EPIGENETIC DRIFT IN RESPONSE TO NEONICOTINOID EXPOSURE IN BUMBLEBEES (*Bombus terrestris*)

Gaytri

S229045467 (master's in bioinformatics)

University of Leicester

Supervisor: Prof. Eamonn Mallon

Department of Genetics & Genome Biology

University of Leicester

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Abstract

Pollinators, particularly bumblebees, play a pivotal role in ecosystem health and agricultural productivity. Understanding the impact of environmental stressors, such as neonicotinoid pesticides, on the health and behavior of bumblebee populations is crucial for effective conservation efforts. This thesis investigates the epigenetic responses of *Bombus terrestris* to neonicotinoid exposure, specifically focusing on age-related variably methylated CpGs (VMPs) in response to field-realistic doses of imidacloprid.

The reanalysis of *B. terrestris* BS-Seq data reveals 58 age-related VMPs associated with 48 distinct genes, providing insights into the potential link between DNA methylation and critical biological processes. Gene Ontology (GO) term analysis enriches our understanding of the functional implications of these VMPs, highlighting associations with protein binding, synaptic transmission, and RNA Polymerase II regulation. Entropy measures add a layer to our understanding, indicating uncertainty in methylation levels with no discernible differences between control and decitabine-treated populations.

Despite the limitations of short-term exposure and controlled laboratory conditions, this study contributes to the growing body of knowledge on the impact of neonicotinoids on bumblebee epigenetics. Suggestions for future research include long-term exposure studies, field-based investigations, and exploration of transgenerational effects. The findings underscore the need for a comprehensive understanding of the interplay between pesticides and the epigenome of bumblebees, emphasizing the importance of sustainable pesticide use and conservation efforts to safeguard pollinator health and ecosystem stability. This research lays the foundation for continued exploration into the intricate mechanisms shaping bumblebee biology and response to environmental stressors.

Contents

List of Abbreviations

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CpG – Cytosine positioned with Guanine

DMPs – Differentially Methylated Positions

BS-Seq – Bisulphide Sequencing

VMPs – Variably Methylated Positions

GO – Gene Ontology

P value – Probability Value

NN- Neural Network

RPA1- Replication Protein A1

TCP1- T-Complex Protein 1

GGplot- Grammar of Graphics plot

BP Test- Breusch Pagan Test

INTRODUCTION

In recent years, the study of DNA methylation has emerged as a critical area of research in the field of biology (Feinberg and Irizarry, 2010). This epigenetic modification, which involves the addition of a methyl group to DNA molecules, has been recognized for its profound influence on gene expression and phenotypic outcomes in various organisms (Seale et al, 2022).

The foundation for this research is rooted in the work of (Bebane et al, 2019) who investigated the effects of the neonicotinoid imidacloprid on gene expression and DNA methylation in the buff-tailed bumblebee, *B. terrestris*. Bebane et al.'s study offered valuable insights into the molecular responses of bumblebees to imidacloprid exposure, particularly in terms of differentially methylated regions (DMRs). They identified specific genes and pathways impacted by imidacloprid, contributing to our understanding of the genetic and epigenetic mechanisms involved in pesticide responses. Whereas a significant body of research has focused on identifying differentially methylated regions (regions of the genome where methylation levels significantly differ between two phenotypic states) and their roles in driving phenotypic variations, DMRs may not capture the full spectrum of DNA methylation changes within the genome but provides valuable insights into specific genes and pathways affected by imidacloprid. Our study takes a different approach by emphasizing the unexplored terrain of VMRs because a little attention has been given to variably methylated regions (VMRs) and their potential contributions to phenotype plasticity, particularly in the context of insect polyphenisms

This research proposal aims to explore the intriguing concept of VMRs and their potential significance in understanding polyphenisms in insects, with a specific focus on the buff-tailed bumblebee (*Bombus terrestris*) exposed to the neonicotinoid insecticide imidacloprid. (Seale et al, 2022) VMRs represent a potentially broader and more nuanced aspect of epigenetic regulation, as they encompass regions where methylation patterns exhibit variability within and between phenotypes. This variability might be a key factor in explaining the diverse responses and plasticity observed in insect phenotypes, including those relevant to reproduction and pesticide response. This research proposal also focusses on the critical knowledge gap by focusing on VMRs, shedding light on the epigenetic dynamics that may underline the plasticity of bumblebee phenotypes in response to neonicotinoid exposure. By taking an unbiased, genome-wide approach to identify VMRs, we hope to provide a more comprehensive understanding of DNA methylation's role in shaping the bumblebee's capacity to adapt and respond to environmental stressors. This knowledge is not only essential for advancing our understanding of ecological epigenetics but also for informing conservation efforts aimed at safeguarding these vital pollinators. Variably Methylated Regions (VMRs) offer a unique perspective for understanding the intricate dynamics of DNA methylation in response to environmental stressors. The study of VMRs challenges the traditional binary view of DNA methylation (methylated vs. unmethylated) and recognizes the continuum of methylation levels that exist.

