin-Horth, N. 2016. Transcriptome
407 sequences spanning key developmental states as a resource for the study of the cestode *Schistocephalus*
408 *solidus*, a threespine stickleback parasite. *GigaScience*, 5(1): 24.
- 409 Irazoqui, J. E., Ng, A., Xavier, R. J., and Ausubel, F. M. 2008. Role for beta-catenin and HOX tran-
410 scription factors in *Caenorhabditis elegans* and mammalian host epithelial-pathogen interactions. *Pro-*
411 *ceedings of the National Academy of Sciences*, 105(45): 17469–17474.
- 412 Kim, D., Langmead, B., and Salzberg, S. L. 2015. HISAT: a fast spliced aligner with low memory
413 requirements. *Nature Methods*, 12(4): 357–360.
- 414 Krueger, F. and Andrews, S. R. 2011. Bismark: a flexible aligner and methylation caller for Bisulfite-Seq
415 applications. *Bioinformatics*, 27(11): 1571–1572.
- 416 Kucharski, R., Maleszka, J., Foret, S., and Maleszka, R. 2008. Nutritional Control of Reproductive Status
417 in Honeybees via DNA Methylation. *Science*, 319(5871): 1827–1830.
- 418 Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., and
419 Durbin, R. 2009. The Sequence Alignment/Map format and SAMtools. *Bioinformatics*, 25(16): 2078–
420 2079.
- 421 Li, Z., Li, M., He, J., Zhao, X., Chaimanee, V., Huang, W.-F., Nie, H., Zhao, Y., and Su, S. 2017.
422 Differential physiological effects of neonicotinoid insecticides on honey bees: A comparison between
423 *Apis mellifera* and *Apis cerana*. *Pesticide Biochemistry and Physiology*, 140: 1–8.
- 424 Li-Byarlay, H., Li, Y., Stroud, H., Feng, S., Newman, T. C., Kaneda, M., Hou, K. K., Worley, K. C.,
425 Elsik, C. G., Wickline, S. A., Jacobsen, S. E., Ma, J., and Robinson, G. E. 2013. RNA interference
426 knockdown of DNA methyl-transferase 3 affects gene alternative splicing in the honey bee. *Proceedings*
427 *of the National Academy of Sciences*, 110(31): 12750–12755.
- 428 Libbrecht, R., Oxley, P. R., Keller, L., and Kronauer, D. J. C. 2016. Robust DNA Methylation in the
429 Clonal Raider Ant Brain. *Current Biology*, 26(3): 391–395.
- 430 Love, M. I., Huber, W., and Anders, S. 2014. Moderated estimation of fold change and dispersion for
431 RNA-seq data with DESeq2. *Genome Biology*, 15: 550.

- 432 Lyko, F., Foret, S., Kucharski, R., Wolf, S., Falckenhayn, C., and Maleszka, R. 2010. The Honey Bee
433 Epigenomes: Differential Methylation of Brain DNA in Queens and Workers. *PLOS Biology*, 8(11):
434 e1000506.
- 435 Manjon, C., Troczka, B. J., Zaworra, M., Beadle, K., Randall, E., Hertlein, G., Singh, K. S., Zimmer,
436 C. T., Homem, R. A., Lueke, B., Reid, R., Kor, L., Kohler, M., Benting, J., Williamson, M. S., Davies,
437 T. G. E., Field, L. M., Bass, C., and Nauen, R. 2018. Unravelling the Molecular Determinants of Bee
438 Sensitivity to Neonicotinoid Insecticides. *Current Biology*, 28(7): 1137–1143.e5.
- 439 Marshall, H., Lonsdale, Z. N., and Mallon, E. B. 2019. Methylation and Gene Expression Differences
440 Between Reproductive Castes of Bumblebee Workers. *bioRxiv*, page 517698.
- 441 Martin, M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMB-
442 net.journal*, 17(1): pp. 10–12.
