PROTEOMIC IMPACTS OF AGROCHEMICAL AND PATHOGEN EXPOSURE IN HIGHBUSH BLUEBERRY POLLINATING HONEY BEES (*APIS MELLIFERA*)

by

Rhonda Thygesen

B.SC., The King's University, 2020

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Biochemistry and Molecular Biology)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

August 2025

The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, the thesis entitled:

Proteomic impacts of agrochemical and pathogen exposure in highbush blueberry pollinating honey bees (*Apis mellifera*)

submitted by Rhonda Thygesen in partial fulfilment of the requirements for

the degree of Master of Science

in Biochemistry and Molecular Biology

Examining Committee:

Leonard Foster, Professor, Biochemistry and Molecular Biology, UBC
Supervisor

Thibault Mayor, Professor, Biochemistry and Molecular Biology, UBC
Supervisory Committee Member

Juli Carrillo, Associate Professor, Applied Biology, UBC
Supervisory Committee Member

Franck Duong, Professor, Biochemistry and Molecular Biology, UBC
Additional Examiner

Additional Supervisory Committee Members:

Katie Marshall, Associate Professor, Zoology, UBC
Supervisory Committee Member

Abstract

Honey bees (*Apis mellifera*) play a critical role in the pollination of highbush blueberry crops, yet exposure to environmental stressors such as agrochemicals and pathogens poses significant risks to their health and colony sustainability. This study aimed to investigate the proteomic responses of honey bees during highbush blueberry pollination, focusing on agrochemical and pathogen stressors. Through large-scale field trials involving honey bee colonies placed near and far from highbush blueberry agriculture, coupled with caged bee trials, differential expression analysis and gene ontology (GO) enrichment analysis were employed to identify proteomic changes. Although no significant proteomic differences were found between exposed and unexposed site types, timepoint and tissue-specific analyses revealed critical changes during the bloom and post-bloom periods, particularly in gut and head tissues. Gut tissues showed extensive metabolic and mitochondrial pathway enrichment, emphasizing their sensitivity to environmental stressors, while head tissues exhibited downregulation in pathways related to cognitive function and DNA repair. Agrochemical residue analysis identified significant pesticide contamination in both exposed and unexposed hives, particularly during bloom, with flupyradifurone and pyrimethanil being the most prominent residues. The study's findings highlight the importance of managing agrochemical applications and addressing pathogen prevalence in blueberry agriculture. Future research should focus on sublethal effects of prominent pesticides, further characterization of pathogen dynamics, and more refined control experiments to better assess stressor impacts on honey bee health. This research provides crucial insights into the molecular mechanisms through which environmental stressors influence honey bee physiology, with broader implications for pollinator conservation and agricultural sustainability.

Lay Summary

Honey bees are vital pollinators for crops like highbush blueberries, a popular product in British Columbia. In agricultural areas, bees are exposed to pesticides and pathogens, which can harm their health. This study examined how honey bees respond to these stressors by analyzing changes in their proteins. We collected bees from colonies near and far from blueberry fields and observed protein changes during the bloom. Bees near blueberry crops had higher pesticide levels, especially during bloom, and even bees from "unexposed" areas showed pesticide residues. The gut tissue showed significant changes in proteins related to energy production, indicating a response to pesticide and pathogen stress. Head tissues, controlling functions like memory and foraging, were also affected. These findings highlight the need for better pesticide management in blueberry farming and further research on the combined effects of pesticides and pathogens on bee health, to support healthier bee populations for crop pollination.

Preface

This thesis is part of the national BeeCSI project, led by Dr. Leonard Foster as one of the principal investigators. The BeeCSI project provided the foundation for the experimental design and much of the data used in this work. I actively participated in the fieldwork associated with this project, specifically focusing on the preparation of all highbush blueberry samples for subsequent proteomic investigation. The cage experiments and associated methods followed protocols established by the BeeCSI project; however, the choice of agrochemicals and pathogens used in the experiments were decisions I made independently, tailored to the objectives of this study. Renata Moravcova developed the protocols for honey bee tissue preparation for proteomic analysis and designed the searching parameters used following the LC/MS-MS analysis. The initial identification and design of the research were completed by Dr. Foster and the BeeCSI team, with modifications and input contributed by myself.

All data analysis and figure generation were completed collaboratively between myself and Yuming Shi. This thesis reflects my contributions to the interpretation of the data, the development of research directions, and the overall execution of the proteomic investigation on honey bees in highbush blueberry pollination environments. Generative artificial intelligence (ChatGPT) was used in a limited capacity to improve sentence clarity and to assist with debugging code.

Table of Contents

List of Tables

List of Figures

List of Multimedia Materials

Multimedia Material 1**.** Apiary log database for the highbush blueberry field trial in 2020 and 2021 with beekeeper's initials.

List of Abbreviations

ABPV	Acute bee paralysis virus
ACN	Acetonitrile
ANOVA	Analysis of variance
B.C.	British Columbia
BCA	Bicinchoninic acid
BQCV	Black queen cell virus
CAA	Chloroacetamide
CBPV	Chronic bee paralysis virus
DEP	Differentially expressed protein
DDA-PASEF	Data-dependent acquisition with parallel accumulation serial fragmentation
DIA-NN	Data-independent acquisition neural network
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
DWV	Deformed wing virus
DWV-A	Deformed wing virus genotype A
DWV-B	Deformed wing virus genotype B
EFB	European foulbrood
FDR	False discovery rate
GLMM	Generalized linear mixed model
GO	Gene ontology
IAPV	Israeli acute paralysis virus
KBV	Kashmir bee virus
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
LMM	Linear mixed models
LSV	Lake Sinai virus
PBS	Phosphate-buffered saline
PCA	Principle component analysis
PCR	Polymerase chain reaction

PSM	Peptide-spectrum match
qPCR	Quantitative polymerase chain reaction
REML	Restricted maximum likelihood
RNA	Ribonucleic acid
RT	Reverse transcription
SBV	Sacbrood virus
SDS	Sodium dodecyl sulphate
TFA	Trifluoroacetic acid
TP1	Timepoint 1
TP2	Timepoint 2
TP3	Timepoint 3
TP4	Timepoint 4
UHPLC	Ultra-high-performance liquid chromatography
VDV	*Varroa destructor* virus

Acknowledgements

I would like to thank Dr. Leonard Foster, who gave me a chance and supported me both as a friend and supervisor the entire time. With his help I would have never gotten to continue my studies and have so many adventures along the way. His patience and kindness with me have been heartwarming during difficult times. I would also like to thank my supervisory committee, Dr. Julie Carrillo, Dr. Katie Marshall, and Dr. Thibault Mayor. It has been a pleasure working with you on this project. Thirdly, I must acknowledge the tireless work of the technical staff in the Foster laboratory, especially Renata Moracova, Jason Rogalski, and Jenny Moon, whose expertise in the lab made this research possible. A special thank you to Yuming Shu who was instrumental in helping with the proteomic data analysis, and Dr. Alison McAfee, for the assistance with the pathogen data analysis. Lastly, thank you to the BeeCSI team for sharing this research with me and to BC Blueberries and the Canadian Association for Professional Apiculturists for providing the financial support necessary to conduct this work. My appreciation extends to the beekeeping community who have heard me speak on this project and have supported me throughout it. I am specifically grateful for Heather Higo and Julia Common, the beekeepers involved in this project and my beekeeping mentors.

Dedication

This thesis is dedicated to my family. Your support and love mean so much to me.

Chapter 1 Introduction

1.1 British Columbia's blueberry and honey industries

1.1.1 Highbush blueberry pollination

Honey bees (*Apis mellifera L.)* contribute up to $7 billion/year to the Canadian economy by providing pollination services to crops, such as highbush blueberries. in British Columbia (B.C.), Canada (Agriculture & Agri-Food Canada, 2023). Managed bees are effective blueberry pollinators and are consequently placed in fields to ensure high fruit set alongside native pollinators, such as the *Bombus* species (Government of British Columbia, 2024). Blueberries are Canada's top fruit export by monetary value, bringing in $280 million/year as recorded in 2023 (Agriculture & Agri-Food Canada). More locally, a significant portion of farmland in the Lower Mainland is dedicated to commercial highbush blueberry cultivation. B.C. reported 27,008 acres of highbush blueberries in 2021, accounting for 88.6% of Canada's total blueberry production (Statistics Canada, 2022.).

Additionally, the increase in highbush blueberry exports over the last five years has seen farmers replace raspberry crops with highbush blueberry, making highbush blueberry one of the most popular crops in the nation (Agriculture & Agri-Food Canada, 2023). As a result, highbush blueberries have emerged as one of the most sought-after crops in the country, with thriving cultivation in regions like Delta, Surrey, Langley, Abbotsford, and Chilliwack. Consequently, this industry provides employment opportunities for tens of thousands Canadian citizens each year (Guarna *et al*., 2019). This not only fuels the local economy, but also contributes to Canada's agricultural diversity, supplying consumers with nutritious and sought-after fruit.

In B.C., the contract pollination system for blueberries is a critical component of the region's highbush blueberry industry. Beekeepers and blueberry growers enter into agreements, known as pollination contracts, to secure the services of honey bee colonies for the pollination of blueberry crops. These contracts are essential because they help ensure the efficient and effective pollination of highbush blueberry fields, leading to increased fruit set and higher crop yields. Beekeepers transport their managed honey bee colonies to blueberry farms during the bloom period, where the bees play a pivotal role in transferring pollen from flower to flower, enabling successful fertilization and fruit development. For beekeepers, pollination contracts are a reliable and high-paying form of income for their business if bee health can be maintained throughout the contract term. Pollination contracts with blueberry growers present a significant financial opportunity for beekeepers, especially as the land area planted to blueberries continues to expand, now competing with apple orchards, raspberry farms, and cranberry farms for the most land covered by a single crop (Agriculture & Agri-Food Canada, 2023). The economic success of blueberry growers and the overall highbush blueberry industry in B.C. is closely intertwined with the collaborative relationship established through these pollination contracts, which support the essential service provided by honey bees.

As an essential component of highbush blueberry pollination, honey bee colonies are typically placed in fields for a period of 2 to 4 weeks during the bloom phase in B.C. This period aligns with the peak flowering time, usually in May, ensuring effective pollination for optimal fruit set and quality (Government of British Columbia, 2024). After this window, colonies are relocated to other crops or returned to their home apiaries to support additional agriculture needs or to recover from the stressors associated with pollination services. While this timeframe allows for efficient pollination, it also exposes honey bees to significant agricultural stressors, including

pesticide applications and limited floral diversity, which can impact their health and colony performance (Graham *et al*., 2021; Bobiwash *et al*., 2018). The intensive management of honey bee colonies for commercial pollination highlights the need for practices that minimize adverse effects during this critical period.

1.1.2 Current concerns between beekeepers and highbush blueberry growers

Recently, B.C. beekeepers have noticed a decrease in the health and strength of their colonies after participating in blueberry pollination. This issue has become increasingly apparent, prompting some beekeepers to reconsider their participation in blueberry pollination contracts altogether. The ramifications of such decisions are far-reaching, potentially having led to substantial financial losses estimated at over $12 million for blueberry growers in 2018 (Grauer, Z., 2019; Azar, P., 2018; King, D., 2019). In summary, this issue highlights the interdependent relationship between the honey and blueberry industries in B.C. and, by extension, Canada, where the health of honey bee colonies directly impacts the success of blueberry crops.

Numerous hypotheses have been put forward to address the complex dynamics of honey bee health within highbush blueberry crops in B.C. One prominent hypothesis revolves around the potential impact of agrochemicals, including fungicides, on honey bee colonies during the pollination season. Honey bees frequently experience prolonged exposure to various fungicides during the pollination of blueberries (Graham *et al*., 2021; Guarna *et al*., 2019; Rondeau *et al*., 2022; Thebeau, J. M., 2023). It is theorized that exposure to these chemicals may lead to adverse effects on bee health, compromising their immune systems, behaviour, and overall vitality (Mayer, D. F. & Lunden, J. D., 1986; Cullen *et al*., 2019). Additionally, the prevalence of pathogens and diseases in blueberry fields, specifically the bacterial infection European foulbrood (EFB) affecting hive larvae, has prompted questions about their role in honey bee

colony decline. Some scientists propose that the close proximity of honey bee colonies to blueberry fields may facilitate the spread of diseases, contributing to weakened colonies (Bristow, P. R. & Martin, R. R., 1999; Grant, K. J., 2021). It is hypothesized locally that climate and weather patterns in the coastal Lower Mainland region of B.C. may also have be impacting bee health (Islam, S., 2022; Moreno-Garcia, S., 2022; Tuell, J. K. & Isaacs, R., 2010). Furthermore, nutritional stressors, stemming from limited forage diversity, reduced pollen collection from blueberries during honey bee foraging, and blueberry pollen alkalinity, have been suggested as a potential factor in the reduced health of honey bees in these crop settings (Graham *et al.*, 2021; Bobiwash *et al*., 2018; Girard *et al*., 2012; Cortés-Rivas *et al*., 2023; Wardell, G. I., 1982).

It is not immediately apparent, however, why these issues should be that much worse in blueberry pollination compared to other crops grown in the same region. More recently, researchers are beginning to explore the impact multiple stressors (rather than single stressors) may have on bee health and blueberry pollination (Thebeau *et al*., 2023; Collison *et al*., 2016; T O'Neal *et al*., 2018). These hypotheses emphasize the varied challenges that honey bee populations face in highbush blueberry pollination and prove the importance of scientific investigation to elucidate the underlying causes of bee health decline to support the highbush blueberry and honey industries.

1.2 Honey bee health

The broader context of honey bee health further supports the urgency of addressing these concerns. Over the past decade, Canadian beekeepers have faced a distressing trend of losing more than a quarter of their colonies each winter. In 2023, the observed national winter loss

stood at 45.5% (Canadian Association of Professional Apiculturists, 2023). Notably, this figure represents nearly double the average annual loss recorded from 2007 to 2021, which was approximately 25.8% (Canadian Association of Professional Apiculturists, 2023). The multifaceted nature of this decline makes it a complex challenge to decipher. Potential factors contributing to hive declines encompass an array of variables, including adverse weather patterns, agrochemical exposure, diseases, and nutritional deficiencies, all of which could impact the health of honey bees (Figure 1.1). For instance, the United States Environmental Protection Agency (EPA) has acknowledged multiple factors influencing the decline of honey bee colonies, such as diseases and pests, poor bee nutrition, lack of genetic diversity, bee management practices, and pesticides (2018). Moreover, research published in the Journal of Economic Entomology highlights pesticides, parasites and pathogens, and nutritional deficiencies as factors implicated in the losses of honey bee populations due to their capacity to exert high stress on bees (Traver *et al*., 2018). Despite industry and government being aware of this issue, there has yet to be a large-scale B.C.-based project dedicated to investigating the specific stressors that may be associated with underperforming hives in highbush blueberry pollination.

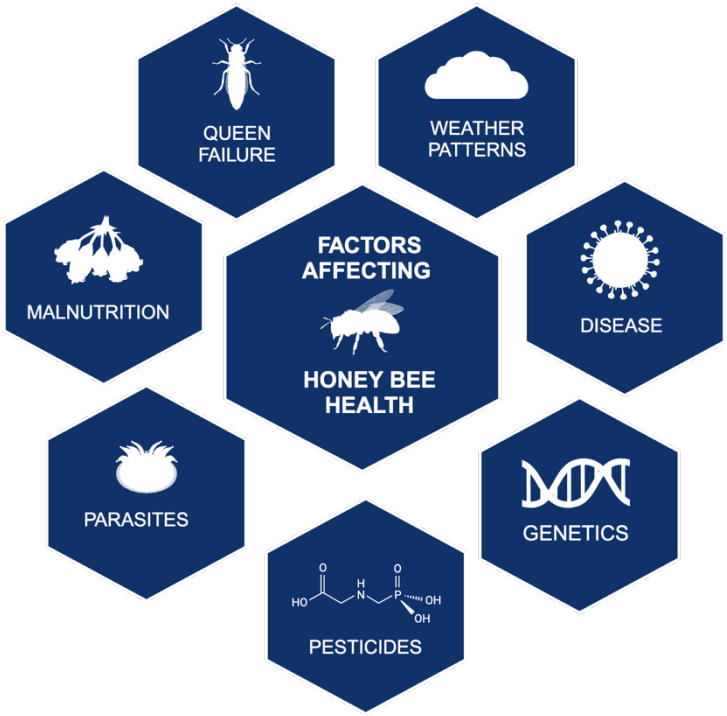


Figure 1.1 Factors impacting honey bee health.

Honey bees play a vital role in pollination, and understanding their biological cycle is crucial for assessing factors that impact their health. Worker bees, the most numerous members of a colony, are responsible for various tasks, including foraging, brood care, and hive maintenance. Foragers are worker bees that venture outside the hive to collect nectar, pollen, and water. Nectar, gathered from flowers, is stored in the bees' honey stomachs and is placed in hive cells where it is later converted into honey, serving as a carbohydrate-rich food source for the colony. Pollen, a protein source, is collected on the bees' hind legs and brought back to feed developing larvae. Bees also produce wax, which is secreted from glands on the abdomen of worker bees and used to build the honeycomb structure within the hive. The queen bee, the sole reproductive female in the colony, is responsible for laying eggs, which she does continuously throughout her life. She produces pheromones that help maintain colony cohesion and regulate

the behavior of worker bees. Brood-nest bees are younger worker bees that remain within the hive to care for the queen's eggs, larvae, and pupae, ensuring the growth of new generations. Worker bees transition from brood care to foraging as they age. Foragers typically fly within a 1.5 km radius of the hive but can travel up to 3-10 km when resources are scarce, allowing them to access a diverse range of flowers and crops.

1.3 Honey bee health stressors

1.3.1 Pathogens

Pathogens have the ability to alter the health of any organism, including insects. Beekeepers are constantly monitoring for pathogens that may be causing a colony's population to dwindle. Parasites, bacterial infections, and viruses are all disruptors of honey bee immunity (Grant, K. J., 2021) and, in severe cases, can kill a honey bee colony. Most pathogens are highly contagious and able to contaminant beekeeping equipment and neighbouring bees leading to more destruction. Commonly a hive will present symptoms for multiple pathogens therefore making diagnosis and treatment difficult for beekeepers.

Varroa destructor, or more commonly known by beekeepers as the *Varroa* mite, is a red-brown external parasite of honey bees (Peck, D. T., 2021). They mainly feed and reproduce on larvae and pupae in the developing bee brood, leading to malformed and weakened adult honey bees (Peck, D. T., 2021). Mites will often sit on the thorax of honey bees and feed on the hemolymph, transmitting viruses and disease while doing so. Perhaps equally as damaging, however, is that the mites also act as a vector for transmitting numerous viruses (Peck, D. T., 2021). *Varroa* mites are currently considered the most damaging pest of honey bees (Currie & Gatien, 2006; Gregorc *et al.*, 2018).

The list of viruses in honey bees is long and exhausting. Deformed wing virus (DWV), Lake Sinai virus (LSV), sacbrood virus (SBV), black queen cell virus (BQCV), Israeli acute paralysis virus (IAPV), *Varroa destructor* virus (VDV), chronic bee paralysis virus (CBPV), acute bee paralysis virus (ABPV), and Kashmir bee virus (KBV), are the viruses monitored and quantified in this study. Each virus targets the immune system of honey bees using a unique mechanism, leading to adverse biological function and behaviours and in severe cases the loss of whole colonies (De Miranda *et al.*, 2013). For example, bees infected with DWV often emerge from their pupal stage with visibly deformed wings, which appear shrunken, crumpled, or underdeveloped. These deformities prevent them from flying and effectively foraging, significantly impacting their survival.

Microsporidian parasites such as *Nosema* and bacterial infections like European foulbrood (EFB) are also common pathogens of honey bees. Nosemosis is caused by two species of microsporidian parasites, *Nosema apis* and *Nosema ceranae*, which reside in the gut of adult honey bees and prevent bees from fully harvesting nutrition from their food (Snow, J. W., 2022). EFB is caused by a non-spore-forming bacterium, *Melissococcus plutonius* and infects the digestive track of honey bee larvae, leaving them as a brown sticky mass in hive cells, rather than white and properly formed larvae (Forsgren, E., 2016). Once infected, most larvae die during the first week of their life (Forsgren, E., 2016).

Pathogen cross-transmission between managed honey bees and native pollinators is increasingly recognized as a significant concern, with implications for both honey bee health and broader ecosystem stability. Honey bees and native pollinators frequently share floral resources, providing opportunities for pathogen spillover through contaminated flowers (Dalmon *et al.*, 2021). As bees forage, they deposit or pick up viral particles, bacteria, and fungal spores on

flower surfaces, facilitating the movement of pathogens between different bee species (Piché-Mongeon & Guzman-Novoa, 2024). Pathogen exchange between managed and wild bees can facilitate the spillback effect, wherein pathogens circulate between wild pollinators and honey bee populations, amplifying their prevalence. For instance, Graystock *et al.* (2013) demonstrated in laboratory settings that DWV can be transmitted from honey bee colonies to bumble bee colonies, which subsequently infected additional honey bee colonies during robbing behaviors.

The increased pathogen load among native pollinator populations, facilitated by pathogen spillover from honey bees, could threaten the diversity and abundance of wild pollinators, ultimately impacting pollination services in both natural and agricultural ecosystems (Graystock *et al.*, 2013; Dalmon *et al.*, 2021; Piché-Mongeon & Guzman-Novoa, 2024). Therefore, understanding the dynamics of pathogen transmission between managed and wild pollinators is crucial for implementing effective disease management practices in apiculture and conserving native pollinator communities. Mitigating the effects of pathogen spillover could involve implementing clean stock programs, requiring health certifications for managed honey bee transport, controlling varroa mites and fungal pathogens, reducing colony density in apiaries, and enhancing wild pollinator habitats through hedgerow planting and field margin restoration to support native bee populations and reduce reliance on managed honey bees (Piché-Mongeon & Guzman-Novoa, 2024).

1.3.2 Pesticides

Highbush blueberry growers in B.C. use a variety of agrochemicals to control pests and diseases that threaten their crops. These agrochemicals, including fungicides, herbicides, and systemic and non-systemic insecticides, are essential for maintaining healthy blueberry bushes and ensuring abundant fruit set. These chemicals are applied directly to the crops at various

stages of growth, particularly during the bloom period when honey bees are actively pollinating. Consequently, honey bees are exposed to these chemicals while foraging on the treated plants. The Government of British Columbia maintains an extensive and informative production guide designed to help growers manage blueberry diseases, insects, and pests effectively by outlining specific agrochemical treatments and application times (2024).

Fungicides are used to control fungal diseases that can affect highbush blueberry and reduce blueberry yields, such as *Botrytis cinerea* (gray mold), *Alternaria spp*. (fruit rot), Athracanose fruit rot, *Armillaria spp*. (root rot), *Godronia cassandrae* (Godronia Canker), and *Monilinia vaccinii-corymbosi* (mummy berry), to name a few (Government of British Columbia, 2024). Fungicides are applied preventively or curatively to protect the plants from fungal infections. Herbicides are essential for weed control in highbush blueberry fields (Government of British Columbia, 2024). Weeds compete with blueberry plants for resources like water and nutrients, and their presence can hinder growth and reduce crop yields. Herbicides are used to manage weed populations and maintain the overall health of the blueberry plants (Government of British Columbia, 2024).

Systemic insecticides are designed to be absorbed by the plant and distributed within the plant so when insects feed on a plant treated with a systemic insecticide, they ingest the chemical along with the plant's nectar (Government of British Columbia, 2024). This is the same case for the systemic insecticides used on blueberry plants that provide protection against a range of insect pests that feed on the crop. They are effective against pests like aphids, Japanese beetles, thrips, and leafhoppers, which can damage blueberry foliage and fruit (Government of British Columbia, 2024). Among these, neonicotinoids have received particular attention due to their widespread use and potential impact on pollinators. These chemicals target the central nervous

system of insects, leading to paralysis and death. However, sublethal exposure can affect honey bees' behavior, such as foraging and navigation, and compromise their immune systems (Schneider *et al.*, 2012; Fischer *et al.*, 2014; Christen *et al.*, 2018). Despite the risks, honey bees possess natural defenses that help them combat certain stressors. For instance, they produce antimicrobial peptides, like defensins, that protect against pathogens by disrupting bacterial cell walls (Zhang *et al.*, 2021).