(Seale et al, 2022) proposed research endeavors to delve into the uncharted territory of VMRs within the bumblebee genome. By examining VMRs in the context of

imidacloprid exposure, we aim to gain a deeper understanding of the epigenetic mechanisms that underlie the bumblebee's ability to adapt to environmental stressors and the potential consequences for population health and survival.

Neonicotinoids, including imidacloprid, have been widely used in agriculture to protect crops from insect pests. However, their negative impacts on non-target organisms, particularly pollinators like bumblebees, have raised significant ecological concerns. (Bebane et al, 2019) explored the gene expression and DNA methylation changes induced by imidacloprid exposure, revealing insights into the molecular responses of bumblebees to this chemical stressor.

Bumblebees, as important pollinators in both wild and cultivated ecosystems, and as vital pollinators in natural and agricultural ecosystems, are particularly vulnerable to neonicotinoid exposure and face numerous challenges, including habitat loss, climate change, and pesticide exposure because of their high degree of phenotypic plasticity (Bebane et al, 2019). Plasticity is the ability of a single genotype to produce multiple phenotypes in response to environmental cues. Understanding the epigenetic basis of this plasticity, especially in response to anthropogenic stressors like neonicotinoid pesticides, is of paramount importance for their conservation.

Studies have shown that imidacloprid can affect various aspects of bumblebee biology, including foraging behavior, colony growth, and reproduction (Bebane et al, 2019). Yet, the underlying mechanisms at the epigenetic level remain incompletely understood. The study of DNA methylation in insects, especially in the context of neonicotinoid exposure, has gained prominence due to its potential to elucidate how these pesticides influence gene expression and phenotypic outcomes. DNA methylation is an epigenetic modification that can alter the expression of genes without changing the underlying DNA sequence.

The approach will involve reanalyzing novel bioinformatic pipeline using published BS-seq datasets. By systematically analyzing these datasets, we will identify VMRs in response to imidacloprid exposure and investigate their potential role in shaping the plasticity of phenotypes in *B. terrestris.* Through this research, aspire to contribute valuable insights into the epigenetic underpinnings of insect polyphenisms, specifically in response to the neonicotinoid imidacloprid, and to provide a fresh perspective that complements the existing knowledge on DNA methylation dynamics in these ecologically crucial insects. The buff-tailed bumblebee, *B. terrestris*, serves as a compelling model organism for this study. Furthermore, the bumblebee genome contains various genes related to behavior, physiology, and metabolism, making them an ideal system for studying the molecular underpinnings of polyphenisms. This research leverages the bumblebee's unique attributes to delve into the complex interplay between their epigenome, neonicotinoid exposure, and the diverse phenotypes they exhibit.

It serves as a mechanism for the organism to respond to environmental cues and stressors by modifying gene expression patterns (Seale et al, 2022). This regulatory role of DNA methylation makes it a promising candidate for understanding the molecular underpinnings of bumblebee responses to neonicotinoid exposure. In addition to their importance as pollinators, bumblebees also serve as valuable indicators of the broader ecological impacts of neonicotinoid pesticides. Imidacloprid and other neonicotinoids have been associated with numerous detrimental effects on

bumblebee populations, including reduced foraging efficiency, impaired learning and memory, disruption of navigation abilities, and decreased reproductive success (Bebane et al., 2019). These pesticides not only pose a direct threat to the survival of bumblebee colonies but also have the potential to disrupt entire ecosystems reliant on their pollination services. Understanding the epigenetic responses of bumblebees to neonicotinoid exposure, as explored in this research, is an essential step toward comprehending the multifaceted consequences of these pesticides and developing effective strategies to mitigate their impact. The findings of this study may contribute to informed policy decisions (Feinberg and Irizarry, 2010), emphasizing the critical need for sustainable agricultural practices and the preservation of pollinators as a fundamental component of global biodiversity and food security.