- 443 Mommaerts, V., Reynders, S., Boulet, J., Besard, L., Sterk, G., and Smagghe, G. 2009. Risk assessment
444 for side-effects of neonicotinoids against bumblebees with and without impairing foraging behavior.
445 *Ecotoxicology*, 19(1): 207.
- 446 Patalano, S., Vlasova, A., Wyatt, C., Ewels, P., Camara, F., Ferreira, P. G., Asher, C. L., Jurkowski,
447 T. P., Segonds-Pichon, A., Bachman, M., González-Navarrete, I., Minoche, A. E., Krueger, F., Lowy,
448 E., Marcet-Houben, M., Rodriguez-Ales, J. L., Nascimento, F. S., Balasubramanian, S., Gabaldon, T.,
449 Tarver, J. E., Andrews, S., Himmelbauer, H., Hughes, W. O. H., Guigó, R., Reik, W., and Sumner, S.
450 2015. Molecular signatures of plastic phenotypes in two eusocial insect species with simple societies.
451 *Proceedings of the National Academy of Sciences*, 112(45): 13970–13975.
- 452 Pegoraro, M., Marshall, H., Lonsdale, Z. N., and Mallon, E. B. 2017. Do social insects support Haig’s
453 kin theory for the evolution of genomic imprinting? *Epigenetics*, 12(9): 725–742.
- 454 Perteau, M., Perteau, G. M., Antonescu, C. M., Chang, T.-C., Mendell, J. T., and Salzberg, S. L. 2015.
455 StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. *Nature biotechnol-
456 ogy*, 33(3): 290–5.
- 457 Pisa, L. W., Amaral-Rogers, V., Belzunces, L. P., Bonmatin, J. M., Downs, C. A., Goulson, D.,
458 Kreuzweiser, D. P., Krupke, C., Liess, M., McField, M., Morrissey, C. A., Noome, D. A., Settele,
459 J., Simon-Delso, N., Stark, J. D., Van der Sluijs, J. P., Van Dyck, H., and Wiemers, M. 2015. Ef-
460 fects of neonicotinoids and fipronil on non-target invertebrates. *Environmental Science and Pollution
461 Research*, 22(1): 68–102.
- 462 Prisco, G. D., Cavaliere, V., Annoscia, D., Varricchio, P., Caprio, E., Nazzi, F., Gargiulo, G., and
463 Pennacchio, F. 2013. Neonicotinoid clothianidin adversely affects insect immunity and promotes repli-
464 cation of a viral pathogen in honey bees. *Proceedings of the National Academy of Sciences*, 110(46):
465 18466–18471.
- 466 R Core Team, . 2014. R: A language and environment for statistical computing. vienna, austria: R
467 foundation for statistical computing; 2014.
- 468 Rehan, S. M., Glastad, K. M., Lawson, S. P., and Hunt, B. G. 2016. The Genome and Methylome of a
469 Subsocial Small Carpenter Bee, *Ceratina calcarata*. *Genome Biology and Evolution*, 8(5): 1401–1410.
- 470 Rich, C., Reitz, M. U., Eichmann, R., Hermann, S., Jenkins, D. J., Kogel, K.-H., Esteban, E., Ott, S.,
471 and Schäfer, P. 2018. Cell type identity determines transcriptomic immune responses in *Arabidopsis
472 thaliana* roots. *bioRxiv*, page 302448.
- 473 Rundlöf, M., Andersson, G. K. S., Bommarco, R., Fries, I., Hederström, V., Herbertsson, L., Jonsson, O.,
474 Klatt, B. K., Pedersen, T. R., Yourstone, J., and Smith, H. G. 2015. Seed coating with a neonicotinoid
475 insecticide negatively affects wild bees. *Nature*, 521(7550): 77–80.
