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

Pshtiwan S.A. Bebane[1*], Benjamin J. Hunt[1*+], Mirko Pegoraro[2*], Alun R.C. Jones[1], Hollie Marshall[1], Ezio Rosato[1] and Eamonn B. Mallon[1]

[1]Department of Genetics and Genome Biology, The University of Leicester
[2]School of Natural Sciences and Psychology, John Moores University, Liverpool
[*]joint first authors
[+]corresponding author bh177@le.ac.uk

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DRAFT

1 **Abstract**

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

14 ***Keywords—*** Epigenetics, Methylome, RNA-seq, BS-seq, Social insects, Pesticide

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15 # Introduction

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

24 Neonicotinoids are nicotinic acetylcholine receptor agonists which disrupt the function
25 of insect neurons and cause paralysis and death. In addition to direct mortality, laboratory and
26 field studies have documented numerous sublethal effects of low doses of neonicotinoids on both
27 honeybees and bumblebees (e.g. Whitehorn *et al.* 2012; Rundlof *et al.* 2015, reviewed in Pisa
28 *et al.* 2015). Sublethal effects at the individual level include reduced fecundity of queens, reduced
29 fertility in males, impaired immune response, impaired navigation and learning, reduced pollen
30 collection and reduced food consumption. Collectively, these effects result in reduced colony
31 growth and colony reproduction performance. The breadth of the effects of neonicotinoids on bees
32 suggests that neonicotinoids have multiple modes of action beyond their designed direct impact on
33 neurotransmission.

34 We hypothesize that some of these effects could be a consequence of epigenetic changes
35 induced by neonicotinoids. Epigenetics is defined as the stable and heritable change in gene
36 expression without any change in the DNA sequence (Goldberg *et al.*, 2007). Environmental
37 contaminants have been found to affect the epigenetics of a diverse range of animal species from

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38 water fleas to polar bears (Head, 2014) and include metals, endocrine disrupting compounds, air
39 pollution, persistant organic pollutants and pesticides (Vandegehuchte and Janssen, 2014), but much
40 ecotoxicology research is centred on a direct link between exposure and response (Head, 2014).
41 Epigenetic changes have the potential to weaken that link, with effects possibly manifesting much
42 later in life or in subsequent generations. Thus if pesticide-induced epigenetic changes were shown
43 to be heritable in bees this would have implications for future ecological risk assessment.

44 In social insect research the role of DNA methylation, an epigenetic marker primarily
45 involving the addition of a methyl group to a cytosine, has come under increasing scrutiny in recent
46 years (Foret *et al.*, 2009; Lyko *et al.*, 2010; Glastad *et al.*, 2013; Amarasinghe *et al.*, 2014; Glastad
47 *et al.*, 2016; Patalano *et al.*, 2015; Libbrecht *et al.*, 2016; Standage *et al.*, 2016; Rehan *et al.*, 2016;
48 Glastad *et al.*, 2017; Arsenault *et al.*, 2018). Methylation has important effects on the biology of
49 bees, including the control of reproductive status (Kucharski *et al.*, 2008; Amarasinghe *et al.*, 2014)
50 and memory (Biergans *et al.*, 2012), behaviours shown to be affected by neonicotinoids (Williams
51 *et al.*, 2015; Stanley *et al.*, 2015). DNA methylation has been linked with alternative splicing in a
52 number of insect species (Lyko *et al.*, 2010; Li-Byarlay *et al.*, 2013; Glastad *et al.*, 2016; Arsenault
53 *et al.*, 2018). In mammals, methylation on gene promoters leads to a reduction in gene expression.
54 The effect of methylation on gene expression in insects is less well understood (Pegoraro *et al.*, 2017),
55 though high levels of methylation have been associated with highly and stably expressed genes
56 (Foret *et al.*, 2012; Bonasio *et al.*, 2012; Wang *et al.*, 2013), while in honeybees hypomethylated
57 genes are associated with caste-specific expression (Elango *et al.*, 2009; Marshall *et al.*, 2019).
58 Gene expression differences due to neonicotinoid exposure have been found in honeyebee larval
59 workers, adult workers and queens (Derecka *et al.*, 2013; Aufauvre *et al.*, 2014; Christen *et al.*, 2016;
60 Chaimanee *et al.*, 2016; Christen *et al.*, 2018).