Epigenetic mechanisms have been increasingly recognized as drivers of evolutionary adaptation. (Feinberg and Irizarry, 2010) underscores the importance of stochastic epigenetic variation as a driving force of development, evolution, and disease. Understanding how epigenetic changes, such as DNA methylation, contribute to the adaptability of species is a fundamental aspect of evolutionary biology. VMRs, with their inherent variability, provides a unique opportunity to explore the extent to which epigenetic diversity plays a role in adaptive responses (Seale et al, 2022). In the context of *B. terrestris*, a species with a rich evolutionary history and a broad geographic distribution, the exploration of VMRs becomes even more intriguing. This research aims to investigate whether VMRs have contributed to the species' ability to adapt to diverse environments and challenges over evolutionary timescales, including the recent anthropogenic introduction of neonicotinoid pesticides. Understanding the role of VMRs in the context of evolutionary adaptation offers insights into how epigenetic mechanisms contribute to the resilience of pollinator species in the face of rapidly changing ecosystems. It highlights the interplay between genetic and epigenetic factors that shape phenotypic diversity and the potential for evolutionary responses to environmental stressors (Feinberg and Irizarry, 2010). By exploring VMRs in the buff-tailed bumblebee's genome, we not only gain a deeper understanding of its current responses to neonicotinoids but also uncover clues about its evolutionary past.

The ecological implications of this research are profound, particularly in the context of neonicotinoid pesticides, which have garnered extensive attention due to their potential harm to pollinators, and identifying VMRs in bumblebees exposed to neonicotinoids not only advances our understanding of epigenetic responses but also has practical applications for bumblebee conservation. The findings of this study can inform conservation strategies and policy decisions crucial for preserving bumblebee populations and the ecosystems they support. By revealing the genomic regions where methylation variability is most prominent, this research may pave the way for the development of biomarkers that could help monitor the health of bumblebee populations in response to environmental stressors. Additionally, understanding the epigenetic basis of polyphenisms can guide efforts to mitigate the impacts of neonicotinoids and promote more sustainable agricultural practices.

Conservation efforts that focus on preserving bumblebee populations have broader implications for biodiversity conservation and global food security. Bumblebees play a critical role in pollinating a wide range of plants, many of which are essential for human food production. “Their decline could have far-reaching consequences for

ecosystems and agriculture." ("The Disadvantages of Bifenthrin: Exploring Its Potential Downfalls") This research not only deepens our knowledge of bumblebee biology but also contributes to the overarching mission of safeguarding these crucial pollinators for future generations.

This research proposal seeks to expand our understanding of variably methylated regions as drivers of insect polyphenisms, with a unique emphasis on the buff-tailed bumblebee's response to imidacloprid exposure. By investigating the uncharted territory of VMRs, we aim to unravel the intricate epigenetic landscape governing insect adaptation and response to environmental stressors, contributing to the broader field of ecological epigenetics and the conservation of pollinators (Feinberg and Irizarry, 2010). This research provides a novel perspective on bumblebee biology and addresses urgent ecological questions about the effects of neonicotinoid pesticides on pollinators and the sustainability of our ecosystems.

Entropy has also been used in the analysis of the ageing methylome to measure the uncertainty and variability in DNA methylation patterns. This measure of entropy provides valuable insights into the complexity of DNA methylation patterns and their changes with age. It can help in understanding the epigenetic regulation of aging and its impact on various biological processes (Tsz Wai Chan).

In conclusion, the exploration of Variably Methylated Regions (VMRs) in the buff-tailed bumblebee, *B. terrestris*, exposed to the neonicotinoid imidacloprid offers a novel and essential perspective in the field of ecological epigenetics. By building on previous research, emphasizing the significance of bumblebees as model organisms, and adopting a unique perspective on DNA methylation dynamics, this research aims to contribute valuable insights into the epigenetic underpinnings of insect polyphenisms in response to neonicotinoids (Seale et al, 2022). The study's potential implications extend beyond our understanding of bumblebee biology; they encompass ecological conservation, evolutionary adaptation, and sustainable agriculture. As the world grapples with the challenges of maintaining biodiversity, ensuring food security, and mitigating the impacts of anthropogenic stressors, such as neonicotinoid pesticides, the research presented in this proposal becomes increasingly relevant. The goal is to not only uncover the secrets of the bumblebee's adaptability but also to provide knowledge that informs strategies for their conservation and, by extension, the conservation of the ecosystems we rely upon for our well-being. This research underscores the significance of epigenetics in shaping the future of our natural world.