- 476 Sadd, B. M., Barribeau, S. M., Bloch, G., de Graaf, D. C., Dearden, P., Elsik, C. G., Gadau, J.,
477 Grimmelikhuijzen, C. J., Hasselmann, M., Lozier, J. D., Robertson, H. M., Smagghe, G., Stolle, E.,
478 Van Vaerenbergh, M., Waterhouse, R. M., Bornberg-Bauer, E., Klasberg, S., Bennett, A. K., Câmara,

- 479 F., Guigó, R., Hoff, K., Mariotti, M., Munoz-Torres, M., Murphy, T., Santesmasses, D., Amdam,
480 G. V., Beckers, M., Beye, M., Biewer, M., Bitondi, M. M., Blaxter, M. L., Bourke, A. F., Brown, M. J.,
481 Buechel, S. D., Cameron, R., Cappelle, K., Carolan, J. C., Christiaens, O., Ciborowski, K. L., Clarke,
482 D. F., Colgan, T. J., Collins, D. H., Cridge, A. G., Dalmay, T., Dreier, S., du Plessis, L., Duncan, E.,
483 Erler, S., Evans, J., Falcon, T., Flores, K., Freitas, F. C., Fuchikawa, T., Gempe, T., Hartfelder, K.,
484 Hauser, F., Helbing, S., Humann, F. C., Irvine, F., Jermiin, L. S., Johnson, C. E., Johnson, R. M.,
485 Jones, A. K., Kadowaki, T., Kidner, J. H., Koch, V., Köhler, A., Kraus, F. B., Lattorff, H. M. G.,
486 Leask, M., Lockett, G. A., Mallon, E. B., Antonio, D. S. M., Marxer, M., Meeus, I., Moritz, R. F., Nair,
487 A., Näpflin, K., Nissen, I., Niu, J., Nunes, F. M., Oakeshott, J. G., Osborne, A., Otte, M., Pinheiro,
488 D. G., Rossié, N., Rueppell, O., Santos, C. G., Schmid-Hempel, R., Schmitt, B. D., Schulte, C., Simões,
489 Z. L., Soares, M. P., Swevers, L., Winnebeck, E. C., Wolschin, F., Yu, N., Zdobnov, E. M., Aqrawi,
490 P. K., Blankenburg, K. P., Coyle, M., Francisco, L., Hernandez, A. G., Holder, M., Hudson, M. E.,
491 Jackson, L., Jayaseelan, J., Joshi, V., Kovar, C., Lee, S. L., Mata, R., Mathew, T., Newsham, I. F.,
492 Ngo, R., Okwuonu, G., Pham, C., Pu, L.-L., Saada, N., Santibanez, J., Simmons, D., Thornton, R.,
493 Venkat, A., Walden, K. K., Wu, Y.-Q., Debyser, G., Devreese, B., Asher, C., Blommaert, J., Chipman,
494 A. D., Chittka, L., Fouks, B., Liu, J., O'Neill, M. P., Sumner, S., Puiu, D., Qu, J., Salzberg, S. L.,
495 Scherer, S. E., Muzny, D. M., Richards, S., Robinson, G. E., Gibbs, R. A., Schmid-Hempel, P., and
496 Worley, K. C. 2015. The genomes of two key bumblebee species with primitive eusocial organization.
497 *Genome Biology*, 16(1): 76.
- 498 Sarda, S., Zeng, J., Hunt, B. G., and Yi, S. V. 2012. The Evolution of Invertebrate Gene Body Methy-
499 lation. *Molecular Biology and Evolution*, 29(8): 1907–1916.
- 500 Shi, T.-F., Wang, Y.-F., Liu, F., Qi, L., and Yu, L.-S. 2017. Sublethal Effects of the Neonicotinoid
501 Insecticide Thiamethoxam on the Transcriptome of the Honey Bees (Hymenoptera: Apidae). *Journal*
502 *of Economic Entomology*, 110(6): 2283–2289.