61 In this study we use whole genome bisulfite sequencing (WGBS/BS-seq) and RNA-seq on

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62 brain tissue of neonicotinoid exposed and control *Bombus terrestris* workers in order to elucidate
63 the effects of the neonicotinoid imidacloprid on the gene expression and methylation status of
64 bumblebee workers.

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Materials and Methods

Beekeeping, experimental design and brain dissection

Six colonies of *Bombus terrestris audax* were purchased from Agralan, UK. Each colony contained a queen and on average ten workers and a small amount of brood. They were kept in wooden nest boxes and maintained under red light at 26°C and 60% humidity on a diet of 50% v/v apiary solution (Meliose-Roquette, France) and pollen (Percie du set, France) (Amarasinghe *et al.*, 2014).

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

BS-seq

Genomic DNA extraction, sequencing and mapping

Six libraries were prepared (3 colonies, control and treatment). For each colony, 10 boxes were reared (5 control and 5 treatment). Each library was generated from 12 pooled brains of non-reproductive workers taken at random from the relevant boxes for a total of 72 brains. Genomic DNA was extracted, using QIAGEN QIAamp DNA Micro Kit following the manufacturer's instruction . The

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86 concentration of genomic DNA was measured using a Qubit® dsDNA BR Assay Kit (ThermoFisher
87 Scientific, USA) and Nanodrop. Sequencing was performed on a HiSeq 2000 machine (Illumina,
88 Inc.) at the Beijing Genomics Institute (BGI), generating 100-bp paired-end reads.

89 Poor quality reads were removed using fastQC v0.11.2 (Andrews *et al.*, 2010) and adapters
90 trimmed using cutadapt V1.11 (Martin, 2011) and trimmomatic V0.36 (Bolger *et al.*, 2014). Bismark
91 v0.18.1 (Krueger and Andrews, 2011) was used to align the reads to the Bter_1.0 genome (Refseq
92 accession no. GCF_000214255.1 (Sadd *et al.*, 2015)) , remove PCR artifacts and extract methylation
93 calls in CpG, CHH and CHG contexts (where H represents adenine, thymine or cytosine). The
94 cytosine report files from Bismark and the *B. terrestris* annotation file (GCF_000214255.1) were
95 combined using the sqldf library (Grothendieck, 2017) in R v3.4.0 (core Team, 2016) to generate the
96 distribution of methylated Cs over genomic features. Cytosines with less than 10X coverage were
97 excluded. For each cytosine the proportion of methylation reads over total reads was calculated.

98 **Methylation differences between treatments**

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

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106 # RNA-seq

107 ## RNA extraction and Illumina sequencing

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

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

125 ## Differential gene expression analysis

126 A table of raw counts was generated using a Python script (`https://github.com/gpertea/`
127 `stringtie/blob/master/prepDE`) and analysed using DESeq2 (Love *et al.*, 2014) in R v3.4.0

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128 (core Team, 2016) to estimate differentially expressed genes using an FDR-adjusted p-value threshold
129 of 0.05 and controlling for colony effects. Genes with less than 10 reads were discarded from
130 analysis. The normalized read counts were $\log_2$ transformed. The quality of replicates was assessed
131 by plotting read counts of samples against one another and assessing the dispersion and presence of
132 any artefacts between samples (Rich *et al.*, 2018). A principal-component analysis was performed
133 to visualize diversity between samples within treatment and between condition.

134 GO term enrichment and KEGG analysis

135 A list of GO terms for the bumblebee were made by annotating the transcriptome using trinotate
136 (default settings) (Hebert *et al.*, 2016) and blast2GO (against RefSeq) (Conesa *et al.*, 2005). These
137 lists were combined, using the pipeline implemented in Amar *et al.* 2014 with a K value of 1. A
138 hypergeometric test was applied and significant GO terms identified after BH correction (p corrected
139 < 0.05) (Benjamini and Hochberg, 1995) using GOstats (Falcon and Gentleman, 2006), with all
140 RNA features in the bumblebee genome used as a background (GCF_000214255.1). We used
141 REVIGO (Supek *et al.*, 2011) to visualise enriched GO terms, selecting the whole UniProt database
142 and SimRel semantic similarity measure.

