

UC Riverside

UC Riverside Electronic Theses and Dissertations

Title

Molecular and Metabolic Biology of Bumble Bees: Advancing Our Understanding of Environmental Stressor Impacts on Bumble Bees

Permalink

<https://escholarship.org/uc/item/7ss7c3nj>

Author

Fischer, Natalie Christine

Publication Date

2023

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA
RIVERSIDE

Molecular and Metabolic Biology of Bumble Bees: Advancing Our Understanding of
Environmental Stressor Impacts on Bumble Bees

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Entomology

by

Natalie Fischer

June 2023

Dissertation Committee:

Dr. S. Hollis Woodard, Chairperson

Dr. Michael Adams

Dr. Nicole Rafferty

Dr. Naoki Yamanaka

Copyright by
Natalie Fischer
2023

The Dissertation of Natalie Fischer is approved:

Committee Chairperson

University of California, Riverside

Acknowledgments

I would like to first thank my mentor, Dr. Hollis Woodard for supporting my goals and guiding me throughout my academic journey as both an undergraduate and graduate student. I would also like to thank Dr. Claudinéia Costa, who was integral in the conception of my third chapter and contributed to data analyses for each chapter. Dr. Costa will be a co-author on all my publications for her contributions. I would also like to thank the community and staff at the Rocky Mountain Biological Laboratory for providing me with field sites and support for my second chapter. Funding for my research was provided by the National Science Foundation Graduate Research Fellowship and the University of California, Riverside National Science Foundation Research Traineeship in Integrated Computational Entomology Fellowship. Additional funding for my second chapter was provided by the Rocky Mountain Biological Laboratory Graduate Fellowships. Lastly, I thank Koppert Biological Systems for generously providing bumble bee colonies for my research in Chapters One and Three.

ABSTRACT OF THE DISSERTATION

Molecular and Metabolic Biology of Bumble Bees: Advancing Our Understanding of Environmental Stressor Impacts on Bumble Bees

by

Natalie Fischer

Doctor of Philosophy, Graduate Program in Entomology
University of California, Riverside, June 2023
Dr. S. Hollis Woodard, Chairperson

Bumble bees (genus *Bombus*) are an important group of bees that perform many complex behaviors and provide vital ecosystem services through plant pollination. Bumble bees, along with many other pollinators, face a variety of stressors in their environment that can lead to global population declines. A deeper understanding of bumble bee molecular biology and physiology is necessary to fill the gap in our understanding of how bumble bees can be impacted by environmental stressors and can elucidate mechanisms underlying some of the responses seen in the current literature. In this dissertation, I explore how molecular mechanisms can be influenced by external stressors, such as neonicotinoid pesticides and food unavailability. I also investigate the molecular mechanisms that are involved in bumble bee foraging behavioral states, of which pollination is byproduct. Bumble bee foraging behaviors are essential for the maintenance of our ecosystems as well as their own survival. Yet environmental stressors threaten the efficiency or performance of these foraging behaviors. In order to understand how bumble bee foraging behavioral states are impacted by environmental stressors, we

must understand what drives these specific behaviors. Within this dissertation, each chapter explores a different facet of bumble bee molecular and metabolic biology, with the intent to fill in the gaps in our comprehensive understanding of the effects, potential or realized, of external factors on different aspects of bumble bee function and fitness. Chapter One explores the effects of both neonicotinoid pesticide exposure and starvation stress on bumble bee energy metabolism. Chapter Two focuses on investigating the molecular mechanisms associated with foraging behavioral states in wild bumble bees. Chapter Three explores how rearing history impacts offspring starvation resilience and further examines the metabolic mechanisms that are involved in enforcing starvation resistance.

Table of Contents

Introduction	1
References.....	3
Chapter 1	5
Introduction.....	5
Material & Methods.....	9
Results.....	17
Discussion.....	18
Conclusion	24
Tables and Figures	26
References.....	34
Chapter 2	44
Introduction.....	44
Methods.....	48
Results.....	52
Discussion.....	55
Conclusion	59
Tables and Figures	61
References.....	66
Chapter 3	70
Introduction.....	70
Methods.....	73
Results.....	79
Discussion.....	80
Conclusion	85
Tables and Figures	87
References.....	94
Conclusion	98

Appendix 1	100
Appendix 1.2.....	107
Appendix 2	108

List of Tables

Chapter 1

Table 1.1: Significant metabolites by pesticide treatment and their pathways.....	28
Table 1.2: ANOVA tests and adjusted p-values (q-values) for each metabolite impacted by imidacloprid treatment alone.....	29
Table 1.3: Significant metabolites by Treatment*Starvation and their pathways.....	30
Table 1.4: ANOVA tests and adjusted p-values (q-value) for each metabolite impacted by the interaction between imidacloprid treatment and starvation duration.....	31
Table A1.1: Sample sizes for the experiment including bees that died.....	100
Table A1.2: Sample sizes sent for Metabolomic Analysis.....	100
Table A1.3: Survival analysis results for exposure period.....	101
Table A1.4: Survival analysis results for starvation period.....	101
Table A1.5: Significant Metabolites by Starvation.....	102