Non-systemic insecticides are contact insecticides that remain on the surface of the plant and are only effective when insects come into direct contact with the treated surfaces, such as leaves or stems (Government of British Columbia, 2024). In blueberry, they provide quick knockdown of insects that feed on plant surfaces and that might otherwise cause significant damage to blueberry leaves and fruits (Government of British Columbia, 2024). Acaricides are another class of agrochemicals, but used by beekeepers, not blueberry growers, and are important in honey bee colonies to control for the *V. destructor* mite (Currie, R. W. & Gatien, P., 2006). These acaracides are used by beekeepers to treat and eradicate *Varroa* mites in the hive and may linger in the colony post-application (Currie, R. W. & Gatien, P., 2006; Gregorc *et al.*, 2018; de Mattos *et al.*, 2017).

Despite their usefulness in controlling blueberry pests and diseases, agrochemicals applied by growers have significant negative impacts on honey bee health. Honey bees encounter these chemicals during pollination by contact or oral exposure, which can affect them in several detrimental ways. Pesticides can cause acute toxicity leading to immediate bee mortality or sub-lethal effects that impact bee behaviour, foraging efficiency, and overall hive productivity (D. F. & Lunden, J. D., 1986; Cullen *et al.*, 2019). Systemic insecticides, like neonicotinoids, have been shown to impact detoxification and metabolism pathways in pollinators, and likely affect their

ability to navigate and return to the hive (Christen *et al.*, 2018; Herbertsson *et al.*, 2022; Stanley *et al.*, 2016). Fungicides, though considered less toxic, can weaken the bees' immune systems and increase their susceptibility to pathogens (Rondeau & Raine 2022). Molecularly, fungicide exposure to commercially available fungicides has been linked to negative impacts on the mitochondria of honey bees (Campbell *et al.*, 2016). Herbicides, while not directly toxic, can reduce the availability of forage plants, leading to nutritional stress (Cullen *et al.*, 2019). Additionally, the synergistic effects of multiple agrochemicals can exacerbate their individual toxicities, leading to more severe impacts on bee health (Biddinger *et al.*, 2013). The widespread use of these chemicals in highbush blueberry cultivation thus poses a significant threat to the sustainability of honey bee populations and the essential pollination services they provide to the crop.

The provincial production guide for blueberry growers does not highlight the toxicity and threat of agrochemicals to honey bees (Government of British Columbia, 2024). Because the guide does not include current research on the negative impacts of agrochemicals in sublethal doses to honey bees, growers may be unaware of the physiological and behavioral damage they are causing the bees pollinating their crop. Growers are likely focused on addressing the issues with their crop first. Other conservation organizations, such as the Pollinator Partnership Canada, continue to work to support highbush blueberry cultivation by providing best practices to protect pollinators, an action guide for growers, and further resources for farmers to protect bee health (2021). Chemical toxins, including various agrochemicals, are subject to ongoing evaluations to assess their potential impact on honey bee physiology, and new research is continually added to the guide (Pollinator Partnership Canada, 2021; Liu *et al.*, 2023). This is an opportunity for the Government of British Columbia to include a similar practice and update their grower guide to

include the level of toxicity to honey bees of the agrochemicals they are applying and focus on the time of pollination when they are applying agrochemicals. More education, awareness, and resources for growers regarding the impacts of agrochemical exposure to honey bee health may positively impact the issue of honey bee health decline in blueberry crops.

Agrochemicals are commonly used in highbush blueberry cultivation to help manage plant health, control diseases, maximize yields, and maintain crop quality. Acaricides are an exception to this, as they are applied by beekeepers to maintain the health of honey bees against pests. However, it is imperative to use these agrochemicals responsibly, adhering to recommended application guidelines. It's crucial to recognize that, even when applied as per label instructions to the plant or plant area, agrochemicals can still find their way to pollinators and their homes, posing a potential risk to their health. Furthermore, pollinators brought in to pollinate highbush blueberries might also forage in other agricultural or natural settings, inadvertently picking up various agrochemical residues from other commercial crops in the surrounding area (Graham *et al.*, 2022 & Averill *et al.*, 2024). This emphasizes the need for continuous strict agrochemical management, which considers the broader ecosystem and the potential impacts of agrochemicals on non-target organisms, such as honey bees, which play a crucial role in the pollination of highbush blueberry flowers.

1.3.3 Nutrition

The nutritional health of honey bees impacts their overall well-being, immune health, and capacity for pollination. In the context of highbush blueberry cultivation, the nutritional value of blueberry pollen is a topic of emerging research interest and concern, particularly regarding its adequacy in supporting honey bee health during intensive pollination periods.

Recent studies have highlighted that while blueberry pollen can be a source of essential nutrients for bees, it may not provide a balanced diet when compared to the diverse forage available in natural habitats (Bobiwash, *et al.*, 2018). Blueberry pollen is relatively low in several essential amino acids crucial for honey bee growth and immune function, particularly isoleucine, valine, leucine, and phenylalanine Bobiwash *et al.*, 2018 & Di Pasquale *et al.*, 2013). This limitation can stress bees, especially when they are confined to monocultural fields where blueberry plants are the primary pollen source (Brodschneider, R. & Crailsheim, K., 2010). Moreover, given a choice, honey bees prefer other food sources over highbush blueberry pollen, likely due to its poor nutritional value (Bobiwash *et al.*, 2018).

Singularly relying on blueberry pollen has been associated with reduced bee longevity and health, leading to concerns about the sustainability of bee populations used for commercial pollination (Zhang *et al.*, 2020). Inadequate nutrition from monoculture pollen sources exacerbates the impact of other stressors such as pathogens and pesticides, thereby compounding the risk to honey bee colonies (Alaux *et al.*, 2017). A balanced diet is essential for the maintenance of the honey bee gut microbiome, which in turn supports digestion and nutrient absorption, and plays a protective role against pathogens (Engel *et al.*, 2016). Insufficiently diverse or nutritionally incomplete pollen intake can disrupt this microbial balance, potentially increasing susceptibility to diseases and decreasing detoxification efficacy against agrochemicals (Castle *et al.*, 2022 & Di Pasquale *et al.*, 2013).

To mitigate the nutritional stress imposed by monofloral foraging on blueberry crops, researchers have recommended supplemental feeding strategies and the conservation of native floral resources in agricultural landscapes (Di Pasquale *et al.*, 2013; Zhang *et al.*, 2023). These management practices are intended to provide bees with a more diverse and complete spectrum

of nutrients, helping to buffer the colonies against other stressors and enhance their hive populations and longevity (St Clair *et al.*, 2020).

1.4 Current honey bee health diagnostic tools

The challenge faced by beekeepers, institutional research, and government regulators in accurately identifying and distinguishing among the diverse stressors impacting honey bee colonies has hindered their ability to effectively monitor and improve honey bee health. The beekeeping industry currently relies on post-mortem analysis to test for the presence of several known pathogens or toxins in dead colonies and bees (University of Guelph, n.d.; Northern Alberta Institute of Technology, n.d.; Reitsma, L., 2023). The search continues for quick and cost-effective disease detection and prevention in Canadian honey bee colonies to aid beekeepers in managing disease, such as those services offered by the Animal Health Laboratory at the University of Guelph, the National Bee Diagnostic Centre with the Northern Alberta Institute of Technology, and more (Reitsma, L., 2023; Hoftyzer, E., 2023). When it comes to blueberry pollination, the specific risk factors, and potential stressors – which may include pesticides and pathogens – that compromise bee health is still not clearly understood. Moreover, rapid testing for these stressors in in hives remains unavailable, making it difficult to determine whether they are acting independently or collectively to disrupt hive health.

This project is part of a larger, multi-year, trans-Canada initiative called BeeCSI, which involves a team of researchers from across the country, including the Foster laboratory. The primary goal of BeeCSI is to enhance honey bee health by developing a novel health assessment and diagnostic tool. This initiative focuses on identifying stressor-specific markers in the microbiome, proteins, and gene expression. BeeCSI utilizes laboratory-controlled cage trials and

data collected from hives of various crop types to find these biomarkers. The project collaborates with industry technology-transfer teams, diagnostic labs, and regional and provincial regulatory agencies to ensure the tools are implemented and available to the beekeeping industry. These tools aim to reduce uncertainty about the factors affecting bee health and colony losses, providing beekeepers with an objective method to diagnose struggling colonies. This project will contribute to the expansion and sustainability of the beekeeping sector, which sustains a multibillion-dollar agroeconomy in Canada (Genome Canada, 2019; Genome BC, 2019; Government of Canada, 2019).

1.5 Proteomics for honey bee health

The application of 'omics approaches, including genomics, transcriptomics, and proteomics, are modern and powerful tools that have and will continue to revolutionize the diagnosis and understanding of stressors affecting honey bee health. Among these tools, proteomics offers a comprehensive view of the protein composition within honey bees. Understanding the proteome – the complete set of proteins expressed in an organism – is pivotal in understanding the biochemical processes that control biological health. Proteins are involved in virtually every aspect of a honey bee's physiology, from immune response to foraging behaviour. It is anticipated that the composition of the honey bee proteome varies significantly with health status, making proteomics a strong tool to pinpoint stressors and their impacts (Chan *et al.*, 2009; McAfee *et al.*, 2021; McAfee *et al.*, 2021; Chapman *et al.*, 2022).

By examining the proteins within honey bee colony populations near and far from highbush blueberry crops, this research aims to elucidate the molecular mechanisms impacted by crop-specific stressors, such as pesticides and pathogens. Specific stressors may manifest at the proteomic level, leading to the discovery of a more nuanced understanding of factors

contributing to hive decline in highbush blueberries. The proteomic analysis conducted in this study will therefore help bridge the gap between the external stressors affecting honey bees and their internal biological responses. As a result, it aims to provide insights that can inform targeted strategies for mitigating these stressors and ultimately bolstering honey bee health, particularly in the context of blueberry pollination.

1.6 Project aims

This project shares data with the larger BeeCSI initiative, which aims to quantify biomarkers and develop accessible diagnostic tools to support Canadian beekeepers in safeguarding honey bee health (Genome Canada, 2019; Genome BC, 2019, Government of Canada, 2019). Highbush blueberries in B.C. are one of several crops used in the BeeCSI study, chosen due to their significance in honey bee pollination, the challenges faced by beekeepers, and their impact on the broader agricultural landscape.

High-quality highbush blueberry pollination is essential for the continued success of the blueberry industry in B.C. Beekeepers have reported declines in colony health following blueberry pollination, a critical concern for both the beekeeping and blueberry sectors. As such, highbush blueberry serves as an ideal model to investigate the stressors affecting honey bee colonies during the pollination period.

While our primary focus is on highbush blueberries, the knowledge gained from this research extends beyond this specific crop because bees are exposed to the same stressors in other crop systems. The findings have the potential to benefit beekeeping practices not only in B.C. but also in regions and for crops facing similar challenges. Understanding the major determinants of honey bee health during blueberry pollination can lead to more effective

strategies for understanding and managing stressors, thereby contributing to the well-being of honey bee colonies and the success of crop pollination services.

This project hypothesizes that the stressors affecting honey bee health in highbush blueberries cause changes in the proteome of brood-nest bees that can be used to diagnose the impact of that stressor. Our specific objectives are to 1) correlate the differences in the proteome of brood-nest bees before, during, and after blueberry pollination with the agrochemicals and pathogens present in real-time blueberry fields and 2) segregate proteome changes caused by individual stressors to define the main causes of stress in blueberry pollination. Validating this hypothesis will shed light on the complex interactions between honey bees, agrochemicals, and pathogens during highbush blueberry pollination. This understanding is essential for implementing science-based practices to safeguard honey bee health and, by extension, enhance the productivity and sustainability of highbush blueberry pollination services.

Chapter 2 Proteomic responses in honey bees to highbush blueberry pollination stressors

2.1 Introduction

Honey bees (*Apis mellifera L.*) are indispensable pollinators in agricultural ecosystems, contributing significantly to global food security and agricultural economies. In B.C., the highbush blueberry industry heavily relies on these insects for the pollination services they provide. These services are estimated to add up to $7 billion annually to the Canadian economy, with highbush blueberries (*Vaccinium corymbosum L.)* being the nation's top fruit export by value (Agriculture & Agri-Food Canada, 2021). Managed honey bees are often placed in blueberry fields to maximize fruit set, complementing the efforts of native pollinators like *Bombus* species (Government of British Columbia, 2024). However, the health of these managed honey bees is becoming a growing concern, particularly in the context of their role in blueberry pollination.

Recent observations by B.C. beekeepers indicate a decline in colony health post-pollination, threatening the symbiotic relationship between beekeeping and blueberry cultivation. These health issues have prompted a re-evaluation of pollination contracts, with some beekeepers declining contracts, potentially leading to significant economic repercussions (Grauer, Z., 2019; Azar, P., 2018; King, D., 2019). The decline in honey bee health has been hypothesized to be multifactorial in blueberry, with agrochemical exposure, pathogen prevalence, and nutritional stressors being prime suspects (Thebeau *et al.*, 2023; Collison *et al.*, 2016; T O'Neal *et al.*, 2018). Amidst these concerns, the Canadian beekeeping industry faces an alarming trend of colony losses, with more than a quarter of hives not surviving the winter for nearly two decades now (Canadian Association of Professional Apiculturists, 2023). The reasons behind this decline are complex, involving multiple stressors that are yet to be fully understood.

To date, a comprehensive investigation into the specific stressors associated with hive performance in highbush blueberry pollination within B.C. has not been conducted.

Pathogens, such as *Varroa* mites and various viruses or bacterial infections, are known to compromise bee immunity and colony health. These pathogens can be highly contagious and are often responsible for weakening and sometimes decimating colonies. The use of agrochemicals in agriculture, including acaricides by beekeepers, is another area of concern. These chemicals, while crucial for crop production, can pose risks to pollinator health in a multitude of ways still being explored in their effects as individual and combined stressors (Stokstad, E., 2021; Almasri *et al.*, 2022; Tesovnik *et al.*, 2020).

The BeeCSI initiative is a collaborative effort to develop new diagnostic tools for honey bee health assessments for Canadian beekeepers using 'omics data from various crops and hives from all over the country (Genome Canada, 2019; Genome BC, 2019, Government of Canada, 2019). This project aims to expand upon BeeCSI's efforts by focusing on the proteomic changes in brood-nest bees exposed to blueberry pollination. Proteomics, the study of the full set of proteins expressed by an organism, offers a window into the internal biological responses of honey bees to external stressors and has the potential to reveal the molecular underpinnings of hive decline as previously proven by research in the Foster lab (Chan *et a*l., 2009; McAfee *et al*., 2021; McAfee *et al.*, 2021; Chapman *et al*., 2022)..

The objectives of this study are twofold: to correlate proteomic changes in brood-nest bees with the presence of agrochemicals and pathogens in blueberry fields and to isolate the effects of individual stressors on the honey bee proteome. Achieving these goals will provide valuable insights into the stressors affecting bee health during blueberry pollination, ultimately

aiding the development of mitigation strategies to support the sustainability of the beekeeping industry and the pollination services it supplies to our agroeconomy in B.C.

2.2 Materials and Methods

2.2.1 Field experimental design

Over two years (2020 and 2021), standard double-brood-box honey bee colonies were distributed randomly to two types of apiaries in the Lower Mainland of B.C. The first type, referred to as exposed sites, was located on or near (within 500 m of) the target crop, highbush blueberry. The second type, known as unexposed sites, were situated far away from the target crop and ideally distant from other agricultural areas. Unexposed hives needed to be 1.5 km away from the target crop, greater than 5 km away from any other crops. See Figure 2.1 for a diagram depiction of exposed and unexposed sites. All but one site was reused in both years for the experiment; one exposed site was moved between 2020 and 2021 due to it no longer being available to us in the 2nd year. Figure 2.2 is a map of the exact field site coordinates and site types for the experiment in the Lower Mainland region of B.C., specifically between Delta and Langley. Specific latitudes and longitudes for all experimental sites are written in Table 2.1.

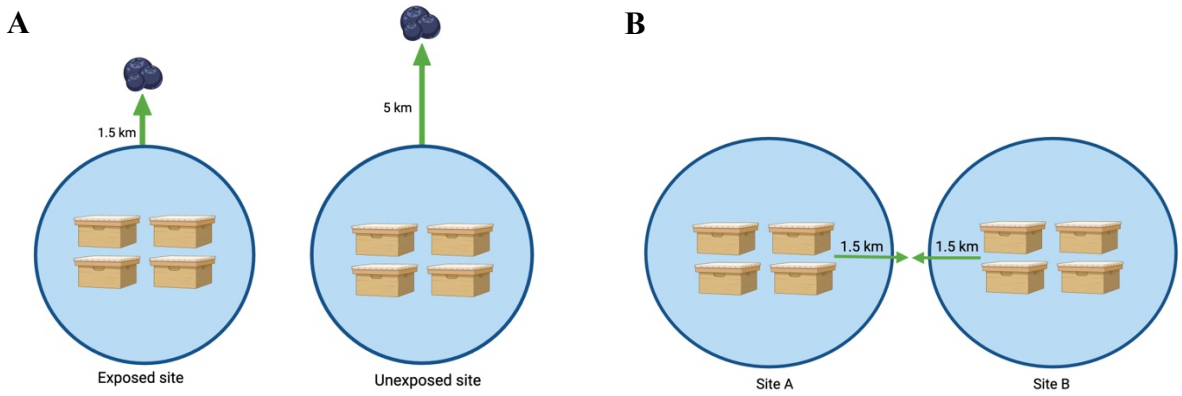


Figure 2. 1 Defining exposed and unexposed sites and organizing quads of hives within sites. a) Finding exposed and unexposed sites. Most bees forage within 1.5 km of their colony. Therefore, unexposed sites should be a minimum of 1.5 km away from crops. However the ideal unexposed site would be greater than 5 km away from any crops. b) Distance between independent quads of hives within the exposed and unexposed sites. Figure made in Biorender.

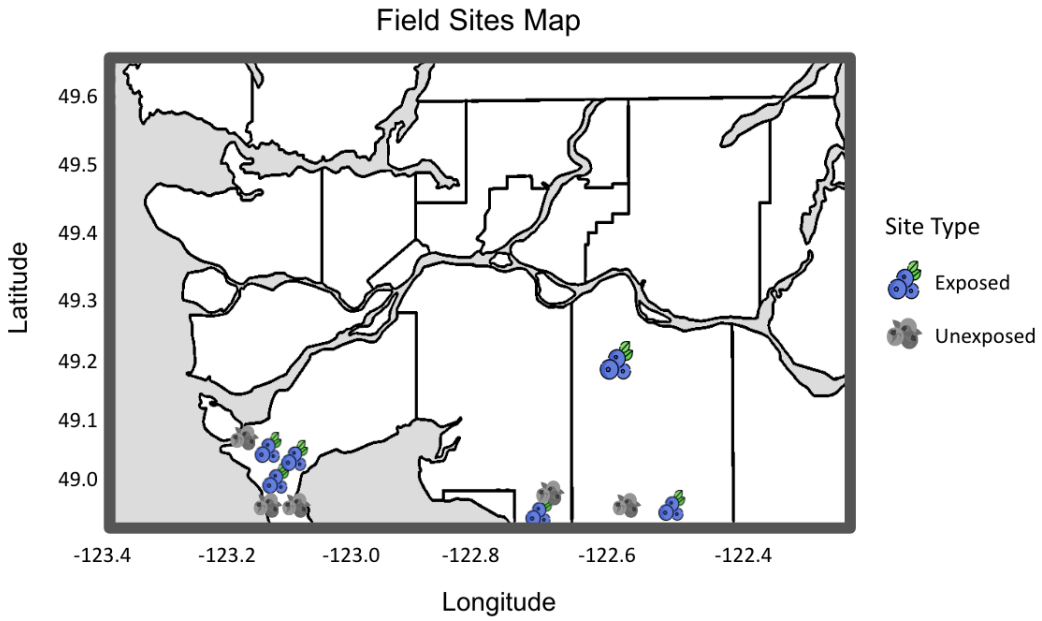


Figure 2. 2 Field site map of colonies exposed and unexposed to highbush blueberry crops in the Lower Mainland of BC. Each year of the two-year experiment there were five exposed (blue berries) and unexposed sites (grey berries). All but one site was reused in the exposed sites in 2021. Figure made in Biorender.

Biological replicates were defined by their site type. Per year there were five exposed and unexposed replicates. In addition to this, technical replicates were used within the biological replicates to capture variance in sampling. Replicates were defined as pallets of hives where each pallet held four individual colonies. See Table 2.1 for a complete breakdown of replicates and site coordinates at TP2 (peak bloom) used in the experiment.

Table 2.1 Biological and technical replicates used in the highbush blueberry field trial and site coordinates at TP2.

Experiment label	Replicate	Site type	Year	Number of colonies	Site coordinates
HBB01e	1	Exposed	2020	1-4	46.0609001, -123.0632941
HBB01u	1	Unexposed	2020	1-4	49.011756, -123.0903459
HBB02e	2	Exposed	2020	1-4	49.0453579, -123.0746929
HBB02u	2	Unexposed	2020	1-4	49.0120709, -123.0519279
HBB03e	3	Exposed	2020	1-4	49.0780343, -123.1142132
HBB03u	3	Unexposed	2020	1-4	49.1011624, -123.1697091
HBB04e	4	Exposed	2020	1-4	49.0105383, -122.6996533
HBB04u	4	Unexposed	2020	1-4	49.032486, -122.6844003
HBB05e	5	Exposed	2020	1-4	49.1186726, -122.581956
HBB05u	5	Unexposed	2020	1-4	49.0208407, -122.5693345
HBB06e	6	Exposed	2021	1-4	49.0184061, -122.4852703
HBB06u	6	Unexposed	2021	1-4	49.0208407, -122.5693345
HBB07e	7	Exposed	2021	1-4	49.0105583, -122.6996533
HBB07u	7	Unexposed	2021	1-4	49.032486, 0122.5693345
HBB08e	8	Exposed	2021	1-4	49.0780343, -123.1142132
HBB08u	8	Unexposed	2021	1-4	49.1011624, -123.1697091
HBB09e	9	Exposed	2021	1-4	49.0609001, -123.0632941
HBB09u	9	Unexposed	2021	1-4	49.011756, -123.0903459
HBB10e	10	Exposed	2021	1-4	49.0453579, -123.0746929
HBB10u	10	Unexposed	2021	1-4	49.0120709, -123.0519279

A total of 40 colonies (four colonies over five exposed and five unexposed sites) were used for each year of the field experiment. Colonies were roughly standardized for strength

based on crop and regional specific practices, and in the case of commercial pollination of blueberries, this meant that all colonies were "double-brood" (two single-brood boxes on top of each other). Colonies were sourced from local beekeepers experienced in blueberry pollination, Heather Higo and Julia Common. Because these hives were sourced from multiple beekeepers, the colonies were split randomly between exposed and unexposed sites to account for genetic diversity. All colonies were required to have a laying queen that was ideally 1 year old, but this criterion was not rigorously monitored for throughout the active experiment. The colonies also needed to have local Canadian genetics (i.e., not recently imported from offshore).

All the colonies in both site types were subjected to the same colony management practices. This meant that if the exposed colonies were fed or treated then the unexposed colonies were similarly fed or treated. Queenless colonies (colonies without a queen) were re-queened, meaning a new, mated queen was placed in the hive. Colonies that needed supers (room for honey production above the colony) were equipped with a super and a queen excluder to allow the bees more room. If a colony was weak or had a small population, it was appropriate to swap a brood frame from a stronger colony from the same group of four colonies at that site, and this change was recorded in the apiary log. If a colony met the criteria for removal from the experiment due to extreme illness or dramatic population decrease, the colony was sampled as per the protocol and then removed from the experiment.