143 The clusterprofiler R package (version 3.8.1) (Yu *et al.*, 2012) identified genes from KEGG
144 pathways using the whole UniProt database. A hypergeometric test was applied and significant
145 KEGG pathways were identified after BH correction (qvalue < 0.05) (Benjamini and Hochberg,
146 1995).

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147 # Results

148 ## Methylation analysis

149 The overall sequence alignment rate was 67.21% ± 1.53% (mean ± standard deviation). The
150 proportion of methylated cytosine reads calculated by Bismark were 0.53% ± 0.05% for CpGs,
151 0.37% ± 0.05% for CHGs, 0.38% ± 0.07% for CHHs and 0.4% ± 0.06% for CNs or CHNs ((H =
152 A, C, or T). While insect methylation levels are often low (Glastad *et al.*, 2017) these methylation
153 levels are lower even than in the honey bee, *Apis mellifera*, estimated at ~1% at the genome level
154 using similar metrics (Feng *et al.*, 2010; Bewick *et al.*, 2017). In a CpG context, across all samples,
155 0.15% ± 0.03 % of loci with a minimum coverage of 10 reads were considered methylated by the
156 mixture of binomial model. The distribution of CpG methylation shows a mild bimodal distribution
157 with the vast majority of sites being not or only modestly methylated and a few fully methylated
158 (Figure S1 A). Methylated CpGs are more abundant in coding regions (seven fold) and exons (five
159 fold) than introns (Figure 1 A). Non-CpG per-loci methylation levels were reported as less than
160 0.001% by the mixture of binomial model. This, in conjunction with the uniformity of non-CpG
161 methylation across genomic features (Figure 1 B,C), led to the conclusion that such levels were
162 indistinguishable from error and as such were excluded from subsequent analysis.

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

164 In total 4,424,986 loci were analysed using the mixture of binomial model, which subsequently
165 identified 6,080 sites to test. No differentially methylated loci were identified using logistic
166 regression at a q-value of 0.05 or 0.1. MethylKit includes an option to pool replicates into single
167 control/treatment samples and use Fisher's exact test; using this approach we identified a small

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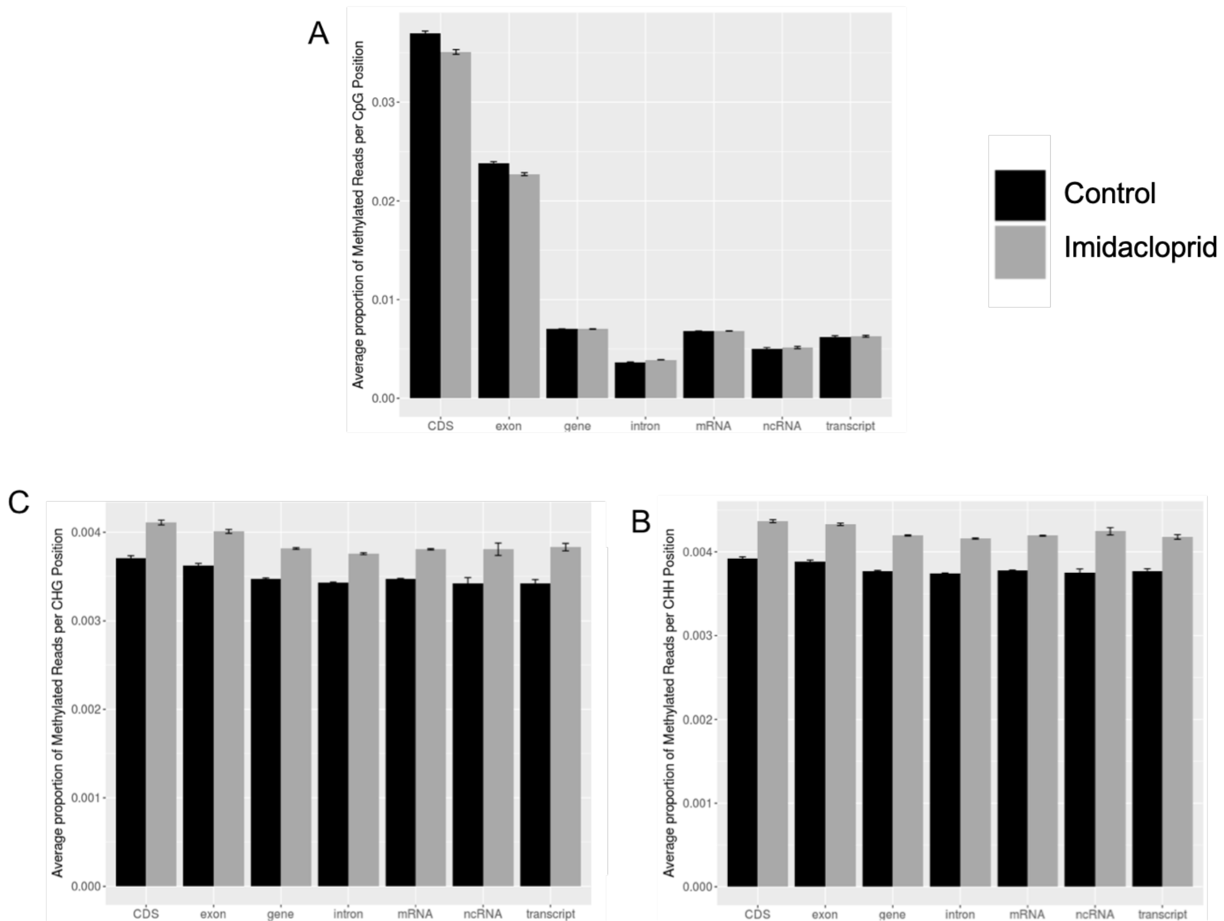