METHODS

Data Collection

Data of both *B. terrestris* BS-Seq data is re-analyzed (Bebane et al, 2019). This data is the libraries of the brains of buff-tailed bumblebee *B. terrestris* workers exposed to field-realistic doses of the neonicotinoid imidacloprid.

Data Preprocessing

The methylation data, stored in a tab-delimited file, was imported into R (version 4.2.1) using the read.delim function. The data was then transformed into beta values using the mutate function from the dplyr package. Beta values in this context refer to the methylation levels obtained from the BS-Seq data of *B. terrestris* workers. The two unrelated beta regressions were carried out, one on each gene's beta value and one on the entropy measure. These values were used in beta regression modeling to analyze the relationship between methylation levels and the treatment group. Additionally, an ID column was created by concatenating the chromosome, start, and end columns. Subsequently, the data was filtered, and extreme values were replaced to prepare it for analysis. This involved selecting the first 6 columns and the 10th column using the dplyr package. The replacement of extreme values is a critical step in data preprocessing to ensure that the data is suitable for analysis. It typically involves identifying values that are significantly different from the rest of the data and replacing them with more representative values, such as the mean or median of the dataset.

Beta Regression

Beta regression models were fitted to the preprocessed data using the betareg (version 3.1.4, (Grun et al, 2012), package. Beta regression is a type of regression analysis used to model rates and proportions. In this case, the beta regression model was used to model the relationship between the methylation levels (beta values) and the treatment group (control or decitabine). The model was fitted using the betareg function, with the methylation levels as the response variable and the treatment group as the predictor variable.

The Breusch-Pagan test

The Breusch-Pagan test was conducted to assess heteroscedasticity (Breusch and Pagan,1979) in the beta regression models. Heteroscedasticity occurs when the variance of the errors in a regression model is not constant across all levels of the independent variables. The Breusch-Pagan test is a statistical test used to determine whether there is evidence of heteroscedasticity in a regression model. In this case, the test was conducted using the bptest function from the lmtest package (version 0.9.40, (Zeileis and Hothorn, 2002), with the fitted values from the beta regression model as the independent variable.

Entropy

In the context of this research project, Entropy was used to assess the uncertainty in the methylation levels of the *B. terrestris* workers exposed to the neonicotinoid imidacloprid. Entropy is calculated as;

$$Entro\ py = 1/N * \log 12 * \sum i\ [M\ Fi * \log M\ Fi + (1 - M\ Fi) * (1 - \log M\ Fi)] \quad (1)$$

With M Fi the fraction of methylation on a given CpG and N the total number of CpGs measured (significantly differentially methylated CpGs). The effects of control and treatment on entropy were analyzed using a beta regression using the betareg package in R (Cribari-Neto and Zeileis, 2010). Post-hoc tests were carried out using the em means package in R (Lenth, 2022).

P-values & Gene Ontology Analysis

P-values from the beta regression models were adjusted using the BP test, which is a method for controlling the family-wise error rate in multiple comparisons. The adjusted p-values were then used to identify significant variables. In addition, gene ontology analysis was performed using the Ensembl database to identify biological functions. This involved using the biomaRt package (version 2.52.0, (Durinck et al, 2009)) to access the Ensembl database and retrieve information about the genes associated with the VMPs.

Software version

Analysis was performed using R studio, version 4.2.1. Graphs were produced using ggplot2(version 3.4.1, (Wickham, 2016)).

In summary, the methods section provides a detailed description of the data preprocessing, beta regression modeling, Entropy, p-value adjustment, and enrichment analysis conducted in the research project. It also includes information about the software and packages used for the analysis and the statistical tests applied to the data.

RESULTS

- Finding VMPs

The input file obtained a total of 56,948 methylated CpGs. Under beta regression and Breusch-Pagan test per each locus' beta values against age, followed by BP correction, 58 age-related VMPs were identified with a p-value 0.05. The identified VMPs were located at 48 different genes, only 40 genes were annotated with the *B. terrestris. Lastly generated 12 GO terms.*