At four times each year, the exposed and unexposed hives were sampled for adult brood-nest bees, pollen, bee-collected nectar, and wax. Before exposure (Timepoint 1, TP1) was shortly before moving colonies to exposed and unexposed sites; at this stage, all colonies were located in their unexposed "home sites." Peak exposure (Timepoint 2, TP2) was when most of the crop was in bloom and colonies were moved either into exposed highbush blueberry fields or designated

unexposed sites. End of exposure (Timepoint 3,TP3) was just prior to moving the colonies out of exposed and unexposed sites. After exposure (Timepoint 4, TP4) was then two weeks after the end of pollination, when colonies were returned to their unexposed "home sites." Figure 2.3 shows a general overview of the field experiment with timepoints for sampling and Table 2 shows exact sampling dates from both years of the experiment.

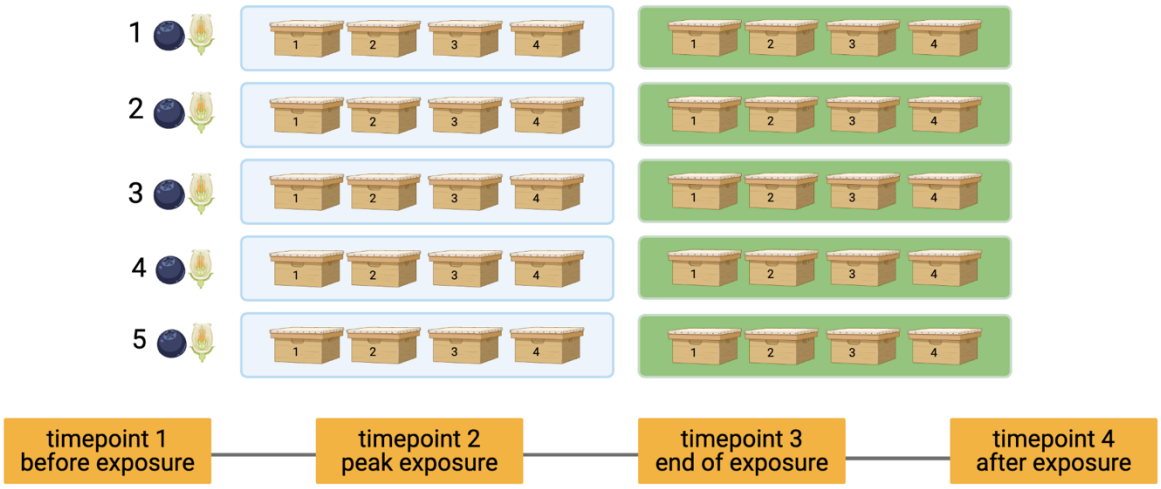


Figure 2. 3 General Overview of the experimental design for the field trial. Hives were either designated as "exposed" or "unexposed" depending on their proximity to the target crop. Over four timepoints, correlated with highbush blueberry pollution, the hives were sampled for brood-nest bees, pollen, bee-collected nectar, and wax. Figure made in Biorender.

Table 2.2 Field sampling dates for brood-nest bees, pollen, bee-collected nectar, and wax over the two years of the experiment.

Sampling date	Time point
2020 April 27	T1
2020 May 11	T2
2020 May 26	T3
2020 June 11	T4
2021 April 23	T1
2021 May 10	T2
2021 May 21	T3
2021 June 2	T4

Brood-nest bees were collected from a hive frame that contained both older, capped and younger, uncapped brood. These frames are typically found near the middle of the hive. An empty 50 mL falcon tube was rolled along the frame at the centre over the capped brood to collect brood-nest bees. Pollen was collected from any frame(s) in the brood boxes. Bee bread was ideally avoided, and fresh pollen was preferred. Approximately 8 mL of pollen from a variety of sources was collected from the cells using a new wooden stir stick that functioned as a scooper. A total of 3 mL of bee-collected nectar was collected from feeder frames with plastic syringes. Wax was only collected during TP3 and a hive tool was used to scrape 6 hive tool-sized pieces (5 X 5 cm approximately) into a freezer bag from the pallet of the four colonies. All samples were places on dry ice in the field after being collected and stored long-term at the laboratory in the -70 °C freezer.

2.2.2 Qualitative assessments of field hives

Each apiary colony was assessed prior to collecting samples. There were several parameters being assessed for during the hive inspection, including the presence or absence of the queen herself and/or the presence of fresh eggs and larvae, any signs of disease, and the

overall strength of the colony's population. The assessment started by going into the bottom brood box of the colony and removing frames one by one to find the queen and to look for fresh eggs and capped brood. It is routine beekeeping practice to also monitor for any signs of disease. If the colony looked strong with a good brood pattern (e.g. capped brood in the centre of the frame with uncapped egg and young larvae on the peripheral) and a healthy population then this was also noted. All comments regarding the strength of the colony, behaviours, or potential signs of disease were put in the comments section of the apiary log. Date of inspection and sampling, site name, colony number, and whether all samples were collected were also noted. In addition to this, the initials of the beekeeper who performed the inspection were also included.

2.2.3 Cage experimental design

In the experimental design of the cage trials, the selection of specific stressors was strategic and informed by both practicality and relevance to the field conditions of honey bees. Pyrimethanil, a fungicide used in highbush blueberry crop, and DWV (A and B strains), a virus in honey bee colonies, were selected for cage experimentation. DWV was chosen as the viral agent due to its presence in the field samples as well as its ease of handling and availability as a pure stock in the lab in both A and B genotype forms. This facilitated the experimental process without the need for additional preparation. It was anticipated that the lab's existing stock of DWV, prepared for a similar BeeCSI experiment by collaborators at the University of Manitoba, provided a consistent and reliable source of the virus, ensuring the reproducibility and comparability of results. The fungicide pyrimethanil was selected based on its high prevalence in the field samples, where it was most frequently detected in the tissues of exposed honey bees as a pesticide residue. This prevalence is indicative of the bees' potential for regular encounter and

uptake of pyrimethanil in highbush blueberry fields, making it an appropriate choice for assessing realistic environmental stressors.

Pyrimethanil and DWV- A and B were fed alone and in combination to brood-nest bees in cage trials. The DWV A and B genotype experiments were independent of each other; thus, the cage trials were performed twice over. Six hives were used as biological replicates, and each replicate was divided into four experimental cages. The experimental cages were control (dO), pyrimethanil exposure (dP), DWV A or DWV B exposure (dD), and pyrimethanil and DWV together (dX). Each cage was filled with 60 brood-nest bees from a brood frame with capped and uncapped brood using soft forceps. Figure 2.4 is a visual outline of the cage experimental design with biological replicates and treatment groups. All hives used for brood-nest bee sourcing were Foster lab research hives located on the roof of the NCE building at the UBC Point Grey campus or at the UBC Farm on the same campus.

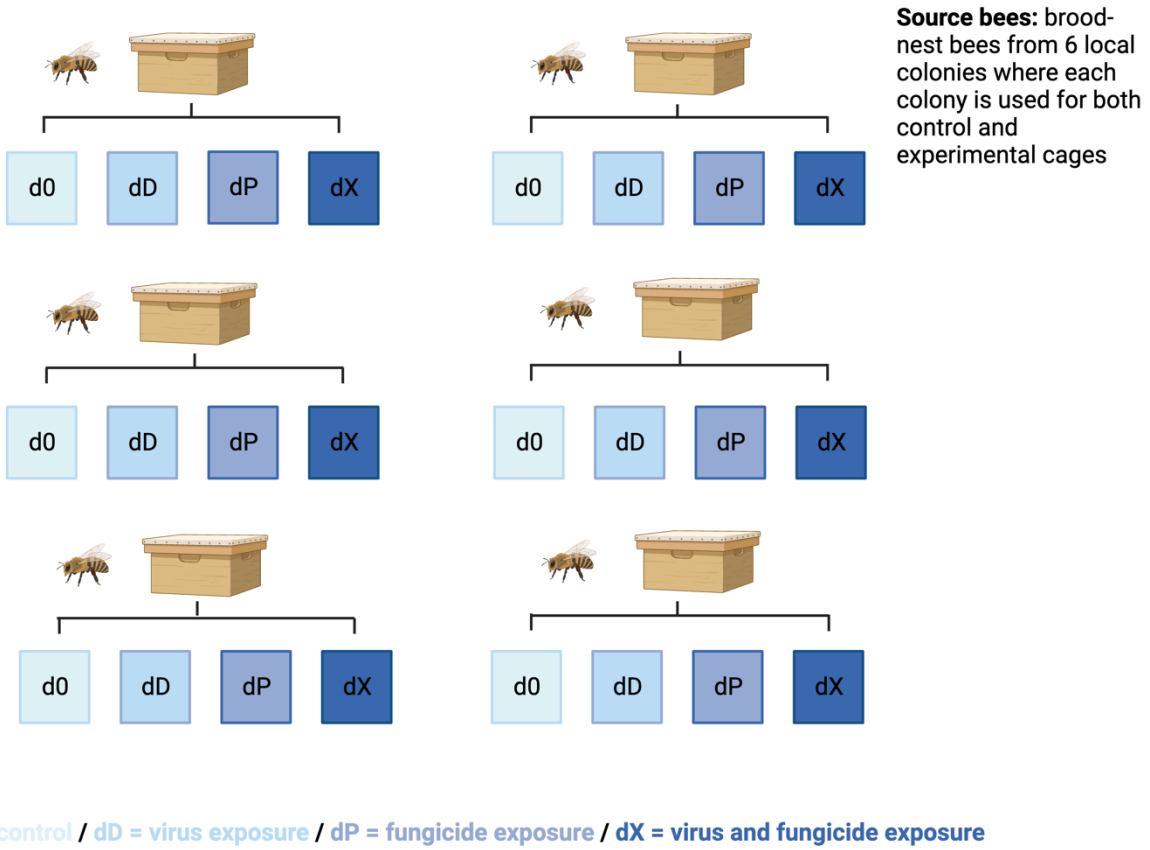


Figure 2. 4 Cage experimental design for pyrimethanil and DWV-A and -B exposure. Brood-nest bees were sourced from six research colonies located at UBC. Each colony was used to fill four cages and was designated as a replicate. The four cages of the experiment indicate what treatment they will receive. The cages were outlined as follows: control (dO), virus exposure only (dD), fungicide exposure only (dP), and virus and fungicide exposure together (dX). Figure made in Biorender.

After collecting brood-nest bees into the cages additional bees were collected to assess the levels of mites and DWV. For the mite count, a standard 50 mL urine vial was used to collect approximately 100 brood-nest bees from frames towards the center of the hive. The urine vial is gently pulled down on the hive frame to allow bees to become unbalanced and collected in the vial. The urine vial was immediately filled with ethanol to drown the bees quickly and to prepare for an alcohol-mite wash, a technique commonly used among beekeepers for mite counts, and immediately taken to the lab for processing. Mite counts were expressed as a percentage of mite infestation, calculated as the number of mites detected per approximately 100 adult honey bees

sampled from a colony. This method provides an estimate of infestation intensity rather than an absolute mite count, enabling comparison across colonies and timepoints. The same methodology was used to collect brood-nest bees for the DWV sampling, except these were collected into a 50 mL conical tube that was stored at -70 °C to preserve ribonucleic acid (RNA) integrity for downstream processing. This subset of bees was used to provide a snapshot of the virus levels within the colony. DWV levels were quantified using real-time polymerase chain reaction (PCR) to measure viral load, expressed as Cq values. This method enabled relative quantification of DWV RNA, offering a practical and reproducible way to estimate viral prevalence and intensity across colonies.

Immediately after collecting the brood-nest bees into their designated cages, the cages were placed in the 37 °C incubator where they stayed until the end of the experiment. All cages were starved for the first 2 h of being in the incubator to ensure that they would be hungry for sugar syrup when it was presented to them. Each cage received one 15 mL tube of water filled to 10 mL of sugar syrup (1:1 sugar to H_2O), which was also replenished every 24 h. Caged bees were exposed to the fungicide, virus, or both via the sugar syrup feeders in sublethal doses (Table 2.3). The pyrimethanil dose was calculated by taking the average amount of pyrimethanil found in the nectar and pollen samples from the field experiment. The pyrimethanil dose was calculated by dissolving 2.06 µL of a 1 mg/mL stock solution into 55 mL of sugar syrup, resulting in a final concentration of 37.5 ppb, based on the average amounts detected in field nectar and pollen samples (Table 2.3).

The experiment lasted several days to ensure incubation of the virus. Following the 2 h starvation period, the bees were fed sugar syrup spiked with DWV-A or -B virus or plain sugar syrup depending on their designated treatment group. The DWV-A and DWV-B doses were

prepared by dispersing the virus aliquots directly into plain sugar syrup to achieve a final titer of 10^8 genomes per bee, with 3 mL of the prepared syrup provided per 60 bees (Table 2.3). The aliquot concentrations used were 1.16×10^9 gene copies/µL for DWV-A and 1.00×10^9 gene copies/µL for DWV-B. All the caged bees were monitored for mortality and fed plain sugar syrup and water for the following 7 d. Pyrimethanil exposure occurred on the final day of the experiment, day eight. Mortality was again assessed at the beginning of day eight and bees were starved for 2 h prior to adding the sugar syrup feeders again, this time spiked with pyrimethanil. Feeders were weighed at the beginning and end of the pyrimethanil exposure period of 6 h. After 6 h, the feeders were removed and mortality for each cage was quantified. Excess plain sugar syrup was fed to the caged bees for 2 h following the experiment. Finally, mortality was assessed again, and the caged bees were collected for proteomics analysis and placed in the -70 °C to await sample processing for proteomics.

Table 2.3 Virus and fungicide concentrations in sugar syrup used in cage trials.

a) DWV-A and -B dose calculations for cages receiving only virus and virus and fungicide combined. Virus was dispersed from the aliquot directly into plain sugar syrup to achieve the desired titre.

Virus	Dose[1]	Titre	Aliquot concentration
DWV-A	3 ml per 60 bees	10^8 genomes/bee[2]	1.16 x 10^9 gene copies/µL
DWV-B	3 ml per 60 bees	10^8 genomes/bee[2]	1.00 x 10^9 gene copies/µL

1. Based on Desai *et al.* 2012.
2. Based on the high dose in Schittny *et al.* 2020, which did not cause significant bee mortality in 6 d but affected survival around day 8 or 10 d. Their medium and low dose were similar in terms of bee survival curves.

b) Pyrimethanil dose calculations for all cages. Pyrimethanil, the fungicide, was administered to cages labelled for fungicide and virus and fungicide. Pyrimethanil was dissolved in acetone and then added to plain sugar syrup. Pyrimethanil experimental dose was calculated using average concentration amounts from the field experiment (ppb).

	Control	Virus	Fungicide	Virus and Fungicide
Pesticide	0 µl	0 µl	2.06 µl of stock solution (1 mg/ml)	2.06 µl of stock solution (1 mg/ml)
Acetone	2.06 µl	2.06 µl	0 µl	0 µl
50% syrup	54.997 ml	54.997ml	54.997 ml	54.997 ml
Total	55 ml	55 ml	55 ml	55 ml

2.2.4 Honey bee dissection and homogenization

Dissection

Proteomic analysis was performed on three honey bee tissues: head, gut, and abdomen (minus the gut) from field and cage bees. For the field replicates, tissues were pooled from all four colonies during dissections. For example, the sample HBB01e_t1 consisted of replicates HBB01e1_t1, HBB01e2_t1, HBB01e3_t1, and HBB01e4_t1. A total of 12 bees were selected for the field samples, meaning three from each of the four replicates. The cage replicates were not pooled and were left as individual samples. For example, DWVAXP01_dO was unique from DWVAXP02_dO. A total of 10 bees were dissected per cage sample.

Bee samples were collected from the -70 °C freezer and kept on wet ice during dissection. Cold 1X PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) wash and a gentle vortex was used to quickly wash wax and other debris from the bees. The gut sample was collected first and consisted of the hindgut and the midgut. Using two sets of forceps and a plastic tray on ice, the thorax was held in place while the bottom section of the abdomen and the stinger were gently pulled to reveal the gut. The stinger and honey crop were removed prior to collecting the gut for sampling. Ruptured guts were not included in the final sample. The remainder of the abdomen was then spliced from the thorax using small scissors. Using the scissors again the antennae were removed from bees. The head was then spliced and collected for

sampling. Steps were repeated on bees until the final number of 12 or 10 bees were reached, depending on whether it was a field or cage sample.

Gut, head, and abdomen tissues were collected separately in 2 mL screw-cap tubes. Each tube also contained three ceramic beads which aid in tissue lyses. All the guts were placed inside one tube. Heads were divided among two tubes and abdomens were split across six tubes for the field samples. For the cage trials five heads were placed in two tubes and two abdomens were divided amongst five tubes. Separating the same tissues into multiple tubes was to accompany the large amount of tissue and buffer combined with space for beads, but after milling, split samples were combined.

To minimize batch effects during proteomic sample preparation, all samples were randomized prior to processing. This included randomizing the order of protein extraction, purification, and subsequent preparation steps to ensure that any potential variation introduced by processing time or equipment use was evenly distributed across all sample groups.

Homogenization

For tissue homogenization 1 mL lysis buffer (4% SDS, 100mM Tris pH 6.8, 1X protease inhibitors) was added to each sample and spun down. Tubes now consisted of honey bee tissue, ceramic beads, and lysis buffer. Samples were homogenized in a bead mill (Precellys 24, Bertin Instruments) for 30 s at 6,500 m/s and left on ice for 1 min to cool down. Samples were spun in the centrifuge at room temperature for 15 s at 14,000 *g*. The process of homogenization was repeated twice more. Samples were boiled at 95 °C for 5 min on the heating block (Eppendorf 5355 Thermomixer R) then centrifuged for 5 min at 14,000 *g* at room temperature. The lysate for gut, head, and abdomen tissue was transferred to 1.5 mL microcentrifuge tubes. Head and

abdomen samples were pooled together here. Two aliquots were created for each sample and tissue and stored in the -70 °C freezer immediately.

2.2.5 Honey bee protein extraction, purification, and quantification

Protein quantification and extraction

A standard bicinchoninic acid (BCA) assay was used to quantify the total protein in the samples. Heads and abdomen were diluted 30 times and gut 50 times with ultra pure water. Diluted samples were prepared with BCA and read at 562 nm using a plate reader (SpectraMax 190, Molecular Devices).

Prior to running the samples on a gel, the protein lysates were suspended in 100 mM Tris pH 8.0 to have 40 µg for head, 20 µg for abdomen, and 60 µg for gut tissue in new Eppendorf tubes. For the reduction step 1.5 µL of 300 mM dithiothreitol (DTT) was added to the protein buffer mixture and allowed to incubate in the dark at 37 °C for 30 min. Following this, 1.5 µL of 500 mM chloroacetamide (CAA) was added to alkylate the protein while incubating for 20 min at 37 °C in the dark. Three microlitres of heated 5X sample loading buffer and 1.5 µL of 300 mM DTT were added to the sample tubes and vortexed briefly. Samples were then heated on the heating block at 90 °C for 5 min and centrifuged briefly.

Most gels were run using precast gels (Mini-PROTEAN TGX, Bio-Rad) and any additional Tris-Glycine SDS gels were made in the laboratory. The protein ladder was loaded into the first well and the samples were loaded in the 2nd well, and so on. Gels were run at 85V constant voltage for about 20 min to ensure all the protein successfully entered the gel. Once the gel was finished it was quickly fixated in a solution containing 45% methanol and 10% acetic acid diluted by dH_20. The gel was then rinsed with dH_20 and then stained with blue-silver gel stain made of 0.12% Coomassie G250, 10% (w/v) $(NH_4)_2SO_2$, 10% phosphoric acid, and 20%

methanol (Coomassie G250). The gel was then washed overnight at room temperature in dH_20 on a belly dancer machine. The following morning the gel was imaged using a BioRad ChemDoc Imaging System. Gels were preserved at 4 °C in the fridge if the next step was continuing that day or kept at -20 °C until the following day.

A clean glass tray and microscope slide were used to cut the stained gel into pieces no bigger than 5 X 5 mm. This means that the vertical lanes were cut out one by one and then cut into smaller pieces. Using the microscopic glass, the pieces from each lane were moved into their respective wells on a 96 deep-well plate. Destaining solution (40% acetonitrile, 30 mM NH_4HCO_3) was added to cover the gel pieces with about 20% excess volume. The plate was covered with a silicon mat and left to shake on a heated, rotating platform (Thermomixer, Eppendorf) at 400 *g* for at least 2 h before changing the destain solution and letting it shake overnight or until all the visible colour had been extracted from the gel pieces. More rounds of destain solution were used if the dye was not coming out. The destain solution was then aspirated using a 9" glass Pasteur tip and replaced with 50% acetonitrile (ACN) and 25 mM NH_4HCO_3 and placed back on the rotating platform. After 30 min, this solution was aspirated from the wells and replaced with 100% ACN and placed on the platform again to shake until the gel pieces were white and opaque. If opaqueness wasn't achieved, another round of ACN was performed. The ACN was aspirated, and the plate was placed in a vacuum centrifuge (SpeedVac, Eppendorf) to dry the gels. The plate was then wrapped with parafilm and stored either at 4 °C for a short period of time or -20 °C if longer storage was required.

When the next steps were to be performed, the plates were removed from storage and placed on wet ice with digestion buffer (40 mM NH_4HCO_3, 1 mM $CaCl_2$, 10% v/v ACN, pH 7.8-8.1). Trypsin was used to perform the in-gel digestion. After diluting the trypsin appropriately

with digestion buffer, 0.32 µg of trypsin was added on top of each well containing the dried gel pieces. Enough digestion buffer to cover the gel pieces was then added. The plate was sealed with a sticky lid, and the plate and ice bucket were placed at -20 °C for 30 min. The plate was then removed, and more digestion buffer was added for the gel pieces to be suspended in and the sticky lid was replaced. The plate was then moved to the rotating platform again for 15 min at 400 g and then transferred to the incubator at 37 °C for 14 to 17 h. After removing the plate from the incubator another 0.128 µg of trypsin was added to each sample and more digestion buffer depending on the leftover volume from previously. The plate was placed in the incubator once again for another 3 to 5 h.

Protein purification

The plate was removed from the incubator and 10 µL of 10% formic acid was added to each well to stop the digestion. The plate was put on the shaker to shake for 20 min at 400 *g*. Liquid solution was removed from the plate and relocated to a clean 1.2 mL 96-depp well plate. The clean plate was put into the vacuum centrifuge (SpeedVac, Eppendorf) to start reducing the volume of the solution without letting it dry completely (~ 1.5 h). Extraction buffer 1 (50% of 0.1% TFA, 50% ACN) was added to the plate containing gel pieces to cover them and this plate was put on the shaker (Eppendorf 5355 Thermomixer R) to shake for at least 2 h at 400 *g*. Extraction buffer 1 was removed from the gel plate and relocated to the new plate that only contained solution with peptides in it. Another round of extraction buffer 1 was repeated on the gel plate until the plate with peptides suspended in solution had enough room for more liquid. Extraction buffer 1 was removed from the gel plate and replaced with extraction buffer 2 (20% of 0.1% TFA, 80% ACN). Extraction buffer 2 was removed from the gel plate and moved to the

new plate. The gel plate was then sealed, wrapped in parafilm, and stored at -20 °C until the end of the experiment. The new plate was placed in the vacuum centrifuge overnight at 30 °C and ran overnight until dryness was achieved.