Figure 1: Methylated Cs distribution. Average proportion of methylation reads ± SD per CpG (**A**), CHH (**B**) and CHG (**C**) positions over genomic features. Control samples in black and Neo treated samples in grey.

168 number of differentially methylated CpGs at q-value < 0.1, including loci within *histone-lysine*
169 *N-methyltransferase 2C*, *histone acetyltransferase p300*, *CXXC1* (a transcriptional activator that
170 binds to unmethylated CpGs), and genes involved with axon formation (supplementary data,
171 diff_meth_fisher).

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172 # Expression analysis

173 Alignment rate to the genome was 93.6% (92.1 to 94.1) and after filtering a total of 10,772 genes
174 were analysed. All libraries from the same treatment showed low variation in their gene expression
175 patterns (Figure S2 , S3) .

176 ## Differential expression

177 A total of 405 genes were differentially expressed: 192 genes upregulated and 213 down-
178 regulated in neonicotinoid samples compared to controls (see supplementary data: differen-
179 tially_expressed_genes). Four cytochrome P450 (CYP) genes were differentially expressed, two
180 upregulated and two downregulated. Upregulated genes in neonicotinoid treated bees also in-
181 clude *apyrase* that hydrolyzes ATP to AMP, the neuropeptide receptor *pyrokinin-1 receptor* and
182 *ionotropic receptor 25a* that is involved in circadian clock resetting in *Drosophila* (Chen *et al.*,
183 2015). Downregulated genes include *neurexin*, involved in synaptic formation and maintenance,
184 *peptide methionine sulfoxide reductase*, involved in repair of oxidation-damaged proteins, and a
185 number of genes related to photoreceptor function. Three genes belonging to the homeotic box
186 gene (Hox) family were downregulated in neonicotinoid treated bees. *lethal(2)essential for life*
187 *(Efl21)* displayed the highest down regulation. We found 228 enriched GO terms (BH corrected p <
188 0.05) associated with differential gene expression (supplementary data: expression_GO and Figure
189 S4). Many of the most significantly enriched terms were associated with energy reserve metabolism.
190 Also enriched were terms associated with synaptic transmission, apoptotic processes, xenobiotic
191 transport and complement activation. No KEGG pathways were over represented for differentially
192 expressed genes (q < 0.05).