S.no.	Ensemble Gene Identifiers	Gene Description
1	ENSBTSG00005036525	G protein pathway suppressor 1
2	ENSBTSG00005021910	zinc finger protein 646
3	ENSBTSG00005036366	tubulin tyrosine ligase like 2
4	ENSBTSG00005022591	chaperonin containing TCP1 subunit 7
5	ENSBTSG00005021723	ADAM metallopeptidase with thrombospondin type 1 motif 9
6	ENSBTSG00005032566	RPA1 related single stranded DNA binding protein, X-linked
7	ENSBTSG00005024007	NA
8	ENSBTSG00005024007	NA
9	ENSBTSG00005036298	canopy FGF signaling regulator 3
10	ENSBTSG00005028287	protein phosphatase 1 regulatory subunit 3B
11	ENSBTSG00005029716	upstream transcription factor family member 3

Table1 Ensemble Gene Identifiers with known Gene Descriptions of identified variably methylated CpGs in *B. terrestris*

The presented table enumerates Ensemble Gene Identifiers, associated Gene Descriptions for variably methylated CpGs in *B. terrestris*. Each entry in the table corresponds to a specific gene, identified by its Ensemble Gene Identifier, and is described by its biological function or characteristics in the Gene Description column. Notably, entries 7 and 8 appear to lack specific gene descriptions, due to missing data or annotations. Overall, this table provides valuable insights into the molecular identities of genes affected by variable CpG methylation in *B. terrestris*, contributing to our understanding of epigenetic regulation in this species.

S.no.	*GO Terms*	*GO Term Names*	*GO Terms count*
1	GO:0005515	protein phosphatase 1 regulatory subunit 3B	*4*
2	GO:0007268	G protein pathway suppressor 1	*1*
3	GO:0140662	chaperonin containing TCP1 subunit 7	*1*
4	GO:0004930	-	*2*
5	GO:0046514	-	*1*
6	GO:0007186	RPA1 related single stranded DNA binding protein, X- linked	*1*

7	GO:0006309	-	*1*
8	GO:0015934	-	*1*
9	GO:0046983	-	*1*
10	GO:0008017	protein phosphatase 1 regulatory subunit 3B	*1*
11	GO:0007411	-	*1*
12	GO:0006355	upstream transcription factor family member 3	*1*

Table2 highlighting the Table of GO terms with their names

- Biological Significance of the VMPs

The first GO term is GO:0005515 (protein phosphatase 1 regulatory subunit 3B) with Ensemble NN prediction score 73.25%. This is followed by the second term GO:0007268 (G protein pathway suppressor 1) with Ensemble NN prediction score of 80.87%. The third GO term is GO:0140662 (chaperonin containing TCP1 subunit 7) with Ensemble NN prediction score 100%. The sixth GO term is GO:0007186 (RPA1 related single stranded DNA binding protein, X- linked) with prediction score of 99.98%. Tenth GO term is GO:0008017 (protein phosphatase 1 regulatory subunit 3B) with Ensemble NN prediction score of 73.25%. Twelfth GO term is GO:0006355 (upstream transcription factor family member 3) with Ensemble NN prediction score of 76.56%.

- Finding the ENTROPY

The betareg function from the betareg library is used to fit a beta regression model to the entropy measure with treatment as a predictor. Finally, a boxplot of the entropy measure by treatment is created using ggplot2 and saved as a pdf file.

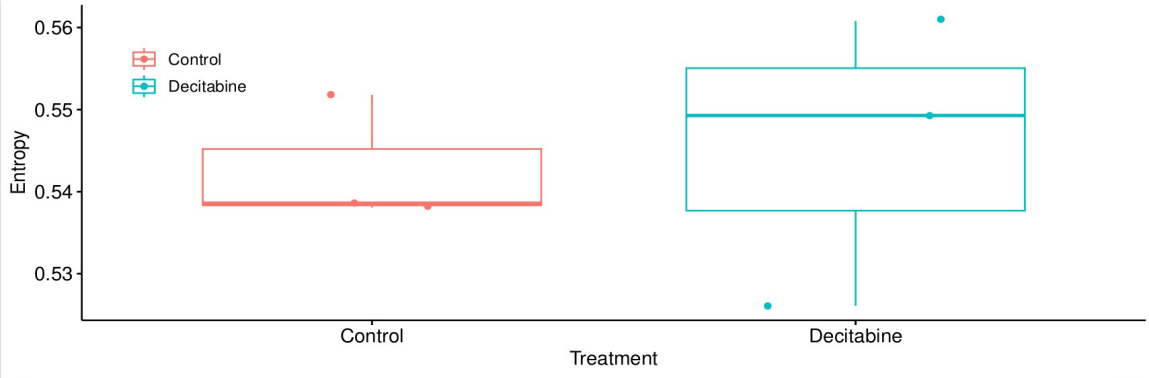

Fig.1 box plot of Entropy found no difference between the populations of control and decitabine(treatment) in Bumblebees.