To desalt and purify the samples, stage-tipping was used. Each sample was assigned a tip that was labelled and contained a C18 membrane (3M Empore, C18 Extraction Disks) hand-prepared in the laboratory with a 14G needle (Hamilton Company, 17 gauge). Small discs of the C18 Empore filter were punched and ejected into P200 pipette tips. All tips were arranged in the correct pattern to be eluted into a new 96-well plate as well as extra tips just in case some stage tips accidentally fully dried in the process. Samples from the vacuum centrifuge (SpeedVac, Eppendorf) were resuspended in 120 µL of wash solution (0.2% TFA) and mixed using gentle pipetting. The plate was put on the shaker (Eppendorf 5355 Thermomixer R) at room temperature at 300 g while preparing the stage tips for sample loading. The stage tips were conditioned with 100% methanol, and this liquid was removed from the tips by centrifuging at 300 *g* for 5 min. A total of 150 µL of hydration solution (80% ACN, 0.2% TFA) was then added to each stage tip and removed by centrifuge at 300 G for around 2 min. It is important to note at this step and the steps that come after that no tips were meant to go dry, and the solutions should not have passed from the top of the C18 membrane. To equilibrate, 150 µL of wash solution (0.2% TFA) was added to each stage tip and centrifuged at 300 G for 2 min. Another 120 µL of wash solution was added and tips were centrifuged for another min. Samples were then loaded to their respective tips. All the sample was taken for abdomen tissue (120 µL), half was taken for head tissue (60 µL) and 1/3 was taken for gut tissue (40 µL). Tips containing the samples were centrifuged at 300 *g* for 2 min. Another 150 µL of wash solution was added to the stage tips containing the samples and centrifuged at 300 *g* until the liquid was at the top of the C18

membrane. This step was repeated once more with the difference that the liquid was removed completely. All liquid collected in the basin from the previous steps was discarded as steps continued.

An elution tool was prepared by stacking and taping two P10 pipette tip holders on top of the Axygen plate to hold the tips above the corresponding wells. To elute the peptides from the C18 membranes, 80 µL of elution solution (40% ACN, 0.1% TFA) was added to each stage tip and the elution tool was centrifuged at 600 *g* for 3 min. This step was repeated once more for a s elution. The Axygen plate containing the samples was then sealed with an aluminum sticky lid and placed in the -70 °C for at least 30 min. Once the solution with the samples were frozen the plate was put in the vacuum centrifuge and dried to completion at room temperature. The samples were then stored with a silicon mat at -20 °C.

2.2.6 LC-MS/MS data collection and analysis

Liquid chromatography

Each combined fraction was reconstituted in a solution containing 0.5% ACN and 0.1% formic acid and subsequently analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) with a total on-column injection of 150 ng. The sample was separated with a NanoElute UHPLC system (Bruker Daltonics) equipped with an Aurora Series Gen2 (CSI) analytical column (25 cm x 75 µm, 1.6 µm particle size, FSC C18, featuring Gen2 nanoZero and CSI fitting; Ion Opticks, Parkville, Victoria, Australia) that maintained a temperature of 50 °C using a Column toaster M (Bruker Daltonics). The LS-MS/MS system was coupled to a timsTOF Pro (Bruker Daltonics) instrument operated in Data-Dependent Acquisition with Parallel Accumulation Serial Fragmentation (DDA-PASEF) mode. During a standard 30-min run, the solvent B gradient rose from 2% to 12% over 15 min, followed by an increase to 33% solvent B

from 15 to 30 min, and finally a rapid change to 95% solvent B over 0.5 min, with a 7.72-min hold at 95% solvent B. Prior to each analysis, the analytical column was conditioned with 4 column volumes of buffer A consisting of 0.1% formic acid and 0.5% ACN, while buffer B consisted of 0.1% formic acid dissolved in 99.4% ACN. The NanoElute thermostat was maintained at 7 °C throughout the analysis, with a constant flow rate of 0.3 µL/min.

Mass spectrometry

The Trapped Ion Mobility – Time of Flight Mass Spectrometer (TimsTOF Pro; Bruker Daltonics, Germany) was configured to operate in Parallel Accumulation-Serial Fragmentation (PASEF) scan mode for Data-Dependent Acquisition (DDA), covering a mass-to-charge ratio (m/z) range of 100 to 1700 with five PASEF ramps. Instrument parameters included a constant capillary voltage of 1800V, a dry gas flow rate of 3 L/min, and a consistent drying temperature of 180°C for recording both MS and MS/MS spectra within the m/z range of 100 – 1700. In Trapped Ion Mobility Spectrometry (TIMS) mode, the settings were an ion mobility range (1/k0) from 0.70 to 1.35 V·s/cm^2, a 100 ms ramp time, 100% duty cycle for accumulation, and a ramp rate of 9.42 Hz, resulting in a total cycle time of 0.64 s.

Linear precursor selection strategies were implemented with a target intensity of 21,000 and an intensity threshold of 2500. Active exclusion was enabled with a 0.4-min release duration. Collision energy was linearly ramped in relation to ion mobility, varying from 27 eV at 1k0 = 0.7 V·s/cm^2 to 55eV at 1/k0 = 1.35 V·s/cm^2. Isolation widths were set at 2.07 m/z for ions with m/z values below 400 and at 3.46 m/z for ions surpassing the 1000 m/z threshold.
For mass accuracy, the typical error of mass measurement is within 3 to 7 ppm without exceeding the upper limit. Ion dimension calibration was performed using ions from the Agilent

ESI-Low Tuning Mix (m/z [Th], 1/k0 [Th]: 622.0290, 0.9915; 922.0098, 1.1986; 1221.9906, 1.3934). The operation of the TimsTOF Pro instrument was controlled using timsControl v.4.1.12 (Bruker), while both the LC and MS were managed using Hystar 6.0 (6.2.1.13, Bruker).

Search

The data was analyzed using the computational platform Fragpipe (v.17.1), with MSFragger (v.3.4), Philosopher[4] (v.3.8) and EasyPQP (v.0.1.27) for building the spectral library. The protein sequence database used for this analysis was the Honey bee (*Apis mellifera*) database from the National Center for Biotechnology Information (NCBI) obtained in 2021. This database contains a total of 37,281 sequences, including common contaminant proteins, and reversed protein sequences incorporated as decoys. During the MSFragger analysis, precursor mass tolerances were set to 50 ppm and fragment mass tolerances were set to 20 ppm. The enzyme specificity was set to "trypsin," allowing for a maximum of 2 missed cleavages. Peptide identification was performed using the DIA-NN (Data-Independent Acquisition Neural Network) software (version). DIA-NN utilizes a deep learning-based approach for robust and accurate peptide identification differing from the traditional DDA method by not discriminating for the most powerful signals.

Following the MS/MS search, Philosopher was used to further process the results, generating final reports that were filtered at a 1% protein False Discovery Rate (FDR) and a 1% FDR for peptide, ion, and peptide-spectrum match (PSM) levels. These files were inputs of EasyPQP to generate spectrum libraries. The ultimate spectral library was filtered to maintain a 1% FDR at both the protein and peptide levels.

2.2.7 Pesticide and pathogen identification

As part of the BeeCSI project, other laboratories conducted the analysis of characterizing and quantifying pesticides and pathogens. Brood-nest bees, nectar, pollen, and wax collected from exposed and unexposed hives throughout the experiment were analyzed for multiple pesticide types and amounts against a screen of frequently used agricultural chemicals. Bees, nectar, and pollen were collected at timepoints TP1, TP2, and TP3. Wax was included only for the highbush blueberry project and was only collected from hives at TP4. This was done in accordance with in-house test methods by the University of Guelph's Agriculture & Food Laboratory, which employed gas chromatography-tandem mass spectrometry (GC-MS/MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) for detection. These methods achieved detection limits ranging from the part per trillion to low part per billion range. The laboratory has the capacity to screen for over 200 active pesticide ingredients, ensuring a broad and comprehensive analysis of potential residues in the samples. However, only pesticides detected above the established detection limits were included in the final analysis and reported.

For the pesticide data, total pesticide residues (measured in parts per billion, ppb) across different sample types (bees, nectar, pollen, and wax) were analyzed to assess temporal and site-specific trends. A two-way analysis of variance (ANOVA) was performed to evaluate the effects of site (exposed vs unexposed), timepoint (TP1–TP3), and their interaction on pesticide residues. Year (2020 vs 2021) was also tested as a potential covariate in an extended ANOVA model, but it was not found to significantly influence pesticide levels. Tukey's Honest Significant Difference (HSD) test was employed post hoc to determine pairwise differences between specific timepoints and site types.

For visual representation, aggregate pesticide totals were plotted using bar plots with error bars representing the standard deviation. Temporal trends and the significant differences

identified in the statistical analyses were annotated on the figures. This statistical approach enabled the identification of critical exposure periods (e.g., during and post-bloom) and sample types with elevated pesticide burdens, providing key insights into pesticide accumulation patterns in highbush blueberry pollination systems.

Brood-nest bees sampled from hives in both exposure groups at the various timepoints were screened for common honey bee bacterial infections and viruses using diagnostic services from the National Bee Diagnostic Centre. This analysis is performed using PCR-based techniques for the identification of the bacterial infections AFB, EFB, and *N. cerenae*. As well as the viral infections ABPV, BQCV, CBPV, DWV, IAPV, KBV, LSV, SBV, VDV.

In this study, field pathogen data were analyzed using two distinct approaches based on the nature of the data. The viral data was continuous but exhibited a problematic structure where values were either zero, 35, or a number much greater than 35. The “35” values arose from qPCR amplification below the lower limit of quantification and were not reliable values (as they have artificially zero variance). For modeling viral loads, “35” and “0” were excluded. To convert to a binomial factor, “0” was considered absent (0), and values >0 (including “35”) were considered as present (1).

For binary variables, such as pathogen detection and classifications of infestation levels (high or low), the analysis was conducted using generalized linear mixed models (GLMMs). These models were fitted using maximum likelihood estimation with Laplace approximation. A binomial distribution with a logit link function was used to suit the binary nature of the data. For continuous integer data, including mite percentage and virus DNA norm copy per bee levels, linear mixed models (LMMs) were used. These models were fitted using restricted maximum likelihood (REML), and Satterthwaite’s method was applied for approximating degrees of

freedom in the t-tests for fixed effects. In all models, site (categorized as exposed vs unexposed), year (comparing 2020 vs 2021), and time (across four timepoints: TP1, TP2, TP3, and TP4) were included as fixed effects. The random effect in each model was attributed to variations among colonies. This approach allowed for the assessment of both fixed and random factors influencing presence and levels across different conditions and time frames.

2.2.8 Statistical analysis of field pesticide and pathogen data

In this study, field pathogen data were analyzed using two distinct approaches based on the nature of the data. The viral data was continuous but did have a problematic structure as values were either zero, 35, or some number much greater than 35. The "35" values arose from qPCR amplification below the lower limit of quantification and were not reliable values (as they have artificially zero variance). For modeling viral loads, "35" and "0" were excluded. To convert to a binomial factor, "0" was considered absent (0) and > 0 (including "35") was considered as present (1).

For binary variables, such as pathogen detection and classifications of infestation levels (high or low), the analysis was conducted using generalized linear mixed models (GLMMs). These models were fitted using maximum likelihood estimation with Laplace approximation. A binomial distribution with a logit link function was used to suit the binary nature of the data. For continuous integer data, including mite percentage and virus DNA norm copy per bee levels, linear mixed models (LMMs) were used. These models were fitted using restricted maximum likelihood (REML), and Satterthwaite's method was applied for approximating degrees of freedom in the t-tests for fixed effects. In all models, site (categorized as exposed vs unexposed), year (comparing 2020 vs 2021), and time (across four timepoints: TP1, TP2, TP3, and TP4) were included as fixed effects. The random effect in each model was attributed to variations among

colonies. This approach allowed for the assessment of both fixed and random factors influencing presence and levels across different conditions and time frames.

2.2.9 Statistical analysis of proteomic field and caged bee tissue data

Statistical modeling was performed to identify differentially expressed proteins between experimental groups using the limma package in R. Model design included covariates relevant to experimental design for field and cage experiments. The variables meaningful to the field analysis included site, year, timepoint, and tissue (exposed vs unexposed; 2020 vs 2021; TP1, TP2, TP3, TP4; abdomen, gut, and head). The blocking factor was replicates. The cage model considered treatment group and again the blocking factor was replicates.

Data preparation

Prior to conducting contrasts, all raw files from the field trials were combined and organized, and common contaminants were removed from the data to only include *A. mellifera* proteins. Following this step, principal component analysis (PCA) plots were used to summarize the information and visualize for outliers in the dataset. Outliers were defined as data points falling significantly outside the main clusters of samples along the principal components, indicating high variance or deviation from the expected biological grouping. Samples were removed because their projections on the PCA plot were isolated far from all other points, showing no overlap or proximity with their corresponding group clusters. These extreme deviations suggested they did not represent the same underlying biological or experimental conditions as the rest of the dataset. Once outliers were identified and removed, the process of identifying differentially expressed proteins could begin using the limma package in R.

A similar approach was applied to the cage trial data. Raw files were prepared by removing common contaminants to focus exclusively on *A. mellifera* proteins. For each dataset, PCA plots were used to assess the distribution of the data and identify any outliers. Additionally, some samples were removed due to experimental issues, such as cage mortality. Following this, the data was processed using the limma package in R to compare protein expressions across different metadata groups.

Model design

Model design for the field samples included covariates relevant to experimental design for field and cage experiments from the metadata. The variables meaningful to the field analysis included site, year, timepoint, and tissue (exposed vs unexposed; 2020 vs 2021; TP1, TP2, TP3, TP4; abdomen, gut, and head). The blocking factor was replicated in all models. For the cage sample models, the only experimental variable to consider contrasting was treatment group. Once again, the blocking factor was replicated for the data.

After fitting the linear models using the limma package in R, empirical Bayes smoothing was applied to estimate moderated t-statistics and adjusted p-values. Differentially expressed proteins (DEPs) were identified based on two criteria: an adjusted p-value (Benjamini-Hochberg method) threshold of ≤ 0.05 and an absolute log2 fold change ($|\log2FC|$) > 1. This corresponds to a fold change of at least 2, ensuring that identified DEPs reflect both statistically significant and biologically meaningful differences.

2.2.10 GO term enrichment of proteomic field and caged bee tissue data

Gene ontology (GO) term enrichment analysis was performed to identify biological processes, cellular components, and molecular functions overrepresented in the proteomic data

from field samples. The analysis was performed using g:Profiler, specifically the gprofiler2 (version 0.2.3) R package.

Enrichment Analysis Parameters

The gprofiler2 package was used to run the enrichment analysis with the default settings, including the correction for multiple testing using the Benjamini-Hochberg false discovery rate (FDR) method. The significance threshold for the GO term enrichment was set to an FDR of less than 0.05.

The gene identifiers used in this analysis were based on the *Apis mellifera* reference set based on GO annotations from NCBI for the organism. The package gprofiler2 annotated these genes with GO terms and assessed the enrichment of these terms in the input gene list, which consisted of differentially expressed proteins identified from the proteomic analysis.

Interpretation of results

The gprofiler2 tool generated a list of GO terms ranked by their enrichment scores. Enrichment scores represent the extent to which specific GO terms were overrepresented in the list of differentially expressed genes compared to the background gene set. GO terms with FDR-adjusted p-values less than 0.05 were considered significantly enriched. These terms were categorized into biological processes, molecular functions, and cellular components to elucidate the potential biological implications of the observed proteomic changes in honey bee tissues.

2.3 Results

2.3.1 Qualitative observations of hives

Systematic assessments of colony health and strength were performed throughout the field study to qualitatively evaluate hive conditions across exposed and unexposed sites. These assessments focused on metrics such as queen health, reproductive stress (e.g., egg-laying and brood viability), and observable symptoms of colony distress or pathogen infestation. Evaluations were conducted at pre-defined sampling points corresponding to the highbush blueberry bloom period (TP1–TP4) from April to June during each study year. Observations were logged systematically, noting the inspector responsible for each evaluation to ensure consistency and accountability.

The colony health observations revealed that incidences of queen absence or egg-laying discontinuity were rare but documented when they occurred. Commentary on apiary inspections ranged from positive evaluations of colony vigor and brood viability to reports of pathogen presence, including chalkbrood, European foulbrood (EFB), and deformed wing virus (DWV). Pathogen infestations varied across colonies, and management interventions were recorded as part of routine hive maintenance. Over the two-year study, one hive from the exposed group and one from the unexposed group were lost. A representative example of an apiary log from 2021 is provided in Table 2.4, with a full record of hive management and health assessments included as Multimedia Material 1. These records demonstrate consistent data collection practices, supported by multiple contributors to the fieldwork, who noted their initials after each inspection.

Overall, qualitative observations indicated generally strong colony health across both exposed and unexposed sites, with limited queen health disruptions or colony losses. However, the presence of pathogens such as DWV and EFB highlights ongoing stressors that may influence honey bee resilience in agricultural landscapes.

Table 2.4 Example apiary log used in highbush blueberry field trials with real data at four sampling timepoints. Full log for the 2020 and 2021 field study can be found in the List of Multimedia Material as Multimedia Material 1.

Date	Site	Col. No.	Q/eggs seen?	Eight samples?	Comments	Initials
Site type & time point: TP1, initial site before moving to experimental sites						
23 April 2021	Jan's	Rep06e1	Yes	Yes	Strong colony	HH
23 April 2021	Jan's	Rep06e2	Yes	Yes	Strong colony	HH
23 April 2021	Jan's	Rep06e3	Yes	Yes	High population, chalkbrood, gave 1 frame food from Rep06u4	HH
23 April 2021	Jan's	Rep06e4	Yes	Yes	Big & strong, solid brood	RT
Site type & time point: TP2, all colonies in experimental sites						
10 May 2021	Lee's blueberry	Rep06e1	Yes	Yes	Capped cells, Q damaged: 2 partial legs, slight chalkbrood, moved 2 frame up from bottom box	BV
10 May 2021	Lee's blueberry	Rep06e2	Yes	Yes	Good colony, healthy, moved 1 frame up, storing honey	BV
10 May 2021	Lee's blueberry	Rep06e3	Yes	Yes	Small colony, no EFB, not much pollen, chalkbrood, moved 1 frame up	HH
10 May 2021	Lee's blueberry	Rep06e4	Yes	Yes	Huge colony, heavy honey super, moved 2 frames up	HH
Site type & time point: TP3, all colonies in experimental sites						
21 May 2021	Lee's blueberry	Rep06e1	Yes	Yes	Supersedure cells, damages queen, chalkbrood	BV
21 May 2021	Lee's blueberry	Rep06e2	Yes	Yes	EFB sampled, strong colony, good food	BV
21 May 2021	Lee's blueberry	Rep06e3	Yes	Yes	Medium size, not much pollen, high chalkbrood	HH
21 May 2021	Lee's blueberry	Rep06e4	Yes	Yes	Huge colony, super heavy, solid brood, no chalkbrood or EFB, needs super	HH
Site type & time point: TP4, all colonies in common sites						

2 June 2021	Barb's	Rep06e1	Yes	Yes	Big, healthy, some chalkbrood	LF
2 June 2021	Barb's	Rep06e2	Yes	Yes	Big, EFB	LF
2 June 2021	Barb's	Rep06e3	Yes	Yes	Good colony, no health issues	RT
2 June 2021	Barb's	Rep06e4	Yes	Yes	Good colony, no health issues	RT

2.3.2 Pesticides found in highbush blueberry fields

Pesticide mass

Pesticide residues were quantified across various hive sample types (bees, nectar, pollen, and wax) using GC-MS/MS and LC-MS/MS. Residues were reported in parts per billion (ppb) to standardize comparisons across sample types with varying capacities for pesticide accumulation. For example, wax tends to retain higher concentrations of lipophilic pesticides compared to other matrices. Aggregate pesticide totals were calculated for each sample type, timepoint, and site to assess overall pesticide burdens. This approach provided insights into trends of pesticide accumulation and identified high-risk periods and sample types, particularly during the bloom period when pesticide applications are typically most intensive.

A total of 32 pesticides—including systemic and non-systemic insecticides, acaricides, fungicides, and herbicides—were detected across all sample types from TP1, TP2, and TP3. Figure 2.5 presents pesticide residue data as an aggregate measure, representing the total pesticide burden across all experimental conditions. Variability matrices (e.g., standard deviation or standard error) were not included, as the values represent cumulative totals rather than averages of individual replicate measurements. Although the laboratory utilized methods capable of detecting over 200 active pesticide ingredients, only those exceeding detection limits were reported. Wax consistently demonstrated the highest pesticide concentrations and diversity,

followed by pollen, bees, and nectar (Figure 2.5). This pattern highlights the strong affinity of wax for pesticide retention and its role as a reservoir for agrochemical exposure in hives.

Residue analysis highlighted the dominance of specific pesticides across sample types. Flupyradifurone, a systemic insecticide, was the most abundant pesticide in unexposed bee samples, while pyrimethanil, a fungicide/herbicide, was most prevalent in exposed bee samples (Figure 2.5). This trend extended to nectar and pollen samples, where flupyradifurone predominated, followed by pyrimethanil. Wax samples from both site types contained elevated levels of pyrimethanil and boscalid, with moderate levels of fluopyram, pyraclostrobin, and metconazole—all categorized as fungicides/herbicides in this study. Imidacloprid, a neonicotinoid insecticide, was detected in wax but at considerably lower levels compared to other pesticides and was not included among the top six pesticides identified.

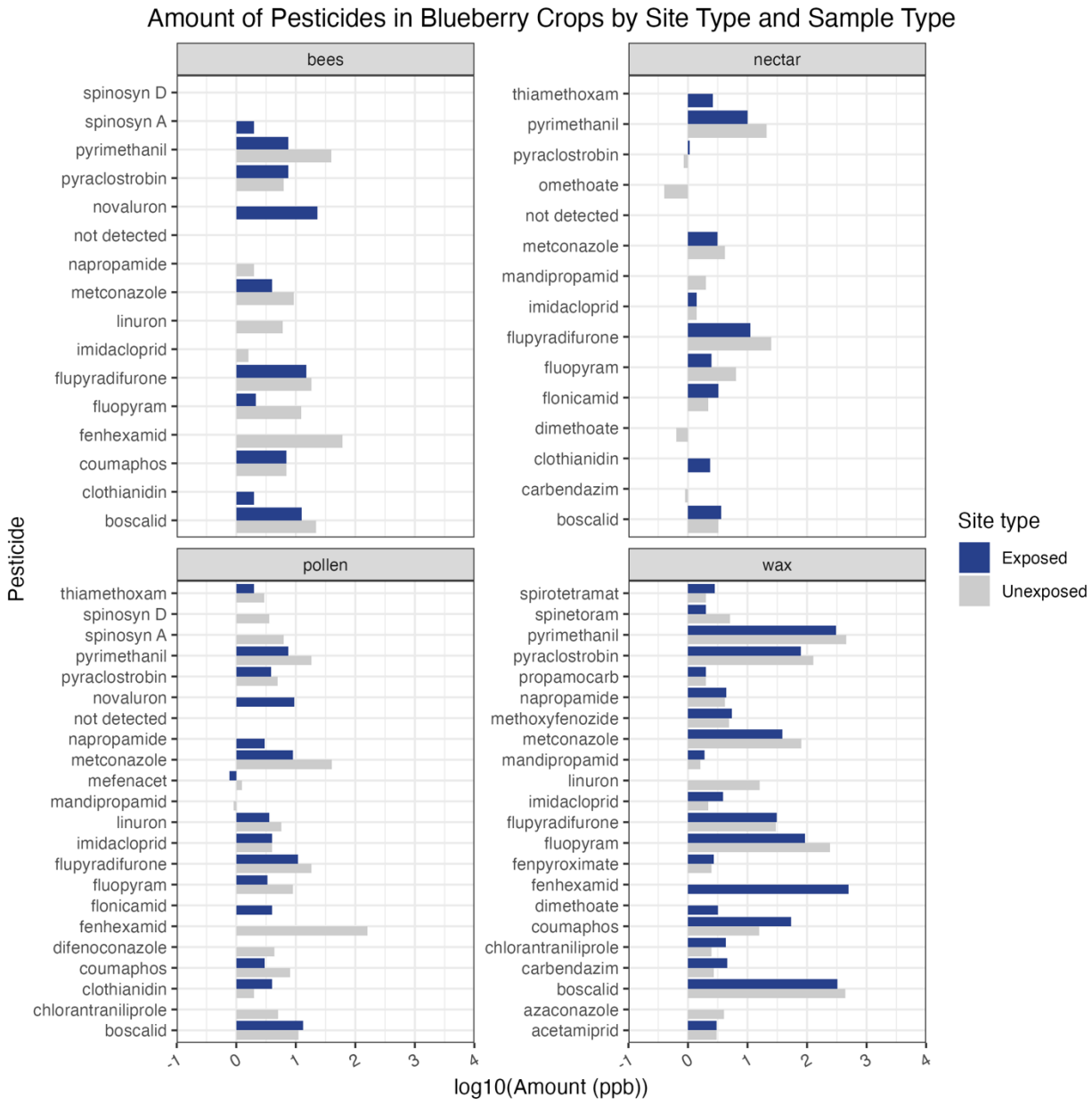


Figure 2. 5 Log-transformed concentrations of pesticide residues detected in hive samples from exposed and unexposed sites during the entire experimental period (T1-T3). Pesticide levels are expressed as log-transformed, summed total amounts measured in parts-per-billion (ppb) to facilitate comparison across a wide range of concentrations. Blue bars represent samples from exposed sites, while grey bars indicate those from unexposed sites. Thicker bars are present for pesticides that were only found in exposed or unexposed samples and not both. The figure illustrates the tendency for higher pesticide residues in wax, with variances in pesticide types and concentrations among the different sample matrices and site exposures. In total, the study identified 32 distinct pesticides across all samples.