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193 # DNA methylation - Expression correlation

194 We calculated the average percentage of methylated reads per gene for the most differentially
195 expressed genes (log2 fold-change > 0.5 or < -0.5) and non-differentially expressed genes (Figure 2),
196 fitting a generalized linear model (GLM) with a quasi binomial error distribution with treatment
197 (control vs neonicotinoid) and expression state (DEG vs. non-DEG) as independent variables. There
198 was no significant interactions between the independent variables (interaction model versus main
199 effects only model: χ^2 = -0.014, d.f. = 1, p = 0.82;). For CpGs, non-differentially expressed genes
200 had more methylation than differentially expressed genes ($z_{1,19673}$=4.641, p<0.001). There was no
201 significant treatment effect on methylation levels ($z_{1,19673}$=-0.772, p=0.692). .

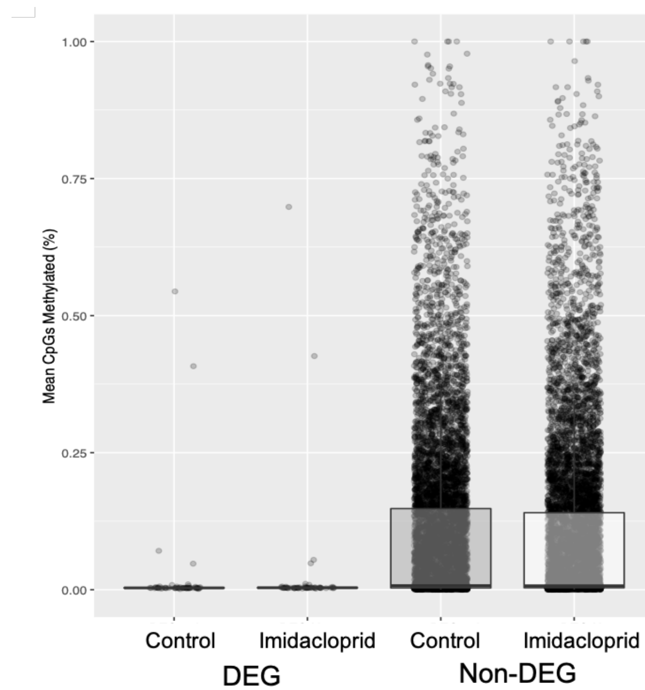

Figure 2: Average percentage of methylated CpG per gene. Differentially expressed genes (DEG) and non differentially expressed genes (nonDEG) are plotted separately. Dots represent genes.

202 To have a more fine scale understanding of the correlation between methylation and expression,

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203 we plotted mean proportion of methylation per gene against ranked expression level (log_{10}fpkm per
204 gene) in 100 bins (from low to high) (Figure 3) fitting a linear model with treatment and expression
205 level as independent variables. There was no significant interaction between expression's and
206 treatment's effects on methylation (interaction model versus main effects only model: $F_{1,189}$ =
207 1.0347, p = 0.3104,). We found a significant effect of expression on methylation ($F_{1,189}$ = 281.654,
208 p = < 2 x 10^{-16}). Neonicotinoid treated bees had comparable levels of CpG methylation to control
209 bees ($F_{1,189}$ = 1.8125, p = 0.1798).