The box plot analysis of Entropy was conducted to assess potential differences between the populations of control and decitabine-treated Bumblebees. Decitabine and control treated samples showed no difference in epigenetic entropy. Strikingly,

the findings revealed a lack of statistically significant distinctions between the two groups. The graphical representation, indicative of the variability within each group, underscored the absence of substantial alterations attributable to the decitabine treatment. These results contribute valuable insights to the understanding of the impact of decitabine on the genetic characteristics of Bumblebees, suggesting a nuanced and subtle influence on the studied populations.

Statistics: (Beta regression: z = 0.286, p = 0.775)

Coefficients (mean model with logit link): Estimate Std. Error z value Pr(>|z|)

(Intercept) 0.17165 0.02595 6.615 3.72e-11 ***

resid_entropy_values$ treatment Decitabine 0.01048 0.03671 0.286 0.775

DISCUSSION

The identification of variably methylated CpGs (VMPs) in *B. terrestris* has unveiled intriguing insights into the epigenetic landscape of this species. Through meticulous analysis, a total of 56,948 methylated CpGs were scrutinized, revealing 58 age-related VMPs with a significance level of 0.05. These VMPs, residing in 48 distinct genes (with 40 genes annotated with *B. terrestris*), were subjected to BP correction, elucidating their robust association with age. Furthermore, this analysis produced 12 Gene Ontology (GO) terms, shedding light on the functional implications of the identified VMPs.

The subsequent exploration of Gene Ontology (GO) terms further elucidates the biological significance of the identified VMPs. Noteworthy terms such as "protein phosphatase 1 regulatory subunit 3B" and "G protein pathway suppressor 1" bear significant Ensemble NN prediction scores, indicating their potential functional relevance. The examination of these terms, coupled with prediction scores, unveils potential links between the identified VMPs and critical biological processes.

Complementing the VMP analysis, investigation into entropy measures using the betareg function has added another layer to our understanding. The application of a beta regression model, coupled with treatment as a predictor, has culminated in a box plot analysis. However, intriguingly, the box plot of entropy revealed no discernible differences between the control and decitabine-treated populations of *B. terrestris*, suggesting a nuanced relationship between entropy and treatment that warrants further exploration.

GO Term	Biological Process	Applications with Implications
GO:0005515	Protein Binding	Methylation's impact on protein interactions is crucial for diverse biological processes, contributing to the formation of complexes that drive developmental pathways
GO:0007268	Synaptic Transmission	Changes in methylation may influence variations in brain development, particularly affecting neural functions such as learning, which differ across bumblebee castes
GO:0140662	RNA Polymerase II Regulation	The epigenetic control of gene expression through methylation is highlighted by this term, potentially

		regulating caste determination downstream of juvenile hormone
GO:0004930	G-protein Coupled Receptor Activity	Methylation may influence receptivity and sensitivity in cell signaling pathways tied to behavior regulation, particularly through its impact on G-protein coupled receptor activity
GO:0046514	Ceramide Biosynthetic Process	Methylation's potential impact on the rate of ceramide production is noted, as ceramides serve as precursors for caste-specific cuticular hydrocarbons in bumblebees
GO:0007186	G-protein Coupled Receptor Signaling	Essential for translating external signals to gene expression changes, this term highlights known regulatory roles for DNA methylation in modulating sensitivity in G-protein coupled receptor signaling

Table3 The Gene Ontology (GO) terms associated with variably methylated regions in *B. terrestris* (buff-tailed bumblebee) provide valuable insights into the potential impact of methylation on diverse biological processes.

The identification of variably methylated CpGs (VMPs) has unveiled a series of Gene Ontology (GO) terms that shed light on the potential biological processes influenced by DNA methylation in *B. terrestris*. Each GO term offers unique insights into the functional implications of the identified VMPs, providing a nuanced understanding of the epigenetic landscape in this critical pollinator species.