Temporal analysis revealed significant differences in pesticide residues between exposed and unexposed hives across all timepoints, with statistical support confirming the higher pesticide burden in exposed sites ($p < 0.05$). An ANOVA was performed to assess the effects of site, timepoint, and their interaction on pesticide residues. Post-hoc comparisons using Tukey's HSD test identified significant differences between exposed and unexposed sites as well as between specific timepoints. At the pre-exposure phase (TP1), pesticide levels were comparable between exposed and unexposed hives, measuring 64.0 and 67.9 ppb, respectively (Figure 2.6). During the bloom period (TP2), pesticide concentrations in exposed hives increased significantly to 6,788 ppb, while unexposed hives remained substantially lower at 1,078 ppb ($p < 0.05$). By the post-bloom period (TP3), exposed hives accumulated 16,874 ppb of pesticides compared to 12,121 ppb in unexposed hives, with TP3 showing a significant increase over TP1 ($p < 0.05$). Wax samples, collected exclusively post-bloom (TP4), revealed the highest total pesticide concentrations, with exposed hives containing 13,127 ppb and unexposed hives 9,798 ppb, further emphasizing wax as a reservoir for pesticide residues. Notably, there was no significant difference in pesticide levels between years (2020 vs. 2021), as determined by an interaction term in the ANOVA (Figure 2.6).

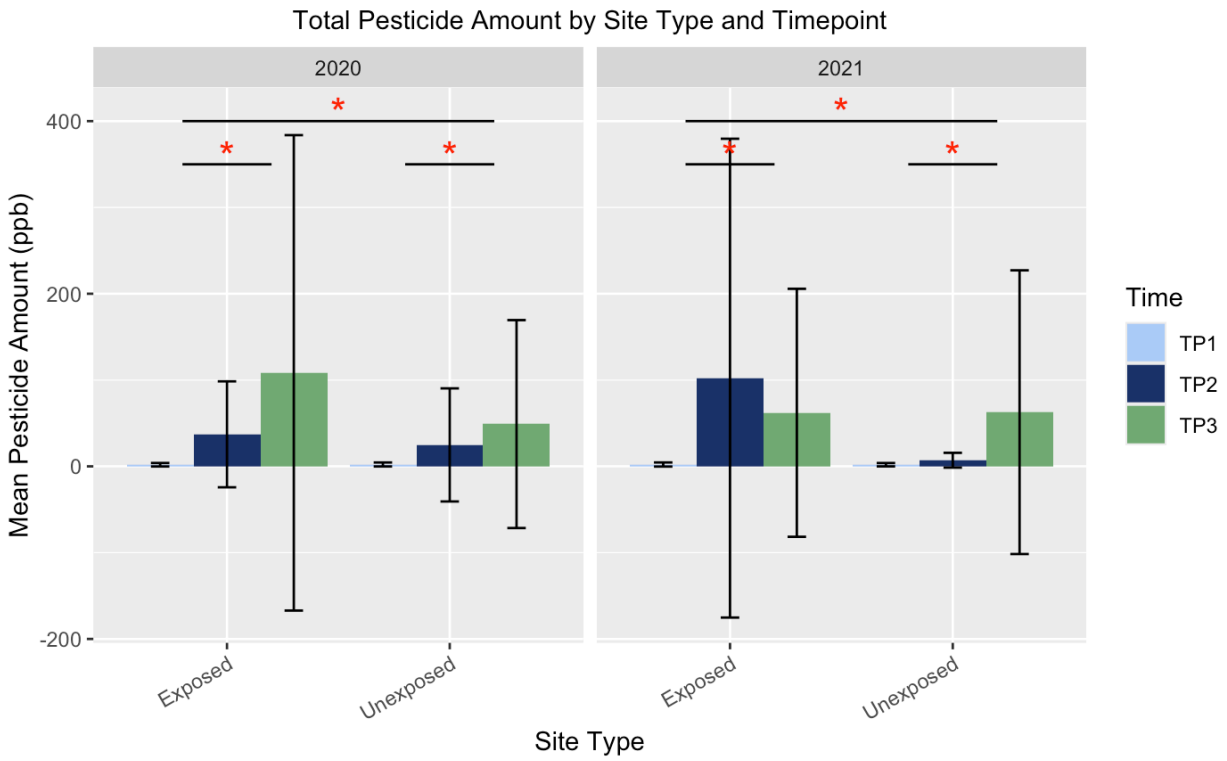


Figure 2. 6 Mean pesticide amount by site type, timepoint, and year in highbush blueberry pollination hives. The mean pesticide amount is measured in parts per billion (ppb) for exposed (blue) and unexposed (green) sites, with error bars representing the standard deviation. Sampling occurred at three timepoints: before exposure (TP1), beginning of bloom (TP2), and end of bloom (TP3)**.** Results are displayed separately for 2020 and 2021. Significance annotations indicate statistically significant differences between exposed and unexposed sites, as well as between TP3 and TP1 ($p < 0.05$). Exposed hive samples consistently showed higher pesticide amounts compared to unexposed hives, with the highest concentrations observed at TP3.

These findings emphasize the substantial pesticide burdens honey bees face in commercial blueberry agricultural systems. The pronounced increase in pesticide concentrations during bloom (TP2) and post-bloom (TP3) in exposed hives highlights the critical periods of exposure. The identification of specific pesticides, particularly flupyradifurone and pyrimethanil, underscores the need for targeted management practices to mitigate risks to honey bee health. Additionally, the consistently higher pesticide levels in wax highlight its importance as an indicator of long-term agrochemical exposure within colonies.

Pesticide risk

To assess pesticide risk, residue concentrations (ppb) detected in hive matrices were paired with LD50 values for honey bees, reflecting the lethal dose required to kill 50% of a test population. Both contact and oral toxicity data were integrated into the analysis to evaluate potential health risks posed by each pesticide. This approach provided a refined perspective beyond aggregate concentrations, accounting for differences in pesticide toxicity and the cumulative risk from concurrent exposures. By incorporating risk quotients alongside total pesticide burdens, this analysis aimed to deliver a more comprehensive understanding of the potential impacts on honey bee populations.

While the aggregate number offers a snapshot of the overall pesticide presence, it may not be the most informative metric regarding potential impacts on honey bee health. This is because different pesticides have varying levels of toxicity to honey bees. A pesticide present at a low concentration might be highly toxic, while another at a higher concentration might be less harmful. Furthermore, pesticides can have concurrent effects when combined, leading to greater harm than when each pesticide is considered individually. Summing the concentrations alone does not capture these interactions. Risk assessments also help in evaluating the overall exposure and its potential consequences, considering the various pathways through which bees come into contact with pesticides. Factors such as foraging behaviour, environmental conditions, and the persistence of pesticides in different matrices (wax, nectar, pollen, and bees) influence the actual risk. Additionally, effective regulation and management of pesticide use in agriculture require a comprehensive understanding of the risks posed by these chemicals. Risk assessments inform guidelines and best practices to mitigate adverse effects on pollinators. By integrating both

pesticide concentrations and their risks, researchers and policymakers can develop more robust strategies to protect honey bee populations and ensure stable pollination services.

The systemic insecticide thiamethoxam was identified as the riskiest pesticide, with a concentration of 0.213 ppb in the sample set. Systemic insecticides, including thiamethoxam, clothianidin, spinosyn A, and imidacloprid, consistently ranked as the most lethal in terms of risk when analyzed by colony, timepoint, sample type, and site type. Interestingly, despite their high associated risks, systemic insecticides such as imidacloprid and spinosyn A were detected at relatively low concentrations compared to other pesticides. This suggests that even trace levels of highly toxic pesticides can have significant implications for honey bee health. The analysis also highlighted the presence of flupyradifurone, a systemic insecticide commonly used in blueberry cultivation, which was less frequently associated with high-risk events in this study. The relative scarcity of certain high-risk systemic insecticides in the sample set suggests that regulatory practices or environmental conditions may influence their prevalence in this agricultural system.

In summary, while aggregate pesticide concentrations offer valuable insights into overall exposure, the integration of risk quotients provides a more nuanced evaluation of potential health impacts. This dual approach underscores the importance of considering both pesticide toxicity and concentration when assessing risks to honey bee populations. By identifying thiamethoxam and other systemic insecticides as key contributors to risk, this analysis emphasizes the need for targeted management strategies to mitigate the impacts of these high-risk pesticides. This combined exposure and risk assessment approach highlights the importance of developing evidence-based regulations to protect pollinators and maintain sustainable pollination services in agricultural systems.

2.3.3 Pathogens found in highbush blueberry fields

The pathogen analysis investigated the prevalence of mites, bacterial infections, and viruses in brood-nest bees sampled across timepoints and sites. GLMMs and LMMs were employed to explore temporal, site-specific, and colony-level influences on pathogen dynamics. While certain pathogens (CBPV, ABPV, KBV) were excluded due to insufficient detection, the analysis focused on mites, EFB, *Nosema*, DWV, SBV, BQCV, LSV, VDV, and IAPV.

Temporal trends were evident across several pathogens, with specific increases in prevalence or levels occurring at later timepoints, particularly TP3 and TP4. For example, EFB detection odds were significantly higher at TP3 ($p = 0.0301$) and TP4 ($p = 0.00818$), while *Nosema* spore counts significantly decreased over time ($p = 0.0252$), despite showing increased odds of infection at TP3 ($p = 0.0183$) and TP4 ($p = 0.00950$). Similarly, DWV and LSV demonstrated notable increases at TP4 (DWV: $p = 0.0127$; LSV: $p = 0.00081$), highlighting the importance of time-dependent pathogen dynamics.

Significant inter-colony variability was observed across all pathogens, highlighting the impact of internal colony dynamics on disease susceptibility. This variability frequently surpassed the influence of site-specific factors, underscoring the importance of individual colony health and management in determining pathogen outcomes.

While site exposure was not a dominant factor for most pathogens, some notable exceptions were observed. SBV levels were significantly higher in exposed colonies at TP4 ($p = 0.00393$), aligning with its association with site exposure. Conversely, pathogens like DWV and VDV showed limited site-specific effects, suggesting that external environmental factors alone do not fully explain pathogen prevalence.

The analysis of mites (*V. destructor*) revealed a significant increase in mite presence in 2021 compared to 2020 ($p = 8.15 \times 10^{-5}$), with higher infestation levels observed at TP4 ($p =$

0.0105). However, mite presence did not vary significantly between exposed and unexposed sites, suggesting that broader temporal factors were more influential. Similarly, BQCV and IAPV were universally present across colonies, showing only minor temporal or site-specific variations.

In summary, temporal factors and internal colony dynamics emerged as critical drivers of pathogen prevalence and levels, while site-specific effects were limited to select pathogens, such as SBV. These findings emphasize the need for honey bee management strategies that address both colony-specific health factors and time-dependent pathogen risks.

2.3.4 Proteomic discoveries from field trials

Differentially expressed proteins

To investigate temporal and tissue-specific responses to environmental stressors during highbush blueberry pollination, we performed differential protein expression analysis across site types (exposed vs. unexposed), tissues (abdomen, gut, and head), and timepoints (TP1 – TP4). Significance was determined using a p-value threshold of 0.05 with replicate blocking.

Overall, no significant differences in protein expression were detected between exposed and unexposed site types when considered alone, suggesting that stressors such as agrochemical drift and pathogens were present in both environments. However, clear patterns emerged when examining timepoint comparisons within each tissue (Figure 2.7).

The heatmap in Figure 2.7 shows the relative imbalance in the number of up- versus downregulated proteins across pairwise timepoint comparisons. Early bloom (TP1 vs. TP2) and post-bloom (TP1 vs. TP4 and TP2 vs. TP3) comparisons demonstrated the most pronounced changes across tissues, with particularly strong shifts in the abdomen and gut. In contrast, the

TP1 vs. TP3 and TP2 vs. TP4 comparisons showed minimal differential expression, indicating relative stability or recovery during peak bloom.

In the abdomen, proteins related to energy metabolism and detoxification were commonly upregulated during early bloom and post-bloom periods, such as cytochrome c oxidase and NADH dehydrogenase (electron transport chain), alongside detoxification enzymes such as cytochrome P450s. Conversely, proteins involved in RNA processing and ribosomal assembly, including 40S ribosomal protein S26, tended to be downregulated during these same timepoints.

In the gut, energy metabolism proteins such as ATP synthase subunits and mitochondrial electron transport components were frequently upregulated, while stress-sensitive enzymes involved in protein synthesis were suppressed. The gut also showed strong temporal variation in digestive and immune-related proteins, including prophenoloxidase and maltase, which were elevated at early bloom before dropping at later timepoints.

The head tissue displayed subtler but still biologically meaningful changes. Proteins involved in neural signaling and sensory function, such as odorant-binding proteins, tended to be downregulated during early bloom and post-bloom periods, whereas stress-response proteins such as heat shock proteins (e.g., HSP40) were elevated. Interestingly, multiple proteins annotated with cell cycle-related functions (e.g., mitotic spindle assembly checkpoint protein MAD1) were detected as differentially expressed in the head tissue; while most neural cells are post-mitotic, these proteins may reflect broader roles in DNA repair, cytoskeletal maintenance, or stress-induced pathways rather than active cell division.

Together, these results highlight that the abdomen and gut were the most dynamically responsive tissues, especially during early bloom (TP2) and post-bloom (TP4) periods, while the

head tissue exhibited fewer but potentially important proteomic changes related to sensory function. These patterns provide a foundation for understanding the biological processes underlying honey bee stress responses during blueberry pollination.

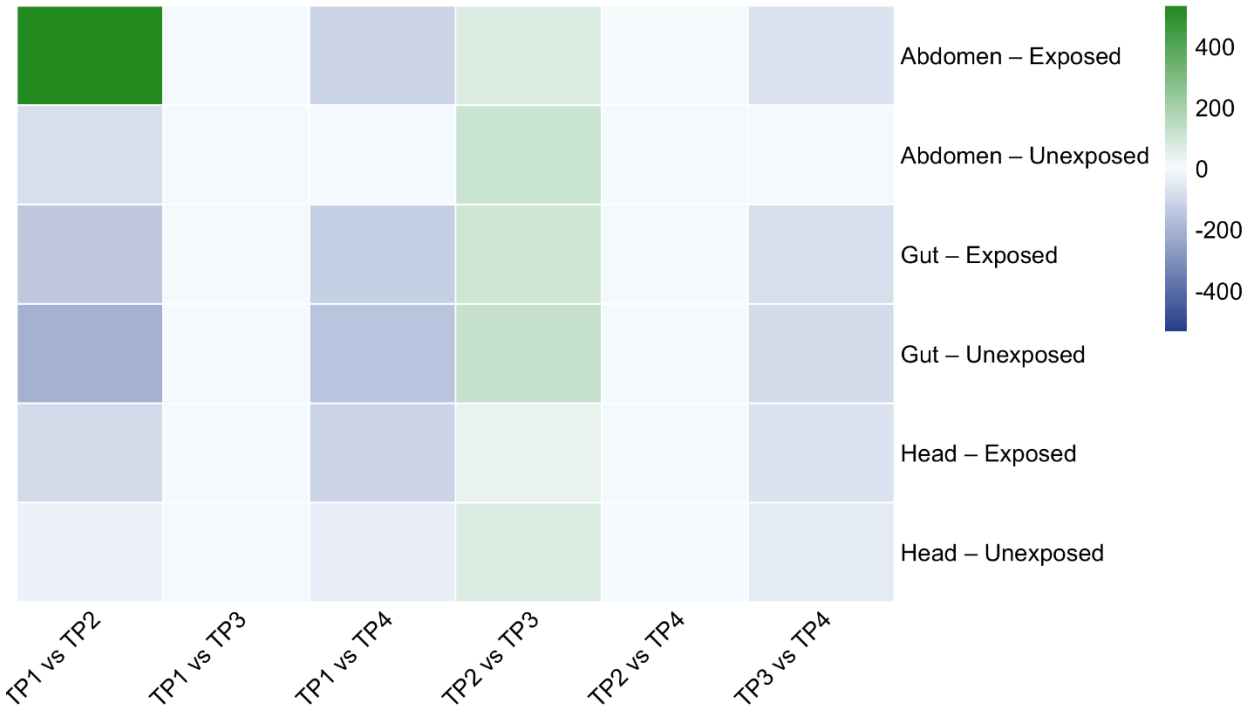


Figure 2. 7 Differentially expressed proteins across tissues, site types, and timepoint comparisons. Rows show tissue-site combinations (Abdomen/Gut/Head x Exposed/Unexposed), and columns show pairwise timepoint comparisons (TP1-TP4). Tile colour encodes the imbalance in direction of change for each cell, calculated as Upregulated – Downregulated DEPs ($p < 0.05$). Green indicates a higher number of upregulated proteins, blue indicates more downregulated proteins, and white indicates a balance or no DEPs.

Functional analysis of differentially expressed genes (GO enrichment)

To interpret the functional consequences of the observed DEPs, we performed a Gene Ontology (GO) enrichment analysis, grouping proteins by biological process, molecular functions, and cellular components. GO terms with a p-value < 0.05 were considered significantly enriched (Figure 2.8).

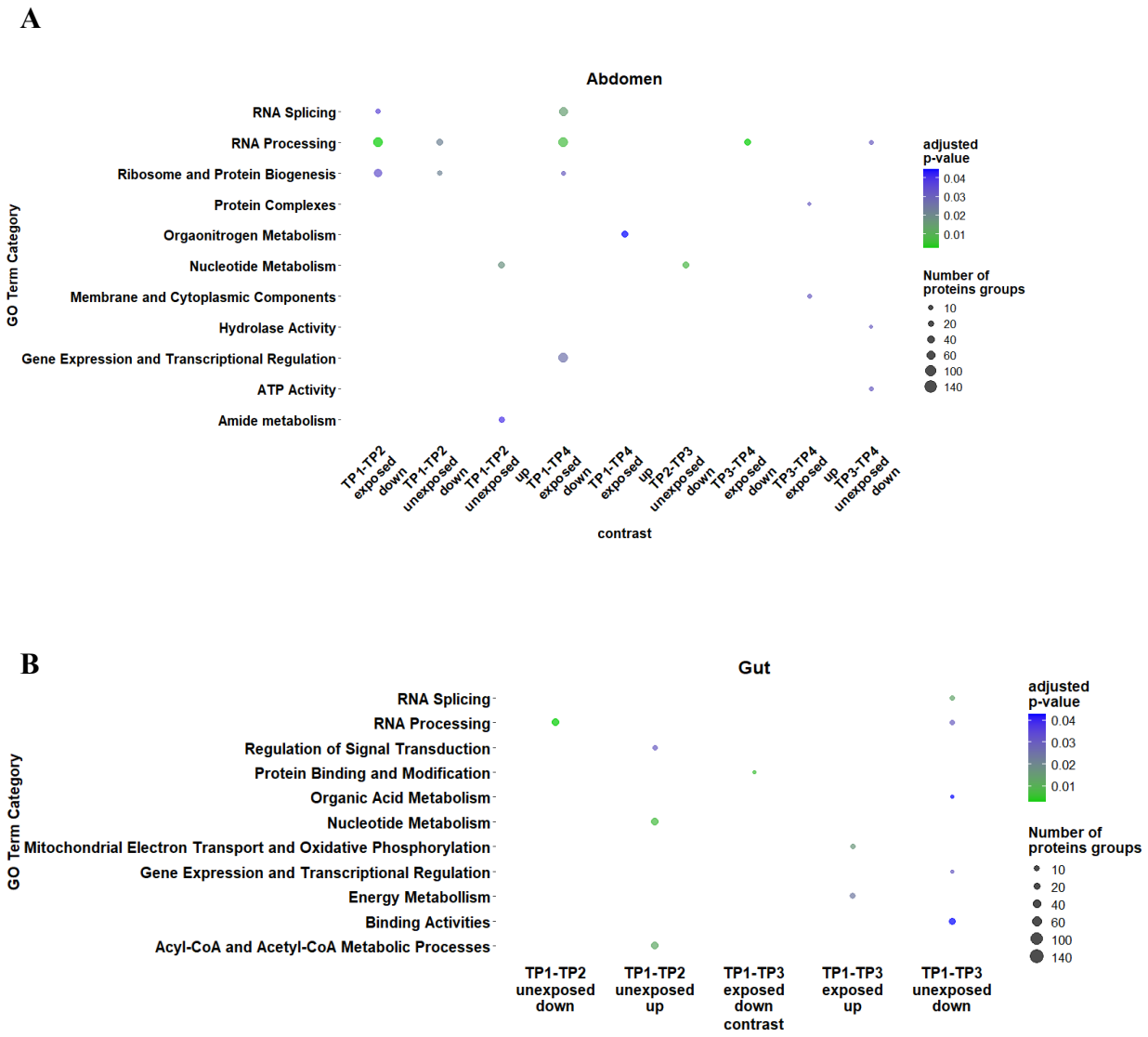
In the abdomen, early bloom and post-bloom comparisons (TP1 vs. TP2 and TP1 vs. TP4) revealed strong downregulation of pathways associated with RNA processing, splicing, and ribosome biogenesis. These disruptions likely impaired the bees' capacity for protein synthesis during periods of heightened stress. In contrast, nucleotide metabolism pathways, including purine and nucleoside bisphosphate biosynthesis, were upregulated in unexposed bees during early bloom, suggesting a more balanced metabolic response compared with their exposed counterparts.

The gut showed pronounced enrichment of energy metabolism and mitochondrial function pathways during early bloom and post-bloom periods. Upregulated processes included oxidative phosphorylation and electron transport chain activity, consistent with the need for increased ATP production during periods of environmental stress. Downregulation of RNA processing pathways was again prominent, highlighting a trade-off between energy production and protein synthesis in this tissue.

In the head tissue, enriched pathways primarily reflected disruptions in core cellular maintenance functions. Early bloom comparisons showed significant downregulation of pathways related to RNA processing and ribosome biogenesis, while post-bloom comparisons revealed suppression of DNA repair processes, including base excision and double-strand break repair. These results suggest that neural tissues may be less able to cope with molecular damage during periods of environmental stress, potentially impairing sensory and cognitive functions.