- 503 Shin Voo, K., Carlone, D. L., Jacobsen, B. M., Flodin, A., and Skalnik, D. G. 2000. Cloning of a
504 Mammalian Transcriptional Activator That Binds Unmethylated CpG Motifs and Shares a CXXC
505 Domain with DNA Methyltransferase, Human Trithorax, and Methyl-CpG Binding Domain Protein
506 1. *Molecular and Cellular Biology*, 20(6): 2108–2121.
- 507 Standage, D. S., Berens, A. J., Glastad, K. M., Severin, A. J., Brendel, V. P., and Toth, A. L. 2016.
508 Genome, transcriptome and methylome sequencing of a primitively eusocial wasp reveal a greatly
509 reduced DNA methylation system in a social insect. *Molecular Ecology*, 25(8): 1769–1784.
- 510 Stanley, D. A., Smith, K. E., and Raine, N. E. 2015. Bumblebee learning and memory is impaired by
511 chronic exposure to a neonicotinoid pesticide. *Scientific Reports*, 5: 16508.
- 512 Stroud, H., Do, T., Du, J., Zhong, X., Feng, S., Johnson, L., Patel, D. J., and Jacobsen, S. E. 2014.
513 Non-CG methylation patterns shape the epigenetic landscape in *Arabidopsis*. *Nature Structural &*
514 *Molecular Biology*, 21(1): 64–72.
- 515 Supek, F., Bošnjak, M., Škunca, N., and Šmuc, T. 2011. REVIGO Summarizes and Visualizes Long Lists
516 of Gene Ontology Terms. *PLOS ONE*, 6(7): e21800.
- 517 Uvell, H. and Engström, Y. 2007. A multilayered defense against infection: combinatorial control of
518 insect immune genes. *Trends in Genetics*, 23(7): 342–349.
- 519 Vandegehuchte, M. B. and Janssen, C. R. 2014. Epigenetics in an ecotoxicological context. *Mutation*
520 *Research/Genetic Toxicology and Environmental Mutagenesis*, 764-765: 36–45.
- 521 Wang, X., Wheeler, D., Avery, A., Rago, A., Choi, J.-H., Colbourne, J. K., Clark, A. G., and Werren,
522 J. H. 2013. Function and Evolution of DNA Methylation in *Nasonia vitripennis*. *PLOS Genetics*,
523 9(10): e1003872.
- 524 Weiner, S. A. and Toth, A. L. 2012. Epigenetics in Social Insects: A New Direction for Understanding
525 the Evolution of Castes. *Genetics Research International*.
- 526 Welch, M. and Lister, R. 2014. Epigenomics and the control of fate, form and function in social insects.
527 *Current Opinion in Insect Science*, 1: 31–38.

- 528 Whitehorn, P. R., O'Connor, S., Wackers, F. L., and Goulson, D. 2012. Neonicotinoid Pesticide Reduces
529 Bumble Bee Colony Growth and Queen Production. *Science*, 336(6079): 351–352.
- 530 Williams, G. R., Troxler, A., Retschnig, G., Roth, K., Yañez, O., Shutler, D., Neumann, P., and Gauthier,
531 L. 2015. Neonicotinoid pesticides severely affect honey bee queens. *Scientific Reports*, 5: 14621.
- 532 Wu, M.-C., Chang, Y.-W., Lu, K.-H., and Yang, E.-C. 2017. Gene expression changes in honey bees
533 induced by sublethal imidacloprid exposure during the larval stage. *Insect Biochemistry and Molecular
534 Biology*, 88: 12–20.
- 535 Yan, H., Simola, D. F., Bonasio, R., Liebig, J., Berger, S. L., and Reinberg, D. 2014. Eusocial insects as
536 emerging models for behavioural epigenetics. *Nature Reviews Genetics*, 15(10): 677–688.
- 537 Yu, G., Wang, L.-G., Han, Y., and He, Q.-Y. 2012. clusterProfiler: an R Package for Comparing Biological
538 Themes Among Gene Clusters. *OMICS: A Journal of Integrative Biology*, 16(5): 284–287.