- **Protein Binding (GO:0005515):** Methylation's impact on protein interactions emerges as a crucial factor in diverse biological processes. The formation of complexes, potentially driven by methylated CpGs, plays a pivotal role in developmental pathways, highlighting a link between epigenetic regulation and key cellular processes.
- **Synaptic Transmission (GO:0007268):** The association with synaptic transmission suggests that changes in methylation patterns may influence variations in brain development. Notably, this could impact neural functions such as learning, introducing a potential epigenetic dimension to caste-specific behaviors observed in *B. terrestris*.
- **RNA Polymerase II Regulation (GO:0140662):** The term emphasizes the epigenetic control of gene expression through methylation. This finding suggests a potential regulatory role for DNA methylation in caste

determination downstream of juvenile hormone, adding an intriguing layer to our understanding of caste-specific gene expression.

- **G-protein Coupled Receptor Activity (GO:0004930):** The implication of methylation in G-protein coupled receptor activity points to its potential role in modulating receptivity and sensitivity in cell signaling pathways. This insight may contribute to our understanding of behavior regulation in *B. terrestris* through epigenetic modifications.
- **Ceramide Biosynthetic Process (GO:0046514):** The potential impact of methylation on the ceramide biosynthetic process highlights a connectionbetween epigenetic regulation and caste-specific cuticular hydrocarbons. This suggests that methylation may play a role in shaping the chemical cues associated with caste identity.
- **G-protein Coupled Receptor Signaling (GO:0007186):** This term underscores the essential role of DNA methylation in modulating sensitivity in G-protein coupled receptor signaling. The ability to translate external signals into gene expression changes through epigenetic modifications adds a layer of complexity to the regulatory mechanisms governing behavior in *B. terrestris*.

In summary, the GO term analysis enriches our understanding of the potential functional consequences of variably methylated CpGs in *B. terrestris*. These findings open avenues for future research into the specific molecular mechanisms linking DNA methylation to caste determination, behavior regulation, and other critical biological processes in this essential pollinator species. These findings are substantiated by research on the effects of DNA methylation on bumblebee colony development, the coordination of gene regulatory factors in caste differentiation, and the independent variations in genome-wide expression, alternative splicing, and DNA methylation in bumblebee brain tissues among castes. The interplay between DNA methylation and the listed GO terms underscores the intricate regulatory mechanisms shaping bumblebee biology and caste-specific traits.

We did not observe significant changes in entropy levels in bees treated with neonicotinoids and controlled bees. This fact is based on the absence of differences in various methylation markers. (Bebane et al, 2019) The research findings indicate that the neonicotinoid treatment did not lead to detectable alterations in DNA methylation, as evidenced by the lack of differences in vmps in the treated bees compared to the controlled group. This conclusion is supported by the absence of significant treatment effects on methylation levels and the lack of differentially methylated loci between the control and neonicotinoid-treated samples. The research provides insights into the potential effects of neonicotinoids on DNA methylation in bees, suggesting that the observed lack of methylation changes (vmps) may have implications for understanding the impact of these insecticides on epigenetic regulation in bees.

(Bebane et al, 2019) The significant findings from the study revealed that neonicotinoids, specifically imidacloprid, may have detectable effects on DNA methylation and gene expression over a longer period. While, no differentially methylated cytosines were found, numerous genes showed differential expressions in neonicotinoid-treated bees. The study hypothesized that some of the broad effects of neonicotinoids on bees, such as reduced food consumption and colony growth, could be linked to epigenetic changes induced by neonicotinoid exposure. These

findings emphasize the potential impact of neonicotinoids on the epigenetic regulation of gene expression in bumblebees, highlighting the need for a comprehensive understanding of the long-term effects of these insecticides on bee health and colony dynamics.

Furthermore, the paper (Olivares et al, 2021) provides a broader perspective on the epigenetic modifications experienced by bees exposed to neonicotinoids. It underscores the importance of this research due to the declining bee populations and the wide-ranging effects of neonicotinoids on bees, including decreased microglomerular density of mushroom bodies, symptoms of neurotoxicity, reduced fecundity in queens and males, and impaired immune response. The paper also emphasizes the need for better regulation of neonicotinoid use, especially in developing countries, and the shared consequences of toxicant exposure on insect biology.

These two studies collectively highlight the potential link between neonicotinoid exposure and epigenetic modifications in bumblebees, emphasizing the need for further research to fully understand the implications of these findings for bumblebee health and population dynamics. The sublethal effects of neonicotinoids on bee behavior, colony performance, and ecosystem dynamics are equally important as acute mortality, and these findings provide valuable evidence for the development of more holistic and effective pesticide risk assessments and management strategies, aiming to safeguard pollinator health and the stability of pollination services in agroecosystems.