Collectively, the GO enrichment analysis reinforces the tissue-specific nature of honey bee stress responses during highbush blueberry pollination. The abdomen and gut tissues appear to prioritize detoxification and energy production at the expense of RNA processing and protein

synthesis, while the head tissue may be particularly vulnerable to cumulative molecular damage during early bloom and post-bloom periods.



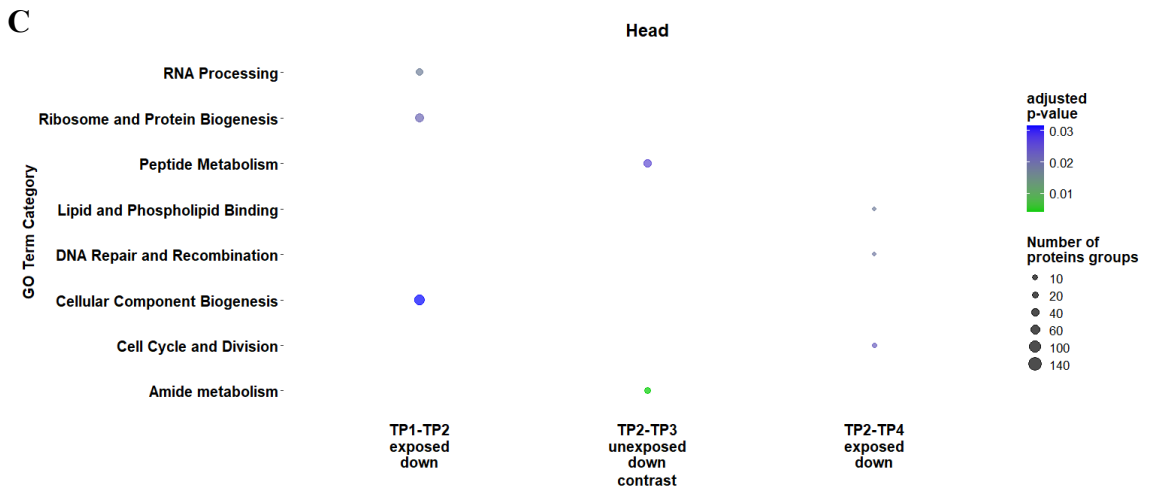


Figure 2. 8 GO term enrichment analysis of differentially expressed proteins in honey bee (A) abdomen, (B) gut, and (C) head tissues across different timepoints and conditions. The x-axis represents the different timepoint comparisons for exposed and unexposed groups, including up- and down-regulated proteins. The y-axis lists the enriched GO term categories, covering biological processes. The size of the dots corresponds to the number of protein groups associated with each GO term, while the color indicates the significance of enrichment, with green denoting the most significant p-values.

2.3.5 Proteomic discoveries from cage trials

Differentially expressed proteins after virus and fungicide exposure

Differential expression analysis of the cage trials, which tested the effects of virus (DWV-A and DWV-B) and fungicide exposure, revealed limited but tissue-specific proteomic changes. Most tissues showed no significant DEPs; adjusted $p < 0.05$) across treatments, particularly in the abdomen and gut for both datasets.

In the DWV-A dataset, no DEPs were detected in abdomen or gut tissues. However, head tissue consistently exhibited upregulation of Cathepsin O across all virus and/or fungicide treatments compared to controls. Cathepsin O plays a key role in protein degradation and turnover, suggesting stress-related effects on neural protein processing.

In the DWV-B dataset, again, no DEPs were detected in the gut tissue. The abdomen showed downregulation of protein "white," a protein involved in ATP binding, in the virus-only treatment. Head tissue revealed downregulation of ethanolamine-phosphate cytidylyltransferase, an enzyme critical for lipid biosynthesis, in the combined virus-fungicide treatment compared to controls.

These findings indicate that while the cage trial identified only a few significant protein-level changes, the responses were localized to specific tissues, most notably the head. This suggests that neural tissues may be particularly sensitive to viral and/or fungicide stress.

2.4 Discussion

The goal of this study was to investigate how honey bee physiology responds to the unique stressors associated with highbush blueberry pollination, including agrochemical exposure and pathogen pressures, by applying proteomics, pesticide analysis, and pathogen detection. Through the integration of these methodologies, this study identified key findings. First, pesticide residue analysis revealed substantial chemical burdens in exposed hives, particularly during the bloom period, with wax samples accumulating the highest pesticide concentrations, followed by pollen, bees, and nectar (Figure 2.5). Flupyradifurone and pyrimethanil emerged as the most prominent pesticides across sample types. Second, pathogen monitoring indicated temporal variations in key honey bee pathogens such as DWV, Nosema, and EFB, with had notable increases at various timepoint and years, however, SBV was the only pathogen with site-related effects. Third, proteomic analysis demonstrated that while no significant differences were found between exposed and unexposed site types alone, timepoint and tissue-specific analyses identified patterns of differential protein expression, particularly in the abdomen, gut, and head tissues.

These patterns included upregulation of proteins involved in energy metabolism, immune defense, and stress response, alongside downregulation in RNA processing and protein synthesis pathways. Together, these findings provide insights into the physiological challenges honey bees face during highbush blueberry pollination and highlight the importance of targeted interventions to mitigate these stressors.

Qualitative observations of field trial hives

In the field trial, careful evaluations of colony health highlighted the importance of beekeeper management for maintaining healthy honey bee populations in agricultural settings. The infrequent occurrences of queen health issues suggest that the colonies largely thrived throughout the field trial in both exposed and unexposed sites. The rare occurrences of queen absence or discontinuities in egg-laying highlight the resilience of queen bees amidst environmental stressors like pesticides and disease, which is supported by research underscoring the queen's pivotal role in colony viability (Amiri *et al.*, 2017). There was no overwhelming qualitative evidence to suggest that hives in highbush blueberry fields were in worse-off compared to the unexposed hives, as some beekeepers and researchers have noticed and attempted to address (McAfee *et al.*, 2018). However, the loss of some hives during the study period highlights the persistent risks faced by colonies today, which may be attributed to factors beyond the scope of the study or beyond the beekeeper's control, as is demonstrated in the most recent research findings which discuss the complexity of unrestricted stressors on honey bee physiology (French *et al.*, 2024; Foster et al, 2024).

Interventions such as brood frame translocations and selective queen replacements appeared effective, as evidenced by the low incidence of hive losses. Strategic interventions like

these reflect a responsive management approach that has been advocated by researchers and the beekeeping community at large to mitigate colony decline throughout all seasons of the year, as thoroughly investigated by Steinhauer *et al.* (2021) in the US and Agrebi *et al.* (2021) in Belgium. The detailed apiary logs, complemented by the initials of the inspectors, not only enhance the traceability of observations but also introduce a human element that may contribute to variability, as each inspector might use slightly different assessment criteria.

The presence of pathogens like chalkbrood, a widespread fungal disease in bee brood (Aronstein, K. A. & Murray, K. D., 2010), and EFB across different sites and timepoints underscores the persistent threat of disease, even in well-managed colonies. The surveillance for behavioural and symptomatic manifestations of distress, alongside signs of obvious pathogen infestation, highlights the complexity of maintaining colony health, aligns with findings that stressors work in networks to disrupt colony health and researchers cannot yet fully predict or explain colony loss (French *et al.*, 2024). The variance in colony strength and pathogen presence, detailed in the apiary logs, may reflect the interplay between environmental stressors like nutrition, climate, genetics, or disease, and beekeeping practices (López-Uribe *et al.*, 2020; French *et al.*, 2024). This study's findings emphasize the importance of continuous monitoring, record-keeping, and proactive management for colony health as suggested by Steinhauer *et al.* (2021), which emphasized that regular monitoring and management are pivotal for maintaining colony health in any apiary.

Pesticides in highbush blueberry pollination hives

As expected, exposed hives from treated blueberry fields contained the most pesticide residue, particularly during the bloom period, which echoes the concerns raised by Tosi *et al.*

(2021) about the heightened risk of pesticide exposure to pollinators during flowering when they are foraging and the impact of these contamination pathways on honey bee physiology. Furthermore, predicting the pesticide exposure source is a difficult task as foraging is a spontaneous event, as explained by Simon-Delso *et al.* (2017). The significant year-to-year increase in pesticide residues also can be explained by site-specific issues concerning blueberry crop health. However, this raises concerns about the cumulative effects of chemicals on bee health and colony resilience over time periods, as more research continues to focus on the long-term impacts of pesticide residues on honey bee behaviour and health in (Tosi *et al.*, 2021; Almasri *et al.*, 2022; Colin *et al.*, 2019; Pilling *et al.*, 2013; Hesselbach *et al.*, 2019)

The presence of a diverse range of pesticides, from systemic and non-systemic insecticides to acaricides and fungicides/herbicides, reflects the multifaceted exposure that bees face in commercial agriculture, aligning with other pesticide residue research that reported a widespread distribution of agrochemicals in bee environments (Zawislak *et al.*, 2019; Traynor *et al.*, 2016). Recent research on pesticides has been focusing on the individual and synergistic effects of pesticides on bee health, and how individual sublethal levels impact honey bee physiology and behaviour (Foster *et al.*, 2024).

The quantification of pesticide residues in this study, measured in ppb, reveals accumulation levels that differ among the sample types, with the highest concentrations found in wax. Additionally, wax had the largest number of different pesticides at 22, followed closely by pollen with 21 types. This is consistent with findings by Mullin *et al.* (2010), which indicate that wax can serve as a reservoir for contaminants because its lipophilic nature tends to accumulate hydrophobic substances such as pesticides. Hive equipment (e.g., frames, hive tools, hive boxes) used in this study were sterilized of bacteria using electron beam technology before beginning

the field trial to avoid pathogen contamination. Agrochemical buildup is less easy to get rid of, especially when reusing frames in hives which is common for the typical beekeeper to reduce equipment costs. Using new frames every season with no pre-existing wax is costly for beekeepers and is not realistic in an industrial setting. Evidence has shown that there is drift of pesticide residue between chemical-contaminated and the bees living in the hive (Fulton *et al.*, 2019). Conflicting research from Payne *et al.* (2019) has shown that previously contaminated wax has no impact on colony health indicators such as brood rearing ability, population size, comb production, food collection, and overwintering survival.

The other samples in the study, bees, pollen, and nectar, contained similar total amounts of pesticide residues, but this was always lower than the amounts found in wax. The detection of chemical residues in bee tissue is a significant concern, as it demonstrates the vulnerability of bees to direct contamination from their environment. It has been found that bees come into contact with pesticides through their activities on plants, including foraging for nectar and pollen (Krupke *et al.*, 2012). These substances adhere to their bodies and can be ingested or absorbed through the exoskeleton (Davidson, 2021). Studies have shown that a variety of pesticides, particularly insecticides and fungicides, can pose significant risk through contact exposure even at low residue amounts (Zaluski *et al.*, 2017; Astolfi *et al.*, 2022).

Twenty-three of the 32 pesticides detected in this study are listed in the 2024 Government of British Columbia Blueberry Production Guide, emphasizing that these exposures align with common agricultural practices. A mix of insecticides, fungicides, and herbicides are outlined to treat common issues in blueberry crops such as pests, fungal infections, and weeds. The 10 remaining agrochemicals not included in the advice given to blueberry growers was a mix of herbicides, insecticides, fungicides, and acaricides, likely coming from other treated crops in the

surrounding agricultural area of the Lower Mainland. Two studies have previously confirmed a large amount of off-farm exposure to agrochemicals linked to pollinating colonies, and this form of chemical-drift could be what is occurring in our study (Averill *et al.*, 2024; Graham *et al.*, 2022). However, exposed and unexposed colonies were interacting with chemicals, yet it was the exposed colonies that reported to have the highest amounts by far between the two groups. These findings suggest that there is definite linkage between the chemicals used to treat blueberry crop threats and the hives of the honey bees provided for blueberry pollination. Additionally, blueberry growers may be using certain agrochemical products that are not listed in the provincial production guide for blueberries (Government of British Columbia, 2024). The results of this work find that there is drift of agrochemicals typically used in blueberry cultivation to surrounding agricultural areas where honey bees are foraging. These findings align with research by Graham *et al.* (2022), who determined that bees face significant pesticide risks from pesticides applied off-site, with exposures mainly occurring through bee-collected pollen.

The predominance of flupyradifurone in unexposed bee samples and pyrimethanil in exposed samples reflects the specific pesticide usage patterns in the surrounding agriculture, which can differ significantly based on the crop's management schedule to treat for pests and fungal threats (Government of British Columbia, 2024). Flupyradifurone is an insecticide used in fruit crops, like blueberry, to protect from sucking pests, such as aphids. It can be applied pre and post bloom of blueberry crops up to once every seven days, as per the blueberry production guide. Current research on flupyradifurone indicates its potential impact on honey bee health, revealing sublethal effects that could be compromising bee behaviour and immunity (Al Naggar *et al.*, 2019; Guo *et al.*, 2021). Pyrimethanil is a common fungicide used in blueberry crops to fight *Botrytis* fruit rot (*Botrytis cinerea*), as noted in the provincial blueberry production guide

(Government of British Columbia, 2024). It is applied at the beginning of bloom and repeated every 7 to 10 d , and possibly during fruit ripening according to the province's blueberry production guide (Government of British Columbia, 2024). Similarly to flupyradifurone, pyrimethanil was found in all samples, suggesting its widespread use in blueberry crops and its potential interaction with pollinators outside of the field of interest. There is growing interest in the effects of fungicides and honey bee physiology as compiled by Rondeau and Raine (2022). Specific research with pyrimethanil has investigated its impact on EFB in honey bee larvae, which found no significant health effects (Wood *et al.*, 2020).

Meanwhile, the other top pesticides found in our samples were, for the most part, associated with a blueberry crop-related threat and are listed in the provincial production guide for blueberries (Government of British Columbia, 2024). For example, pyraclostrobin, fluopyram, boscalid, and metconazole are fungicides used to treat Anthracnose fruit rot (*Colletotrichum fioriniae*), Botrytis fruit rot, mummy berry (*Monilinia vaccinii-corymbosi*), and Phomopsis canker (*Phomopsis vaccinii*) in highbush blueberry. Insecticides like imidacloprid and flonicamid are used against aphids in the crop. Linuron is a herbicide used for management of large-leafed weeds in berry crops. Finally, coumaphos is an acaricide used applied to honey bee hives for treatment against the *Varroa* mite which dominates in the spring and fall, it is unsurprising to find ruminants of this treatment in the beekeeper hives used in this study.

Although fungicides are not considered highly toxic to honey bees, recent research has been able to link fungicide exposure, specifically by pyraclostrobin and boscalid, to early foraging, reduced worker lifespans, and reduced colony populations (Fisher *et al.*, 2021). Recent research has highlighted the mitochondrial effects of fungicides on honey bees (Campbell *et al.*, 2016). Sub-lethal doses of fungicides such as pyrimethanil have been associated with decreased

energy metabolism and altered foraging behavior (Prado *et al.*, 2019). It is likely from this research that exposure to fungicides like pyrimethanil, pyraclostrobin, and boscalid in large amounts as found in this study is having an impact on bee behaviour and health status impacting their performance in highbush blueberry pollination.

The analysis of pesticide residues provides insights into the types of agrochemicals bees are exposed to in the commercial blueberry agricultural setting, as opposed to an organic blueberry farm. The detection of a wide array of synthetic pesticides, including insecticides, fungicides, herbicides, and miticides, highlights the extensive chemical inputs used in conventional blueberry farming systems. While most detected pesticides are incompatible with organic farming practices, trace amounts of Spinosyn A and D, which are approved for use in organic agriculture, were found in bee samples. Although these compounds were present in nearly untraceable amounts, their potential toxicity to honey bees—particularly when residues are wet—raises concerns about their safe application and timing. This finding underscores that even organic-approved pesticides can pose risks to pollinators and highlights the importance of careful pesticide management in both conventional and organic farming systems.

Pesticide risk

When considering pesticide risk, the application of risk quotients that incorporate the relative toxicity of each compound has been shown to provide a more discerning assessment of potential impacts on bee health (Graham *et al.*, 2022). Systemic insecticides, such as thiamethoxam, are among the most lethal classes of pesticides for honey bees (Shi *et al.*, 2017; Tesovnik *et al.*, 2020). The identification of thiamethoxam as the pesticide with the highest oral risk in this study, despite its relatively low concentration in samples, highlights the importance of

considering evaluating toxicological profiles alongside residue levels. This is also supported by the findings of Sanchez-Bayo and Goka (2014) who emphasized that the risk to bees from pesticides is not solely a function of the quantity but also of the intrinsic toxicity of the compounds involved, highlighting the need for comprehensive evaluations that consider both the individual and synergistic effects of pesticide residues.

In terms of overall concentration, the cumulative pesticide levels detected in exposed hives in this study were higher than in unexposed hives, but still relatively low compared to levels reported in studies of crops with more intensive pesticide use, such as almonds (e.g., Mullin *et al.*, 2010). This suggests that although risk was elevated in exposed bees, the absolute quantities of pesticides may not have reached acute toxicity thresholds, possibly explaining the absence of strong protein-level changes in some tissues or timepoints.

This resonates with the findings of Sanchez-Bayo and Goka (2014), who emphasized that pesticide risk to bees is not solely a function of contration, but also of the intrinsic toxicity of compounds and potential synergistic effects. These findings collectively necessitate a nuanced approach to pesticide management in agricultural landscapes, considering not only the direct lethal effects but also the sub-lethal and chronic exposures that bees endure across different hive matrices.

Exposure to sub-lethal doses of thiamethoxam has been documented to cause changes in foraging, gene expression, organ development, and increased viral loads in honey bees (Tavares *et al.*, 2017; Christen *et al.*, 2018; Shi *et al.*, 2017; Tesovnik *et al.*, 2020; Frial *et al.*, 2017; Coulon *et al.*, 2019). Thiamethoxam was found predominantly in our study's pollen and nectar samples, both of which are key sources of food for bees and are commonly processed into honey. Although honey was not analyzed in this study, it is possible that thiamethoxam residues could

accumulate in honey stores over time, depending on the duration and concentration of exposure. This warrants further investigation, particularly given consumer and regulatory interest in pesticide residues in bee products.

As discovered by Graham *et al.* (2022), thiamethoxam is detected in highbush blueberry field samples in North American despite its limited reported use in blueberry crops. Thiamethoxam research in the context of highbush blueberry pollination is limited and deserves greater recognition in future studies as a potential source of hive health disturbance due to its high oral toxicity and sub-lethal effects on honey bee physiology.

Pathogens in highbush blueberry pollination hives

The pathogen landscape within the apiaries of our field trial was diverse, comprising one parasite, two bacterial infections, and nine viruses, reflecting the disease-fraught ecosystem honey bees navigate. The presence of multiple varieties of pathogens, sometimes all found within one hive, is demonstrative of the complex nature of pathogen-host interactions within colonies. This finding resonates with research by Cornman *et al.* (2012) suggesting that hives are susceptible to a range of pathogens and that simultaneous infections can interact negatively to disrupt hive health. Conversely some pathogens like CBPV, ABPV, and KBV, were not prevalent enough in the experimental hives for inclusion in statistical modeling, a finding that resonates with the results of previous studies which have reported variable incidences of these viruses in bee populations (Berényi *et al.*, 2006).

V. destructor, a well-known menace of bee colonies, showed a significant decrease in 'high' infestations in 2021 in exposed and unexposed colonies. This was likely due to colony-level differences since the hives used in 2020 were unique to 2021. Research has shown that

natural hive tendencies, or phenotypic traits like mite resistance or improved hygienic behaviour, can improve a colonies outcome with *Varroa* mites (Mondet *et al.*, 2020; Lefebre *et al.*, 2024). However, the increased likelihood of detecting *Varroa* mites overall indicates a paradoxical rise in their presence, suggesting that while fewer colonies were experiencing high infestation rates, mites remained widespread throughout colonies in that year. Furthermore, the LMM analysis revealed an increase in mite infestation percentages at the end of the experiment (TP4) in site types, which is consistent with findings by Van Dooremalen *et al.* (2012) that *Varroa* populations tend to peak as bees prepare for the winter. The lack of a site effect on mite infestations is unsurprising and aligns with research indicating that *Varroa* management is multifaceted and highly dependent on individual colony characteristics such as genetics (Mondet *et al.*, 2020; Lefebre *et al.*, 2024; Ramos-Cuellar *et al.*, 2022). No evidence from this study suggests that *Varroa* mite infestation rates in colonies are impacted by blueberry pollination.

Nosema spore counts showed a significant decrease over time and years, with notable reductions at TP2, TP3, and TP4 compared to the baseline. Site exposure did not significantly affect spore counts, indicating that there is no indication of a relationship between *Nosema* prevalence and blueberry pollination. As a binary outcome, *Nosema* showed increased odds of infection at later timepoints, TP3 and TP4, and in 2021. Similar to spore counts, site exposure did not significantly impact infection likelihood, emphasizing temporal and annual variations over site-specific factors in predicting *Nosema* infection rates. The high variability among colonies in both site types suggests diverse responses to *Nosema*, potentially due to genetic susceptibility in colonies unrelated to blueberry pollination.

EFB presence was significantly influenced by time, particularly at TP3 and TP4 with increased infection later in the summer. Most commonly EFB is found in the early spring,

however, our findings show detection throughout the blueberry season. In both years of the study, exposed colonies experiences higher incidences of EFB outbreak at the end of pollination, echoing the findings of Grant *et al.* (2021) who's study hives also had increased levels of EFB immediately following blueberry pollination. This study's lack of site influence on EFB detection is in contrast with previous findings and concerns over the high incidences of EFB disease in colonies pollinating blueberry crops (Wardell, 1982; Guarna *et al.*, 2019; Thebeau *et al.*, 2022). It was hypothesized because of these concerns around EFB that the bacterial infection would be increased in exposed sites, however, results of this study did not find that one site favored EFB detection.

DWV levels were somewhat sporadic throughout the experiment, highlighting this virus' prevalence in colonies. Detection increased over time throughout the study, with the highest detection at TP4 in 2021, suggesting progressive virus accumulation within colonies. The substantial colony-level variability and the higher infection rates in colonies in 2021 compared with the year 2020 suggests that DWV dynamics are influenced by factors unique to each colony. No site-specific relationship was determined with DWV and blueberry pollination in this study.

SBV was detected the most during the study at TP3 but had the highest viral loads at TP4. This increase in viral loads at TP4 was only found in the exposed group. This high level of SBV associate with blueberry pollination, could be a major contributor to the health issues beekeepers report after their bees pollinate this crop, instead of the previously-held belief that EFB was the main driver of pathogen stress following blueberry pollination (McAfee *et al.* 2024).

LSV experienced increased levels towards the end of blueberry pollination at TP3 and TP4 suggesting that the virus was accumulating in small amounts over time, but site and year were not significant predictors of this virus for the experiment. VDV was not found to have any significant relationship to time, site, or year in this study. BQCV and IAPV were universally present in all samples from experimental hives, denoting their pervasive nature as viruses in North American colonies. In general, the levels of LSV, VDV, BQCV, and IAPV found in colonies was likely unique to hive characteristics as suggested by the large amount of variability found in our statistical results.

Overall, while individual pathogens showed temporal and colony-level variation throughout the blueberry pollination period, there was no consistent or statistically significant evidence that hives exposed to blueberry pollination experienced increased pathogen loads compared to unexposed hives. This finding challenges the assumption that blueberry pollination directly contributes to elevated pathogen pressure and instead suggests that other factors, such as colony genetics, inter-colony transmission, and broader environmental stressors, may play more substantial roles in shaping pathogen dynamics during pollination periods.

Proteomic discoveries from highbush blueberry field trials

Proteomic studies are critical for uncovering how honey bees respond to environmental stressors, particularly in agricultural landscapes where they are frequently exposed to pesticides and pathogens. This study focused on examining proteomic changes across different tissues and timepoints to understand honey bee physiological responses during highbush blueberry pollination, a system known for its challenges in maintaining bee health.