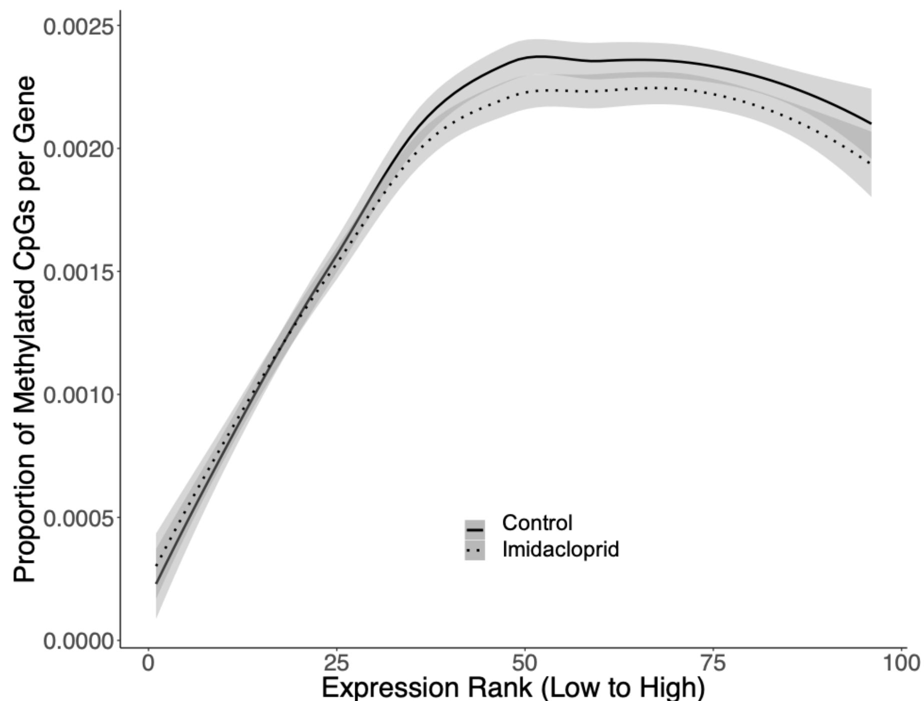

Figure 3: The proportion of methylated CpGs is plotted against gene expression rank. One hundred "bins" of progressively increasing level of expression were generated and genes with similar level of expression have been grouped in the same bin. Solid lines represent control samples and dotted lines neonicotinoid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

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210 # Discussion

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

216 Four cytochrome P450 (CYP) genes were identified as differentially expressed, in line with
217 other studies assessing the impact of insecticides on honeybees (Shi *et al.*, 2017; Li *et al.*, 2017;
218 Derecka *et al.*, 2013; Wu *et al.*, 2017; Christen *et al.*, 2018). Two were upregulated (CYP6k1
219 and 4c3) and two downregulated (28d1 and 9e2). CYP6, 9 and 28 genes are linked to xenobiotic
220 metabolism and resistance to insecticides (Feyereisen, 2006) and CYP6 genes specifically have been
221 found to be upregulated in honeybees after treatment with sublethal doses of the neonicotinoid
222 Thiamethoxam (Shi *et al.*, 2017), as has CYP4C1 after treatment with the neonicotinoid Clothianidin
223 (Christen *et al.*, 2018). The CYP9Q subfamily were recently shown to be responsible for bee
224 sensitivity to neonicotinoids (Manjon *et al.*, 2018).

225 The preponderance of differentially expressed genes associated with synaptic transmission is
226 to be expected, given that we used brain tissue and given the known target effects of neonicotinoids.
227 The identification of a downregulated *neurexin* gene aligns with the results of Shi *et al.* (2017). The
228 effects seen here on metabolic pathways has also been found in honeybees, with GO term enrichment
229 for catabolic carbohydrate and lipid metabolism (Christen *et al.*, 2018). These authors suggested
230 that due to the intensive energy demands of the brain, negative effects on metabolic pathways could
231 affect brain function and therefore behaviour. *Efl21*, the most downregulated gene identified, has
232 been found to be involved in foraging behaviour in bees (Hernández *et al.*, 2012). Downregulation

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233 of carbohydrate metabolism pathways has also been shown in honeybee larvae (Derecka *et al.*, 2013;
234 Wu *et al.*, 2017). Also downregulated were three hox genes. This may be indicative of an impaired
235 immune system, as hox genes have been found to play a role in invertebrate innate immune responses
236 (Uvell and Engström, 2007; Irazoqui *et al.*, 2008). Hox genes have been found to be downregulated
237 in response to insecticide treatment in honeybees (Aufauvre *et al.*, 2014). The bumblebee visual
238 system may also be impacted by imidacloprid treatment, given the downregulation of genes such as
239 protein scarlet, protein glass and ninaC.