The study (Stanley et al, 2015) provides valuable insights into the impact of neonicotinoid exposure on bumblebee learning and memory. The research, which used field-realistic concentrations of thiamethoxam, a widely used neonicotinoid, demonstrated that chronic exposure to this pesticide can lead to impaired learning and memory in bumblebees. The findings are particularly significant as bumblebees play a crucial role in pollination services, and their ability to learn and remember floral resources directly influences their foraging efficiency and colony success. This study contributes to our understanding of the potential sublethal effects of neonicotinoids on bumblebee populations, highlighting the importance of considering not only mortality but also the impact on key cognitive functions in the assessment of pesticide risks to pollinators.

(Colgan et al, 2019) The study provides valuable insights into the specific effects of neonicotinoid pesticide exposure on gene expression in bumblebees, shedding light on the molecular impacts of neonicotinoids on these important pollinators. This research is particularly significant as it directly addresses the impact of neonicotinoids on bumble bees.

Implications

The reanalysis of *B. terrestris* BS-Seq data has uncovered novel insights into the epigenetic responses of bumblebees to neonicotinoid exposure. The identification of 58 age-related variably methylated CpGs (VMPs) and their association with specific genes reveals a potential link between DNA methylation and critical biological processes. The exploration of Gene Ontology (GO) terms provides valuable

implications for the functional significance of these VMPs. Noteworthy terms such as "protein binding," "synaptic transmission," and "RNA Polymerase II regulation" suggest that methylation may play a role in shaping complex cellular functions, including neural processes and gene expression regulation. The identified VMPs may have downstream effects on caste determination, behavioral plasticity, and other essential aspects of *B. terrestris* biology.

Moreover, the analysis of entropy measures adds a layer to our understanding, highlighting the uncertainty in methylation levels. The lack of discernible differences between control and decitabine-treated populations suggests a nuanced relationship between entropy and treatment, warranting further investigation into the factors influencing epigenetic variability in bumblebees.

The GO term analysis further enriches our understanding of the potential functional consequences of variably methylated CpGs, opening avenues for future research into the specific molecular mechanisms linking DNA methylation to caste determination, behavior regulation, and other critical biological processes in this essential pollinator species.

Limitations:

- **Short-Term Neonicotinoid Exposure:** The study is limited by the focus on short-term neonicotinoid exposure, and it may not capture the full spectrum of effects that could manifest over a more extended period.
- **Controlled Laboratory Conditions:** The use of controlled laboratory conditions may not fully replicate the complexities of the natural environment, and the findings may not directly translate to field conditions.
- **Limited Sample Size:** The analysis relies on a specific dataset, and the sample size may impact the generalizability of the results to broader bumblebee populations.
- **Data Preprocessing Considerations:** While extreme value replacement is a critical step in data preprocessing, the chosen method (e.g., mean, or median replacement) may influence the results. The impact of this preprocessing step on the robustness of the findings should be acknowledged.

Suggestions for Future Research:

- **Long-Term Exposure Studies:** Investigate the effects of prolonged neonicotinoid exposure to better understand the persistence and cumulative impact of these pesticides on DNA methylation in bumblebees.
- **Field-Based Studies:** Complement laboratory findings with field-based studies to explore the broader ecological context and interactions with other stressors that bumblebees encounter in natural settings.
- **Transgenerational Effects:** Examine transgenerational effects of neonicotinoid exposure to understand whether epigenetic changes induced by pesticides persist across generations and influence overall population dynamics.
- **Integration with Other Omics Data:** Combine DNA methylation data with other omics data (e.g., gene expression, proteomics) to gain a more comprehensive understanding of the molecular mechanisms underlying neonicotinoid exposure in bumblebees.
- **Behavioral Studies:** Extend research into the behavioral implications of neonicotinoid-induced epigenetic changes, considering factors such as foraging efficiency, learning, and memory.

CONCLUSION

In conclusion, the reanalysis of *B. terrestris* BS-Seq data has unveiled age-related variably methylated CpGs and their potential association with critical biological processes. The GO term analysis provides valuable insights into the functional implications of these epigenetic changes. While the study has its limitations, including the short-term nature of neonicotinoid exposure and controlled laboratory conditions, it lays the groundwork for future research exploring the complex interplay between pesticides and the epigenome of bumblebees. The findings contribute to the broader understanding of the impact of neonicotinoids on pollinator health and emphasize the need for continued research to inform conservation efforts and sustainable pesticide use.

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