The absence of significant differences between exposed and unexposed sites suggests that environmental stressors, such as agrochemical drift and pathogens, may be pervasive across the highbush blueberry pollination landscape. Notably, in other crop systems studied under the BeeCSI project, there was a more pronounced distinction between colonies near and far from the crops, which was not observed in the blueberry system. The absence of a difference between site types in this study could reflect a true lack of biological difference or, alternatively, that variability within site types was too high to detect meaningful differences. . Despite efforts to establish unexposed control sites, it is likely that both site types experienced similar environmental stressors, such as agrochemical drift and pathogens, as supported by our data. This highlights the pervasive nature of these stressors, even in supposedly "unexposed" areas, particularly in a highly agriculturally active region like the Lower Mainland. Future studies should aim to use more controlled unexposed sites and consider factors known to impact honey bee health like climate, nutrition, queen health, and genetic diversity when linking proteomic changes to specific agricultural environments.

Timepoint comparisons across abdomen, gut, and head tissues revealed clear temporal patterns in honey bee stress responses. Early bloom (TP2) and post-exposure (TP4) periods were the most critical periods, as indicated by the high number of DEPs at these timepoints (Figure 2.7). Fewer DEPs were observed at TP3 (peak bloom); however, functional enrichment results suggest that TP3 was still a physiologically important period, particularly for energy metabolism in the gut. These findings suggest that bees face acute stress response early in the pollination period and again at post-exposure, while TP3 may represent a metabolic bottleneck where bees attempt to meet heightened energy demand while suppressing other cellular functions.

In honey bees, different tissues serve specialized functions, which is reflected in the biological processes and proteins they express. The abdomen, housing critical organs such as the fat body, and digestive and reproductive organs, plays a significant role in the detoxification and stress response, the immune response, and RNA regulation. Functional enrichment analysis revealed significant downregulation of critical pathways during early bloom (TP2) and post-exposure (TP4), particularly in processes related to RNA processing, splicing, and ribosomal biogenesis. These findings suggest a disruption in the gene expression and transcription machinery, further supported by the downregulation of key proteins such as the 40S ribosomal protein S26 and the 39S ribosomal protein L11, which are essential for the assembly and function of ribosomes. Additionally, RNA processing components like the U3 small nucleolar ribonucleoprotein protein, a key player in pre-rRNA processing, and proteins involved in RNA splicing, such as ribonucleases, were also significantly impacted. This disruption likely affects the bees' ability to synthesize proteins efficiently while exposed to agrochemicals and pathogens. Additionally, the upregulation of heat shock proteins (HSP) like HSP40 at these timepoints indicates an acute stress response at the cellular level helping to refold damaged proteins or assist in their degradation if they are beyond repair, reflecting the biological demand on the abdomen to manage toxins and other stressors in the blueberry environment.

In the unexposed abdomen comparison, a similar disruption was observed, but with differences in the impacted pathways. Key processes related to nucleotide metabolism, such as purine and nucleoside bisphosphate biosynthetic processes, were upregulated during early bloom (TP2) indicating a focus on energy production and cellular maintenance. While the unexposed bees also showed downregulated of RNA processing pathways, including ribonucleoprotein complex biogenesis and RNA splicing, the enrichment of nucleotide metabolism suggests a more

balanced physiological state, potentially allowing for recovery or adaptation and suggesting that bees in less stressed conditions may have better preserved their energy production and RNA metabolic capacity. Additionally, the unexposed bees exhibited increase amide metabolic processes, which could be tied to protein degradation or recycling under stress, which may help these bees maintain cellular homeostasis despite external stressors.

Gut tissue, central to nutrient absorption and energy production, showed significant proteomic shifts throughout the highbush blueberry pollination period, especially during peak bloom (TP3). The upregulation of pathways related to mitochondrial function, such as oxidative phosphorylation, mitochondrial electron transport, and cellular respiration, suggests a heightened energy demand in the gut tissue. This upregulation aligns with the gut's role in generating ATP, which is crucial for detoxification and managing cellular stress. Key proteins such as NADH dehydrogenase and cytochrome c oxidase, essential components of the electron transport chain, were overexpressed, reinforcing the idea that the gut tissue is working harder to meet the increased metabolic demands likely caused by agrochemical exposure. Similar findings have been observed in other studies (e.g., Nicodemo *et al.*, 2020), where sub-lethal exposure to fungicides impaired mitochondrial function in bees, highlighting the gut's susceptibility to environmental stressors during pollination.

The downregulation of RNA processing in gut tissue (e.g. RNA splicing and ribonucleoprotein complex assembly), seen in both exposed and unexposed bees during early bloom and post-exposure, suggests that the bees may be prioritizing energy production over protein synthesis under periods of stress. Similar patterns have been reported in studies where bees under stress shift their metabolic pathways to manage detoxification and maintain energy production at the expense of other cellular processes. This trade-off could impair long-term gut

health and protein synthesis, making the bees more susceptible to extended stress over time. The upregulation of TOR signaling pathways in TP4 further indicates an adaptive response, where bees attempt to regulate growth and metabolism in response to environmental stressors. However, this comes at the cost of suppressing critical pathways, such as RNA processing and cellular repair. The downregulation of these pathways, paired with the overexpression of stress-response proteins such as heat shock proteins (HSP40) and NADH dehydrogenase, indicates that the gut was experiencing acute stress, with limited capacity for normal protein synthesis and repair. This acute stress response, particularly visible through the overrepresentation of oxidoreductase activity and electron transfer pathways, emphasizes the gut's role in detoxifying reactive oxygen species generated by chemical stress. Together, these findings indicate that the gut tissue undergoes a complex response to stress, balancing heightened energy production with impaired protein synthesis. This makes it one of the tissues most vulnerable to the environmental stressors encountered during highbush blueberry pollination, particularly at peak bloom and post-exposure periods.

In the head tissue, which includes the brain and sensory organs, the most significant changes were observed in pathways associated with the cell cycle, DNA repaid, and lipid metabolism (Figure 2.8). Key proteins likely to be affected include neurotransmitter receptors, synaptic proteins, and odorant-binding proteins, all of which are essential for cognitive functions, sensory processing, and communication. Functional enrichment analysis revealed significant downregulation of pathways related to RNA processing, ribosome biogenesis, and cellular component organization, particularly during the early bloom period (TP2). This suggests that stressors may impair the bee's ability to produce and synthesize proteins necessary for neural activity, ultimately affecting cognitive and sensory functions. For example, ribosomal proteins

(e.g., 39S ribosomal protein L11) involved in ribosome biogenesis were downregulated, highlighting potential disruptions in protein synthesis.

Pathways related to DNA repair in head tissue, such as base excision repair and double-strand break repair, were also downregulated, particularly during the post-exposure period (TP4). This suggests that the head tissue may be less able to cope with DNA damage caused by environmental stressors such as agrochemicals. The downregulation of cell cycle and division pathways, including mitotic cytokinesis and DNA recombination, further suggests that the bees' ability to replenish and maintain neural cells may be compromised, which could impair cognitive function over time. Interestingly, lipid and phospholipid binding pathways were also downregulated during the post-exposure period, indicating potential disruptions in cell membrane integrity and signaling. While this response may be linked to pesticide exposure, it is also possible that certain compounds or nutrient imbalances present in blueberry pollen contributed to this effect. Previous studies have shown that pollen composition can influence honey bee physiology, and thus chemical and dietary factors should be considered in interpreting these results (Vaudo *et al.*, 2016; Tosi *et al.*, 2022).

Additionally, the downregulation of lipid and phospholipid binding pathways in exposed bees suggests potential disruptions in neural membrane integrity, which could further impair sensory processing and communication. In unexposed bees, the upregulation of mitophagy at early bloom indicates that bees in less stressful conditions may be clearing damaged mitochondria more efficiently, potentially preserving their neural function better than their exposed counterparts. These findings indicate that while the head tissue showed fewer differentially expressed proteins compared to the abdomen and gut, the consistent changes across timepoints suggest it is still moderately impacted by stressors. The downregulation of critical

pathways involved in protein synthesis, DNA repair, and cell division could affect the bees' ability to maintain cognitive and sensory functions, ultimately impairing their ability to respond to environmental challenges during pollination.

Throughout this study, timepoint comparisons across different tissues revealed varying degrees of differential expression and functional pathway enrichment, which provided insights into the temporal dynamics of honey bee responses to stressors during highbush blueberry pollination. Initially, differential expression analysis suggested that TP2 and TP4 were the most critical periods, showing the highest number of differentially expressed proteins (DEPs) across tissues. These timepoints likely represent the immediate response to early exposure (TP2) and the sustained or delayed response post-exposure (TP4), highlighting broad proteomic changes in response to environmental stressors. However, the functional enrichment analysis painted a more nuanced picture. Early bloom (TP2) appears to be a critical period of disruption, particularly in RNA and protein synthesis pathways across tissues. This is followed by a metabolic shift during peak bloom (TP3), where energy production pathways, especially in gut tissue, become central. Post-exposure (TP4) maintains significant disruptions, particularly in the abdomen and head, where bees face ongoing impairments in protein synthesis, DNA repair, and energy metabolism. Despite TP3 showing fewer DEPs, the functional enrichment suggests this period is critical for energy production, particularly in gut tissue. This highlights the possibility of a metabolic bottleneck, where the bees' capacity to cope with environmental stressors is most strained. While TP2 and TP4 may represent times of acute stress, TP3 reflects a critical phase where the bees attempt to meet heightened metabolic demands while possibly suppressing other vital functions like RNA processing.

This findings of this study support the need for targeted interventions during specific pollination periods, particularly early bloom and post-exposure. Strategies aimed at supporting energy metabolism and mitigating oxidative stress in intense agricultural landscapes like highbush blueberry - such as supplementing bees with nutrition or antioxidants during these periods - could help improve honey bee resilience during pollination. Future research should focus on refining unexposed control sites, considering the potential for agrochemical drift even in areas designated as "control" sites. Additionally, more controlled experiments should investigate how genetic diversity, queen health, and nutritional factors interact with proteomic responses to provide a more comprehensive understanding of honey bee health in agricultural landscapes. In particular, further studies on gut tissue responses could reveal more about the trade-offs bees face when balancing energy production and protein synthesis under stress.

Proteomic discoveries from cage trials

The intention for the findings of the cage experiments was to shed light on the proteomic responses of honey bees to viral infection, fungicide exposure, and their combination. A secondary goal was for the data discovered from the cage trials to be such that could either validate or dismiss the findings from the field trial proteomic analysis.

The differential expression analysis of the DWV-A and DWV-B datasets revealed limited changes in protein expression across the abdomen, gut, and head tissues of honey bees subjected to different treatments. In the abdomen and gut tissues no DEPs were identified across any of the treatment comparisons (dO vs dD, d, dX). This suggests that the DWV-A treatment had minimal or no impact on protein expression levels in these tissue types. One protein, cathepsin O, was significantly upregulated in the head tissue when comparing the control group (dO) to each of

the treated groups (dD, dP, and dX). Cathepsin O is known for its role in protein degradation and turnover as a protease, which may indicate an increased need for protein degradation processes in response to the treatments of virus, fungicide, and virus and fungicide combined. This suggests that the viral and/or fungicide stress may induce heightened protein degradation and turnover as a cellular response in the head tissues of honey bees.

In contrast, the DWV-B dataset displayed slightly more nuanced changes. In the abdomen tissue, the protein “white”, involved in ATP binding and cellular energy metabolism, was downregulated in honey bees exposed to the virus alone (dD) compared to the control (dO). This suggests a possible disruption in energy metabolism pathways, which could have broader implications for the energy demands of the honey bees under viral stress. Additionally, in head tissues, ethanolamine-phosphate cytidylyltransferase, a key enzyme in lipid biosynthesis, was significantly downregulated in the virus and fungicide treatment group (dX) compared to the control (dO). This down-regulation may point to an altered lipid metabolism, possibly affecting membrane integrity or signalling pathways. Similar to the DWV-A dataset, no differentially expressed proteins were identified in the gut tissue across any treatment comparisons. This indicates a lack of significant impact on protein expression in the gut tissue from the virus and fungicide treatments.

These findings allude to the idea that abdomen and gut tissues generally did not show significant changes in protein expression across treatments, except for the downregulation of the “white” protein in the abdomen tissue in the DWV-B dataset. Head tissues exhibited more pronounced changes, with cathepsin O being upregulated in response to DWV-A virus and fungicide treatments, and ethanolamine-phosphate cytidylyltransferase being downregulated in response to DWV-B treatment combined with fungicide. These findings suggest that head tissue

might be more responsive to the stressors applied in the experiments, particularly in processes related to protein degradation and lipid metabolism.

None of the differentially expressed proteins identified in tissue and treatment specific comparisons from the cage trials overlapped with the proteomic findings from the field study. This indicates that the cage trials did not validate the specific protein-level changes observed in honey bees pollinating highbush blueberries. However, distinct changes in head tissue proteins, particularly in response to fungicide-containing treatments, suggest that field-realistic doses of pyrimethanil may still have a biological impact on individual proteins such as cathepsin O and ethanolamine-phosphate cytidylyltransferase. One limitation of the cage trials is that pesticide residues were not quantified in bee tissues, making it difficult to confirm whether exposure levels were comparable to those in the field. Future trials would benefit from verifying pesticide residues, increasing DWV levels, and rigorously testing viral stock purity to better replicate field conditions and enhance the reliability of results Additionally, conducting experiments with virus-free bees and increasing replicate numbers would help clarify treatment effects. Despite current discrepancies, the tissue and treatment specific proteomic patterns observed in this study suggest that this approach holds promise for dissecting honey bee responses to agrochemical and pathogen stressors under controlled conditions.

Chapter 3 Conclusion

3.1 Summary of findings

In this body of work, the primary goal was to investigate the proteomic responses of honey bees to stressors associated with highbush blueberry pollination, including agrochemical exposure and pathogen prevalence. The study aimed to correlate these proteomic changes with specific stressors to identify potential impacts on honey bee health. This work was done using a large-scale field trial with honey bee colonies near and far from highbush blueberry agriculture, as well as with caged bees and specific stressor treatments inferred from field trial data, specifically agrochemicals and pathogens. Proteomics were performed to elucidate protein markers from these stressors and statistical techniques such as differential expression analysis and gene enrichment analysis aided in elucidating the biological impacts of agrochemical and pathogen stressors on honey bees in the blueberry agricultural landscape.

The large-scale field trial revealed that pesticide residues were highest during the bloom period, and even unexposed hives experienced significant contamination, raising concerns about the effectiveness of control sites. The presence of 32 pesticides, with 23 linked to blueberry farming, highlighted the widespread impact of agricultural practices on honey bee health. Notably, systemic insecticides like flupyradifurone and fungicides like pyrimethanil, commonly used during the early bloom period, were found in brood-nest bee samples, suggesting sublethal effects on behavior and immunity.

The pathogen analysis found that Sacbrood Virus (SBV) was significantly associated with exposed hives, particularly at TP4, indicating increased stress in colonies near highbush blueberry crops. Conversely, European Foulbrood (EFB), which had been considered a concern

for bees pollinating blueberries, showed no significant correlation with site type, suggesting variability in pathogen prevalence.

The proteomic analysis indicated that timepoints and tissue types were more influential factors than proximity to highbush blueberry crops in determining protein expression changes. While no significant differential expression was observed between exposed and unexposed hives, the study revealed important time and tissue-specific differences. Abdomen and gut tissues were particularly sensitive to stressors, with notable changes in energy metabolism and cellular function during the bloom period, reflecting the dynamics of foraging near highbush blueberry crops.

Initial differential expression results suggested that abdomen tissue might be the most responsive to stressors, showing the highest number of DEPs, likely linked to detoxification and immune processes. However, functional analysis revealed that gut tissue underwent more extensive and consistent metabolic changes across both exposed and unexposed sites, particularly in pathways critical for energy production and oxidative phosphorylation. These findings underscore the gut's heightened sensitivity to the highbush blueberry environment. Although fewer DEPs were detected in the gut, the proteins involved were functionally significant, suggesting that this tissue may be more critically impacted by agrochemical and pathogen stressors than previously thought.

The controlled cage trials allowed for a more refined exploration of specific stressors, such as viruses and fungicides. However, the proteomic responses observed in the cage trials did not fully align with those from the field studies, indicating that the experimental design may require refinement. Differences between field and cage results demonstrate the complexity of replicating environmental conditions in controlled settings.

3.2 Future research directions

Based on the findings, several future research directions are suggested to better understand environmental stressors on honey bee health in highbush blueberry agriculture. Investigating the long-term effects of flupyradifurone and pyrimethanil on behavior and immunity is crucial, as these chemicals may have broader impacts on colony health. Further research should explore interactions between agrochemicals and pathogens, such as SBV and EFB, to understand their combined effects on bee physiology.

A key limitation of this study was the difficulty in isolating control sites from agrochemical exposure. Future studies should use control hives located farther from agricultural activities to ensure more accurate comparisons. Improved cage trials with verified virus-free bees, quality virus stocks, and larger sample sizes would also enhance the reliability of findings, helping to clarify the effects of stressors in controlled settings. Longer field trials, particularly around bloom and post-exposure periods, are needed to identify when honey bees are most vulnerable, guiding the development of targeted interventions. Additionally, further research into potential biomarkers identified in this project, alongside the BeeCSI project, could solidify specific proteomic markers to be used as potential health indicators to help develop diagnostic tools for beekeepers. Genetic studies focused on stress response pathways could also be valuable as they may identify traits for breeding bees with enhanced resilience to colony distress.

In summary, advancing knowledge of pesticide effects, pathogen dynamics, and genetic factors will support better management practices for honey bee health in highbush blueberry farming and other agricultural settings.

3.3 Limitations of this study

Several limitations were encountered during this study. The control sites, though designated as "unexposed," still experienced significant pesticide contamination, limiting the ability to compare exposed versus unexposed colonies accurately. Additionally, the cage trials did not fully replicate field conditions, leading to discrepancies between field and cage trial results. The controlled conditions may have oversimplified stressor interactions, which are more complex in natural settings. The limited number of virus-free bees and sample sizes also reduced the generalizability of findings related to viral and fungicide impacts.

Furthermore, the study was conducted over two pollination seasons, which may not fully account for year-to-year variability in agrochemical exposure and bee health. Extending the research across multiple seasons would provide more robust data on the cumulative effects of stressors. Geographically, the study focused on a specific region, and findings may not represent other areas with different environmental conditions or agricultural practices.

Another limitation lies in the scope of stressors examined. The study primarily focused on a limited range of agrochemicals and pathogens, excluding other potential stressors such as additional pests, nutritional deficiencies, or climate-related factors. Variability in how and when agrochemicals were applied by different growers could also impact the level of exposure, making it challenging to generalize results across different farming practices.

The temporal resolution of the data was another constraint. While timepoints were designed to capture critical exposure periods, more frequent sampling might have provided greater insights into the dynamics of agrochemical exposure and pathogen infection. The depth of proteomic analysis also had its limits, potentially missing low-abundance proteins that could play significant roles in stress responses. Additionally, potential confounding factors, such as

pre-existing colony health and environmental conditions, may have influenced the outcomes, complicating the isolation of individual stressor effects.

Overall, while the findings of this study are valuable, addressing these limitations in future research will help to refine our understanding of honey bee health in agricultural systems.

Bibliography

Agriculture and Agri-Food Canada (AAFC). (2023). Statistical Overview of the Canadian Honey and Bee Industry – 2023. Retrieved from https://agriculture.canada.ca/en/sector/horticulture/reports/statistical-overview-canadian-honey-and-bee-industry-2023

Alaux, C. *et al.* (2017). Nutritional Stress and Ecological Factors Affect Honeybee Health. *Journal of Apicultural Research,* 56(4), 392-405.

Almasri, H., Liberti, J., Brunet, J. L., Engel, P., & Belzunces, L. P. (2022). Mild chronic exposure to pesticides alters physiological markers of honey bee health without perturbing the core gut microbiota. *Scientific Reports*, *12*(1), 4281.

Al Naggar, Y., & Baer, B. (2019). Consequences of a short time exposure to a sublethal dose of Flupyradifurone (Sivanto) pesticide early in life on survival and immunity in the honeybee (*Apis mellifera). Scientific Reports*, *9*(1), 19753.

Amiri, E., Strand, M., Rueppell, O., & Tarpy, D. (2017). Queen Quality and the Impact of Honey Bee Diseases on Queen Health: Potential for Interactions between Two Major Threats to Colony. Health. *Insects*, *8*(2), 48. https://doi.org/10.3390/insects8020048

Aronstein, K. A., & Murray, K. D. (2010). Chalkbrood disease in honey bees. *Journal of Invertebrate Pathology*, *103*, S20-S29.

Astolfi, A., Kadri, S. M., de Castro Lippi, I. C., Mendes, D. D., Alonso, D. P., Ribolla, P. E. M., & de Oliveira Orsi, R. (2022). Field relevant doses of the fipronil affects gene expression in honey bees *Apis mellifera*. *Apidologie*, *53*(6), 69.

Averill, A. L., Eitzer, B. D., & Drummond, F. A. (2024). Pesticide Contamination in Native North American Crops, Part I—Development of a Baseline and Comparison of Honey Bee Exposure to Residues in Lowbush Blueberry and Cranberry. *Insects*, *15*(7), 489.

Azar, P. (2018, March 29). Local blueberry crop in trouble: Why some beekeepers are staying away. CTV News Vancouver. Retrieved from https://bc.ctvnews.ca/local-blueberry-crop-in-trouble-why-some-beekeepers-are-staying-away-1.3868735

Berényi, O., Bakonyi, T., Derakhshifar, I., Köglberger, H., & Nowotny, N. (2006). Occurrence of six honeybee viruses in diseased Austrian apiaries. *Applied and Environmental Microbiology*, *72*(4), 2414-2420.

Bobiwash, K., Uriel, Y., & Elle, E. (2018). Pollen foraging differences among three managed pollinators in the highbush blueberry (*Vaccinium corymbosum*) agroecosystem. *Journal of Economic Entomology*, *111*(1), 26-32.

Bristow, P. R., & Martin, R. R. (1999). Transmission and the role of honeybees in field spread of blueberry shock ilarvirus, a pollen-borne virus of highbush blueberry. *Phytopathology*, *89*(2), 124-130.

Brodschneider, R., & Crailsheim, K. (2010). Nutrition and health in honey bees. *Apidologie*, *41*(3), 278-294.

Campbell, J. B., Nath, R., Gadau, J., Fox, T., DeGrandi-Hoffman, G., & Harrison, J. F. (2016). The fungicide Pristine® inhibits mitochondrial function in vitro but not flight metabolic rates in honey bees. *Journal of Insect Physiology*, *86*, 11-16.

Canadian Association of Professional Apiculturists (CAPA). (2023). Statement on honey bee wintering losses in Canada for 2023. Retrieved from https://capabees.com/capa-statement-on-honey-bees/

Castle, D., Alkassab, A. T., Bischoff, G., Steffan-Dewenter, I., & Pistorius, J. (2022). High nutritional status promotes vitality of honey bees and mitigates negative effects of pesticides. *Science of The Total Environment*, *806*, 151280.

Chan, Q. W., Melathopoulos, A. P., Pernal, S. F., & Foster, L. J. (2009). The innate immune and systemic response in honey bees to a bacterial pathogen, *Paenibacillus larvae*. *BMC Genomics*, *10*, 1-9.

Chapman, A., Amiri, E., Han, B., McDermott, E., Rueppell, O., Tarpy, D. R., ... & McAfee, A. (2022). Fertility costs of cryptic viral infections in a model social insect. *Scientific Reports*, *12*(1), 15857.

Christen, V., Schirrmann, M., Frey, J. E., & Fent, K. (2018). Global transcriptomic effects of environmentally relevant concentrations of the neonicotinoids clothianidin, imidacloprid, and thiamethoxam in the brain of honey bees (*Apis mellifera*). *Environmental Science & Technology*, *52*(13), 7534-7544.