240 No differentially methylated loci between control and treatment were identified using a logistic
241 regression model, and we suggest that if acute neonicotinoid exposure does alter methylation status
242 in *B. terrestris* it is subtle and the data reported here may be underpowered to detect it due to low
243 per-sample coverage. A small number of differentially methylated loci were identified by pooling
244 replicates and using Fisher's exact test (supplementary data: diff_meth_fisher), but unlike logistic
245 regression this approach cannot control for covariates and the results should be treated with caution.
246 Using this approach a CpG loci in *CXXC-type zinc finger protein 1* was identified as hypermethylated
247 in neonicotinoid-treated bees; this gene also was upregulated in that group. In mammals, CXXC1 is
248 a transcriptional activator that binds to unmethylated CpGs to regulate gene expression (Shin Voo
249 *et al.*, 2000). Other loci identified by pooling were located within *histone acetyltransferase p300* and
250 *histone-lysine N-methyltransferase 2C*. These findings raise the possibility that neonicotinoids may
251 have a more detectable effect on methylation and subsequent gene expression over a longer period
252 through a cascade of epigenetic processes. A study on the effects of imidacloprid on bumblebees
253 found no effect on mortality or reproduction over 11 weeks using 10 ppb when workers were not
254 required to forage for food, while 20 ppb affected mortality and foraging was impaired at both doses
255 (Mommaerts *et al.*, 2009). It may therefore be that a higher dose or longer exposure time might have
256 a detectable impact on CpG methylation, and further work investigating chronic rather than acute

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257 exposure to imidacloprid at different doses would be valuable. Also worthy of investigation is the
258 potential effect on epigenetic processes other than DNA methylation, such as histone modification,
259 which has been found to have a similar, but non-redundant, association with gene expression in the
260 ant *Camponotus floridanus* (Glastad *et al.*, 2015).

261 We found patterns of CpG methylation to be in line with other insect species. It is mainly
262 focused in exons (Glastad *et al.*, 2017), and high CpG methylation was associated with highly
263 expressed genes (Figure 3) (Arsenault *et al.*, 2018; Bonasio *et al.*, 2012; Glastad *et al.*, 2013;
264 Libbrecht *et al.*, 2016; Patalano *et al.*, 2015; Wang *et al.*, 2013), and non-differentially expressed
265 genes showed higher levels of methylation (Glastad *et al.*, 2013, 2016; Libbrecht *et al.*, 2016; Sarda
266 *et al.*, 2012). Neonicotinoids appear to have no effect on overall levels of CpG methylation (see
267 Figures 2 and 3).

268 Non-CpG methylation plays a role in gene silencing in flowering plants (Stroud *et al.*, 2014)
269 and to a lesser extent, in mammals (Dyachenko *et al.*, 2010). Wang *et al.* 2013 stated that the jewel
270 wasp's (*Nasonia*) genome lacked non-CpG DNA methylation , and in this study, while we identified
271 a very small number of loci showing methylation in CHG/CHH contexts, we could not exclude the
272 possibility that much of it was noise, as bisulfite sequencing is prone to false positives from sources
273 such as incomplete bisulfite conversion, miscalled bases and SNPs. Overall, we conclude that there
274 is no notable methylation of non-CpG cytosines in *B. terrestris*, as with *Nasonia* and the honeybee
275 (Lyko *et al.*, 2010) In contrast to the preponderance of CpG methylation in exons, we found that
276 CHH and CHG methylation was uniformly spread throughout genes (Figure 1) a pattern which
277 would be consistent with the idea that there is no significant methylation in these contexts.

278 Recently, it has become clear that epigenetics can play a role in the interplay between
279 man-made chemicals and natural ecosystems, and their constituent species (Vandegehuchte and
280 Janssen, 2014). Hymenopteran insects (ants, bees and wasps) are ideal models to study this. They are

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both strongly affected by man-made chemicals and are important emerging models for epigenetics (Glastad *et al.*, 2011; Weiner and Toth, 2012; Welch and Lister, 2014; Yan *et al.*, 2014). However, on the evidence of this study, imidacloprid does not appear to have epigenetic effects. This finding is important in the context of future legislation for pesticide control, as it is evidence suggesting a potential lack of transgenerational effects on *B. terrestris* with the use of imidacloprid.

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Data accessibility

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

Authors' contributions

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

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297 # Supplementary material

298 Supplementary figures are availible in the supplementary figures file. Supplementary data is
299 available at `https://doi.org/10.6084/m9.figshare.6796802.v5`.

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