Collison, E., Hird, H., Cresswell, J., & Tyler, C. (2016). Interactive effects of pesticide exposure and pathogen infection on bee health–a critical analysis. *Biological Reviews*, *91*(4), 1006-1019.

Colin, T., Meikle, W. G., Paten, A. M., & Barron, A. B. (2019). Long-term dynamics of honey bee colonies following exposure to chemical stress. *Science of the Total Environment*, *677*, 660-670.

Cornman, R. S., Tarpy, D. R., Chen, Y., Jeffreys, L., Lopez, D., Pettis, J. S., ... & Evans, J. D. (2012). Pathogen webs in collapsing honey bee colonies.

Cortés-Rivas, B., Monzón, V. H., Rego, J. O., & Mesquita-Neto, J. N. (2023). Pollination by native bees achieves high fruit quantity and quality of highbush blueberry: a sustainable alternative to managed pollinators. *Frontiers in Sustainable Food Systems*, *7*, 1142623.

Coulon, M., Schurr, F., Martel, A. C., Cougoule, N., Bégaud, A., Mangoni, P., ... & Dubois, E. (2019). Influence of chronic exposure to thiamethoxam and chronic bee paralysis virus on winter honey bees. *PLoS One*, *14*(8), e0220703.

Cullen, M. G., Thompson, L. J., Carolan, J. C., Stout, J. C., & Stanley, D. A. (2019). Fungicides, herbicides and bees: A systematic review of existing research and methods. *PLoS One*, *14*(12), e0225743.

Currie, R. W., & Gatien, P. (2006). Timing acaricide treatments to prevent *Varroa destructor* (Acari: Varroidae) from causing economic damage to honey bee colonies. *The Canadian Entomologist*, *138*(2), 238-252.

Dalmon, A., Diévart, V., Thomasson, M., Fouque, R., Vaissière, B. E., Guilbaud, L., ... & Henry, M. (2021). Possible spillover of pathogens between bee communities foraging on the same floral resource. *Insects*, *12*(2), 122.

Davidson, G. (2021). Honey bee pharmacology. *Honey Bee Medicine for the Veterinary Practitioner*, 135-148.

de Mattos, I. M., Soares, A. E., & Tarpy, D. R. (2017). Effects of synthetic acaricides on honey bee grooming behavior against the parasitic *Varroa destructor* mite. *Apidologie*, *48*, 483-494.

De Miranda, J. R., Bailey, L., Ball, B. V., Blanchard, P., Budge, G. E., Chejanovsky, N., … & Steen, J. J. M. (2013). Standard methods for virus research in *Apis mellifera*. *Journal of Apicultural Research,* 52: 1 - 56.

Desai, S. D., Eu, Y. J., Whyard, S., Currie, R. W. (2012). Reduction in deformed wing virus infection in larval and adult honey bees (*Apis mellifera L.*) by double-stranded RNA ingestion. *Insect Molecular Biology,* 21: 446 - 455.

Di Pasquale, G., Salignon, M., Le Conte, Y., Belzunces, L. P., Decourtye, A., Kretzschmar, A., ... & Alaux, C. (2013). Influence of pollen nutrition on honey bee health: do pollen quality and diversity matter?. *PloS One*, *8*(8), e72016.

El Agrebi, N., Steinhauer, N., Tosi, S., Leinartz, L., de Graaf, D. C., & Saegerman, C. (2021). Risk and protective indicators of beekeeping management practices. *Science of the Total Environment*, *799*, 149381.

Engel, P., Kwong, W. K., McFrederick, Q., Anderson, K. E., Barribeau, S. M., Chandler, J. A., ... & Dainat, B. (2016). The bee microbiome: impact on bee health and model for evolution and ecology of host-microbe interactions. *MBio*, *7*(2), 10-1128.

Fischer, J., Müller, T., Spatz, A. K., Greggers, U., Grünewald, B., & Menzel, R. (2014). Neonicotinoids interfere with specific components of navigation in honeybees. *PloS One*, *9*(3), e91364.

Forsgren, E. (2010). European foulbrood in honey bees. *Journal of invertebrate pathology*, *103*, S5-S9.

Foster, L. J., Tsvetkov, N., & McAfee, A. (2024). Mechanisms of pathogen and pesticide resistance in honey bees. *Physiology*.

French, S. K., Pepinelli, M., Conflitti, I. M., Jamieson, A., Higo, H., Common, J., ... & Zayed, A. (2024). Honey bee stressor networks are complex and dependent on crop and region. *Current Biology*, *34*(9), 1893-1903.

Friol, P. S., Catae, A. F., Tavares, D. A., Malaspina, O., & Roat, T. C. (2017). Can the exposure of *Apis mellifera* (Hymenoptera, Apiadae) larvae to a field concentration of thiamethoxam affect newly emerged bees?. *Chemosphere*, *185*, 56-66.

Fulton, C. A., Huff Hartz, K. E., Reeve, J. D., & Lydy, M. J. (2019). An examination of exposure routes of fluvalinate to larval and adult honey bees (*Apis mellifera*). *Environmental Toxicology and Chemistry*, *38*(6), 1356-1363.

Genome British Columbia. (2019) BeeCSI: 'Omic tools for assessing bee health. Retrieved January 29, 2024, online, from https://www.genomebc.ca/projects/beecsi-omic-tools-for-assessing-bee-health

Girard, M., Chagnon, M., & Fournier, V. (2012). Pollen diversity collected by honey bees in the vicinity of *Vaccinium* spp. crops and its importance for colony development. *Botany*, *90*(7), 545-555.

Government of British Columbia. (2024) Blueberries – Production Guides. Retrieved from https://www2.gov.bc.ca/gov/content/industry/agriservice-bc/production-guides/berries/blueberries

Government of Canada. (2019). BeeCSI: 'Omic tools for assessing bee health. Retrieved January 29, 2024, online, from https://grdi.canada.ca/en/projects/beecsi-omic-tools-assessing-bee-health

Graham, K. K., Milbrath, M. O., Zhang, Y., Baert, N., McArt, S., & Isaacs, R. (2022). Pesticide risk to managed bees during blueberry pollination is primarily driven by off-farm exposures. *Scientific Reports*, *12*(1), 7189.

Graham, K. K., Milbrath, M. O., Zhang, Y., Soehnlen, A., Baert, N., McArt, S., *et al.* (2021). Identities, concentrations, and sources of pesticide exposure in pollen collected by managed bees during blueberry pollination. *Scientific Reports*, *11*(1), 16857.

Grant, K. J., DeVetter, L., & Melathopoulos, A. (2021). Honey bee (*Apis mellifera*) colony strength and its effects on pollination and yield in highbush blueberries (*Vaccinium corymbosum*). *PeerJ*, *9*, e11634.

Grauer, Z. (2019, July 3). B.C. beekeepers worry blueberries are making their bees sick. The Star. Retrieved from https://www.thestar.com/vancouver/b-c-beekeepers-worry-blueberries-are-making-their-bees-sick/article_e213c9a1-2889-5c80-ac8c-dd7aaa378f3d.html

Graystock, P., Blane, E. J., McFrederick, Q. S., Goulson, D., & Hughes, W. O. (2016). Do managed bees drive parasite spread and emergence in wild bees?. *International Journal for Parasitology: Parasites and Wildlife*, *5*(1), 64-75.

Gregorc, A., Alburaki, M., Sampson, B., Knight, P. R., & Adamczyk, J. (2018). Toxicity of selected acaricides to honey bees (*Apis mellifera*) and *Varroa* (*Varroa destructor* Anderson and Trueman) and their use in controlling *Varroa* within honey bee colonies. *Insects*, *9*(2), 55.

Gregorc, A., & Sampson, B. (2019). Diagnosis of *varroa* mite (*Varroa destructor*) and sustainable control in honey bee (*Apis mellifera*) colonies—a review. *Diversity*, *11*(12), 243.

Guarna, M. M., Higo, H., Foster, L., Pernal, S. F., & Wolf, V. P. (2019). Bee health and blueberry pollination. HiveLights, 32, 14.

Guo, Y., Diao, Q. Y., Dai, P. L., Wang, Q., Hou, C. S., Liu, Y. J., ... & Gao, J. (2021). The effects of exposure to flupyradifurone on survival, development, and foraging activity of honey bees (*Apis mellifera L.*) under field conditions. *Insects*, *12*(4), 357.

Herbertsson, L., Klatt, B. K., Blasi, M., Rundlöf, M., & Smith, H. G. (2022). Seed-coating of rapeseed (Brassica napus) with the neonicotinoid clothianidin affects behaviour of red mason bees (Osmia bicornis) and pollination of strawberry flowers (Fragaria× ananassa). *Plos One*, *17*(9), e0273851.

Hesselbach, H., Seeger, J., Schilcher, F., Ankenbrand, M., & Scheiner, R. (2020). Chronic exposure to the pesticide flupyradifurone can lead to premature onset of foraging in honeybees *Apis mellifera*. *Journal of Applied Ecology*, *57*(3), 609-618.

Hoftyzer, E. (2023, May 26). New CRISPR-based diagnostic test detects viruses in honey bees. Retrieved from https://www.pharmacy.utoronto.ca/news-announcements/new-crispr-based-diagnostic-test-detects-viruses-honeybees

Islam, S. (2022, June 21). BC honey bee colonies decline 32% over winter: Dr. Leonard Foster comments on higher-than-normal losses. Retrieved from https://lsi.ubc.ca/2022/06/21/bc-honey-bee-colonies-decline-32-over-winter-dr-leonard-foster-comments-on-higher-than-normal-losses/

King, D. (2019, October 7). Alberta beekeepers boycott B.C. blueberries. Abbotsford News. Retrieved from https://www.abbynews.com/news/alberta-beekeepers-boycott-b-c-blueberries-1773410

Krupke, C. H., Hunt, G. J., Eitzer, B. D., Andino, G., & Given, K. (2012). Multiple routes of pesticide exposure for honey bees living near agricultural fields. *PLoS One*, *7*(1), e29268.

Lefebre, R., Broeckx, B. J., De Smet, L., Peelman, L., & de Graaf, D. C. (2024). Population-wide modelling reveals prospects of marker-assisted selection for parasitic mite resistance in honey bees. *Scientific Reports*, *14*(1), 7866.

Lester, P. J., Felden, A., Baty, J. W., Bulgarella, M., Haywood, J., Mortensen, A. N., ... & Smeele, Z. E. (2022). Viral communities in the parasite *Varroa destructor* and in colonies of their honey bee host (*Apis mellifera*) in New Zealand. *Scientific Reports*, *12*(1), 8809.

Little, G. (2018, April 4). Beekeepers refuse to put thousands of colonies in Fraser Valley blueberry fields. Vancouver Sun. Retrieved from https://vancouversun.com/news/local-news/beekeepers-refuse-to-put-thousands-of-colonies-in-fraser-valley-blueberry-fields

Liu, Y. J., Hou, C., Guo, R., & Darrouzet, E. (2023). Agrochemical use impact on bee physiology. *Frontiers in Physiology*, *14*.

Mayer, D. F., & Lunden, J. D. (1986). Toxicity of fungicides and an acaricide to honey bees (Hymenoptera: Apidae) and their effects on bee foraging behavior and pollen viability on blooming apples and pears. *Environmental Entomology*, *15*(5), 1047-1049.

McAfee, A. (2018, October 10). The blueberries and the bees. Michael Smith Laboratories. https://www.msl.ubc.ca/the-blueberries-and-the-bees/

McAfee, A., Chapman, A., Pettis, J. S., Foster, L. J., & Tarpy, D. R. (2021). Trade-offs between sperm viability and immune protein expression in honey bee queens (*Apis mellifera*). *Communications Biology*, *4*(1), 48.

McAfee, A., French, S. K., Wizenberg, S. B., Newburn, L. R., Tsvetkov, N., Higo, H., ... & Foster, L. J. (2024). Higher prevalence of sacbrood virus in *Apis mellifera* (Hymenoptera: Apidae) colonies after pollinating highbush blueberries. *Journal of Economic Entomology*, toae119.

McAfee, A., Tarpy, D. R., & Foster, L. J. (2021). Queens, but not their stored sperm, are resilient to temperature stress. PLOS ONE, 16(8), e0255381. https://doi.org/10.1371/journal.pone.0255381

Mondet, F., Beaurepaire, A., McAfee, A., Locke, B., Alaux, C., Blanchard, S., Danka, B. & Le Conte, Y. (2020). Honey bee survival mechanisms against the parasite *Varroa destructor*: a systematic review of phenotypic and genomic research efforts. *International Journal for Parasitology*, *50*(6-7), 433-447.

Moreno-Garcia, S. (2022, February 22). BC heat waves threaten survival of bee colonies. Retrieved from https://beyond.ubc.ca/bc-heat-waves-threaten-survival-of-bee-colonies/

Mullin, C. A., Frazier, M., Frazier, J. L., Ashcraft, S., Simonds, R., VanEngelsdorp, D., & Pettis, J. S. (2010). High levels of miticides and agrochemicals in North American apiaries: implications for honey bee health. *PloS One*, *5*(3), e9754.

Nicodemo, D., Mingatto, F. E., De Jong, D., Bizerra, P. F. V., Tavares, M. A., Bellini, W. C., ... & de Carvalho, A. (2020). Mitochondrial respiratory inhibition promoted by pyraclostrobin in fungi is also observed in honey bees. *Environmental Toxicology and Chemistry*, *39*(6), 1267-1272.

Northern Alberta Institute of Technology. (n.d.). National Bee Diagnostic Centre. Retrieved from https://www.nwpolytech.ca/nbdc/

O'Shea-Wheller, T. A., Rinkevich, F. D., Danka, R. G., Simone-Finstrom, M., Tokarz, P. G., & Healy, K. B. (2022). A derived honey bee stock confers resistance to *Varroa destructor* and associated viral transmission. *Scientific Reports*, *12*(1), 4852.

Payne, A. N., Walsh, E. M., & Rangel, J. (2019). Initial exposure of wax foundation to agrochemicals causes negligible effects on the growth and winter survival of incipient honey bee (*Apis mellifera*) colonies. *Insects*, *10*(1), 19.

Peck, D. T. (2021). The parasitic mite *Varroa destructor*: history, biology, monitoring, and management. *Honey Bee Medicine for the Veterinary Practitioner*, 235-251.

Piché-Mongeon, V., & Guzman-Novoa, E. (2024). Pathogen spillover from honey bees (*Apis mellifera L.*) to wild bees in North America. *Discover Animals*, *1*(1), 33.

Pilling, E., Campbell, P., Coulson, M., Ruddle, N., & Tornier, I. (2013). A four-year field program investigating long-term effects of repeated exposure of honey bee colonies to flowering crops treated with thiamethoxam. *PLoS One*, *8*(10), e77193.

Pollinator Partnership Canada. (2021). Blueberry pollinator guide. Retrieved from https://pollinatorpartnership.ca/assets/generalFiles/Blueberry-Pollinator-GUIDE_digital.pdf

Prado, A., Pioz, M., Vidau, C., Requier, F., Jury, M., Crauser, D., ... & Alaux, C. (2019). Exposure to pollen-bound pesticide mixtures induces longer-lived but less efficient honey bees. *Science of the Total Environment*, *650*, 1250-1260.

Ramos-Cuellar, A. K., De la Mora, A., Contreras-Escareño, F., Morfin, N., Tapia-González, J. M., Macías-Macías, J. O., ... & Guzman-Novoa, E. (2022). Genotype, but not climate, affects the resistance of honey bees (*Apis mellifera*) to viral infections and to the mite *Varroa destructor*. *Veterinary Sciences*, *9*(7), 358.

Ray, A. M., Gordon, E. C., Seeley, T. D., Rasgon, J. L., & Grozinger, C. M. (2023). Signatures of adaptive decreased virulence of deformed wing virus in an isolated population of wild honeybees (*Apis mellifera*). *Proceedings of the Royal Society B*, *290*(2009), 20231965.

Reitsma, L. (2023, February 28). U of S researchers devise strategies for faster disease detection in honey bees. Retrieved from https://wcvmtoday.usask.ca/articles/2023/03/usask-researchers-devise-strategies-for-faster-disease-detection-in-honey-bees.php

Rondeau, S., & Raine, N. E. (2022). Fungicides and bees: a review of exposure and risk. *Environment International*, *165*, 107311.

Sanchez-Bayo, F., & Goka, K. (2014). Pesticide residues and bees–a risk assessment. *PloS One*, *9*(4), e94482.

Schittny, D., Yañez, O., & Neumann, P. (2020). Honey bee virus transmission via hive products. *Veterinary Sciences*, *7*(3), 96.

Schultz-Cherry, S. (2015). Viral interference: the case of influenza viruses. *The Journal of Infectious Diseases*, *212*(11), 1690-1691.

Schneider, C. W., Tautz, J., Grünewald, B., & Fuchs, S. (2012). RFID tracking of sublethal effects of two neonicotinoid insecticides on the foraging behavior of *Apis mellifera*. *PloS One*, *7*(1), e30023.

Stokstad, E. (2021, November 22) Pesticides can harm bees twice: larvae and adults. Retrieved from https://www.science.org/content/article/pesticides-can-harm-bees-twice-larvae-and-adults

Shi, T. F., Wang, Y. F., Liu, F., Qi, L., & Yu, L. S. (2017). Sublethal effects of the neonicotinoid insecticide thiamethoxam on the transcriptome of the honey bees (Hymenoptera: Apidae). *Journal of Economic Entomology*, *110*(6), 2283-2289.

Simon-Delso, N., San Martin, G., Bruneau, E., Delcourt, C., & Hautier, L. (2017). The challenges of predicting pesticide exposure of honey bees at landscape level. *Scientific Reports*, *7*(1), 3801.

Snow, J. W. (2022). *Nosema apis* and *N. ceranae* Infection in Honey Bees: A Model for Host-Pathogen Interactions in Insects. In *Microsporidia: Current Advances in Biology* (pp. 153-177). Cham: Springer International Publishing.

Stanley, D. A., Russell, A. L., Morrison, S. J., Rogers, C., & Raine, N. E. (2016). Investigating the impacts of field-realistic exposure to a neonicotinoid pesticide on bumblebee foraging, homing ability and colony growth. *Journal of Applied Ecology*, *53*(5), 1440-1449.

Statistics Canada. (2022). Mushroom, greenhouse and highbush blueberry farming play pivotal role in British Columbia. Government of Canada. https://www150.statcan.gc.ca/n1/pub/96-325-x/2021001/article/00010-eng.htm

St. Clair, A. L., Zhang, G., Dolezal, A. G., O'Neal, M. E., & Toth, A. L. (2020). Diversified farming in a monoculture landscape: effects on honey bee health and wild bee communities. *Environmental Entomology*, *49*(3), 753-764.

Steinhauer, N., & Saegerman, C. (2021). Prioritizing changes in management practices associated with reduced winter honey bee colony losses for US beekeepers. *Science of The Total Environment*, *753*, 141629.

Tavares, D. A., Dussaubat, C., Kretzschmar, A., Carvalho, S. M., Silva-Zacarin, E. C., Malaspina, O., ... & Belzunces, L. P. (2017). Exposure of larvae to thiamethoxam affects the survival and physiology of the honey bee at post-embryonic stages. *Environmental Pollution*, *229*, 386-393.

Tesovnik, T., Zorc, M., Ristanić, M., Glavinić, U., Stevanović, J., Narat, M., & Stanimirović, Z. (2020). Exposure of honey bee larvae to thiamethoxam and its interaction with *Nosema ceranae* infection in adult honey bees. *Environmental Pollution*, *256*, 113443.

Thebeau, J. M., Cloet, A., Liebe, D., Masood, F., Kozii, I. V., Klein, C. D., ... & Wood, S. C. (2023). Are fungicides a driver of European foulbrood disease in honey bee colonies pollinating blueberries?. *Frontiers in Ecology and Evolution*, *11*, 1073775.

T O'Neal, S., Anderson, T. D., & Wu-Smart, J. Y. (2018). Interactions between pesticides and pathogen susceptibility in honey bees. *Current Opinion in Insect Science*, *26*, 57-62.

Tosi, S., Nieh, J. C., Brandt, A., Colli, M., Fourrier, J., Giffard, H., ... & Simon-Delso, N. (2021). Long-term field-realistic exposure to a next-generation pesticide, flupyradifurone, impairs honey bee behaviour and survival. *Communications Biology*, *4*(1), 805.

Traver, B. E., Feazel-Orr, H. K., Catalfamo, K. M., Brewster, C. C., & Fell, R. D. (2018). Seasonal effects and the impact of in-hive pesticide treatments on parasite, pathogens, and health of honey bees. *Journal of Economic Entomology*, *111*(2), 517-527.

Traynor, K. S., Pettis, J. S., Tarpy, D. R., Mullin, C. A., Frazier, J. L., Frazier, M., & Vanengelsdorp, D. (2016). In-hive Pesticide Exposome: Assessing risks to migratory honey bees

from in-hive pesticide contamination in the Eastern United States. *Scientific Reports*, *6*(1), 33207.

Tuell, J. K., & Isaacs, R. (2010). Weather during bloom affects pollination and yield of highbush blueberry. *Journal of Economic Entomology*, *103*(3), 557-562.

United States Environmental Protection Agency. (2018, July 30). Understanding how pesticide exposure affects honey bee colonies. Retrieved from https://www.epa.gov/sciencematters/understanding-how-pesticide-exposure-affects-honey-bee-colonies

University of Guelph. (n.d.). Bees at the Animal Health Laboratory. Retrieved from https://www.uoguelph.ca/ahl/bees

Van Dooremalen, C., Gerritsen, L., Cornelissen, B., van der Steen, J. J., van Langevelde, F., & Blacquiere, T. (2012). Winter survival of individual honey bees and honey bee colonies depends on level of *Varroa destructor* infestation. *PloS One*, *7*(4), e36285.

Vaudo, A. D., Tooker, J. F., Grozinger, C. M., & Patch, H. M. (2016). Erratum: Bee nutrition and floral resource restoration (Current Opinion in Insect Science (2015) 10 (133-141. *Current Opinion in Insect Science*, *15*, 145.

Wardell, G. I. (1982). *European foulbrood: Association with Michigan blueberry pollination, and control*. Michigan State University.

Xerces Society for Invertebrate Conservation. (n.d.). Systemic insecticides: A reference and overview. Retrieved from https://xerces.org/systemic-insecticides-reference-and-overview

Zaluski, R., Justulin Jr, L. A., & Orsi, R. D. O. (2017). Field-relevant doses of the systemic insecticide fipronil and fungicide pyraclostrobin impair mandibular and hypopharyngeal glands in nurse honeybees (*Apis mellifera*). *Scientific Reports*, *7*(1), 15217.

Zawislak, J., Adamczyk, J., Johnson, D. R., Lorenz, G., Black, J., Hornsby, Q., ... & Joshi, N. (2019). Comprehensive survey of area-wide agricultural pesticide use in southern United States row crops and potential impact on honey bee colonies. *Insects*, *10*(9), 280.

Zhang, G., Murray, C. J., St. Clair, A. L., Cass, R. P., Dolezal, A. G., Schulte, L. A., ... & O'Neal, M. E. (2023). Native vegetation embedded in landscapes dominated by corn and soybean improves honey bee health and productivity. *Journal of Applied Ecology*, *60*(6), 1032-1043.

Zhang, G., St. Clair, A. L., Dolezal, A., Toth, A. L., & O'Neal, M. (2020). Honey bee (Hymenoptera: *Apidea*) pollen forage in a highly cultivated agroecosystem: limited diet diversity and its relationship to virus resistance. *Journal of Economic Entomology*, *113*(3), 1062-1072.

Zhang, Q., Fu, L., Cang, T., Tang, T., Guo, M., Zhou, B., ... & Zhao, M. (2021). Toxicological effect and molecular mechanism of the chiral neonicotinoid dinotefuran in honeybees. *Environmental Science & Technology*, *56*(2), 1104-1112.