Chapter 2

Table 2.1: Gene ontology enrichment analysis results showing the significant terms using GO q-values for each behavioral pairwise comparison.....	64
Table 2.2: Gene Ontology enrichment analysis results showing significant terms using p-value for comparison between legitimate foragers and switchers only.....	65
Table A2.1: Gene Ontology enrichment analysis results showing significant terms using GO p-values for behavioral pairwise comparisons.....	108

Chapter 3

Table 3.1: Total number of hemolymph samples collected across all treatment groups.....	88
Table 3.2: Total number of hemolymph samples used for glucose and trehalose analysis.....	89

List of Figures

Chapter 1

Figure 1.1: Exposure period during experiment.....	26
Figure 1.2: Starvation period during experiment.....	27
Figure 1.3: Key metabolites impacted after all bees were nutritionally stressed.....	32
Figure A1.1: Additional key metabolites impacted after all bees were nutritionally stressed.....	106

Chapter 2

Figure 2.1: Spaghetti plot showing observation times for each tactic or switching behavior.....	61
Figure 2.2: Bar plot showing proportion of foraging workers per foraging tactic.....	62
Figure 2.3: Heatmap showing relative levels of differentially expressed genes between the foraging behavioral states.....	63

Chapter 3

Figure 3.1: Flow diagram of treatment groups.....	87
Figure 3.2: Boxplot representing queen-reared and worker-reared offspring body size.....	90
Figure 3.3: Histogram showing how long queen-reared (red) and worker-reared (blue) offspring survived under starvation treatments.....	91
Figure 3.4: Glucose titers for queen-reared and worker-reared offspring across all starvation treatment groups.....	92
Figure 3.5: Trehalose titers for queen-reared and worker-reared offspring across all starvation treatment groups.....	93

Introduction

The physiology and behavior of individual bees can ultimately impact entire ecosystems, by influencing plant population dynamics and community interactions (Simpson and Raubenheimer, 2012). Bumble bees (genus *Bombus*) are an important group of bees that perform many complex behaviors and provide the vital ecosystem service of plant pollination. Bumble bee foraging behaviors often result in pollination services in both natural and agricultural systems (Corbet et al., 1991; Fontaine et al., 2006; Rao and Strange, 2012). However, bumble bees, along with other pollinators, face many stressors in their environment, such as pesticides and nutritional stress that can lead to global population declines (reviewed in Goulson, 2013 and Goulson et al., 2015). It is necessary to examine how environmental stressors impact bumble bees from the molecular level to the population level to better understand their resilience and how we can best protect them.

Bumble bee foraging behaviors are essential for the maintenance of our ecosystems as well as their own survival. Yet environmental stressors threaten the efficiency or performance of these foraging behaviors either directly (Feltham et al, 2014; Stanley et al., 2016; Woodard et al., 2017) or indirectly through susceptibility to other stressors that impact health (Brown et al., 2003; Tyler et al., 2006; Goulson et al, 2015; Costa et al., 2022). In order to understand how bumble bee foraging behavioral states are impacted by environmental stressors, we must understand what drives these specific behaviors. Many studies have examined the external, ecological and social factors that influence, or in

some cases drive, bumble bee foraging behaviors (see references in Chapters 2). However, few studies have examined the molecular or physiological mechanisms underlying foraging behavioral states. A deeper understanding of bumble bee molecular biology and physiology will provide further comprehension of how bumble bee foraging behavioral states evolved and how they might be impacted by environmental stressors.

In this dissertation, I explore how molecular mechanisms can be influenced by external stressors and I also investigate the molecular mechanisms that are involved in bumble bee foraging behavioral states. Specifically, Chapter One examines the effects of a neonicotinoid pesticide, imidacloprid, as well as starvation on bumble bee energy metabolism. Chapter Two focuses on investigating the molecular mechanisms associated with foraging behavioral states in wild bumble bees. Chapter Three explores how rearing history impacts offspring starvation resilience and further examines the metabolic mechanisms that are involved in enforcing starvation resistance. Each chapter explores a different facet of bumble bee molecular and metabolic biology, with the intent to fill the gap in our comprehensive understanding of the effects, or potential effects, of external factors on different aspects of bumble bee fitness.

References

- Brown, M.J.F., Loosli, R., Schmid-Hempel, P. Condition-dependent expression of virulence in a trypanosome infecting bumblebees. *Oikos Advancing Ecology*. **91**: pp. 421-427. (2003).
- Corbet, S.A., Williams, I.H., Osborne, J.L. Bees and the pollination of crops and wild flowers in the European community. *Bee World*. **72**: pp. 47–59. (1991).
- Costa, C.P., Leza, M., Duennes, M.A., Fisher, K., Vollaro, A., Hur, M., Kirkwood, J.S., Woodard, S.H., 2022. Pollen diet mediates how pesticide exposure impacts brain gene expression in nest-founding bumble bee queens. *Science of the Total Environment* **833**: 155216. (2022).
- Feltham, H., Park, K. & Goulson, D. Field realistic doses of pesticide imidacloprid reduce bumblebee pollen foraging efficiency. *Ecotoxicology* **23**: pp. 317–323. (2014).
- Fontaine C, Dajoz I, Meriguet J, Loreau M. Functional diversity of plant–pollinator interaction webs enhances the persistence of plant communities. *PLoS Biology* **14**(1): e1. (2006).
- Goulson, D. REVIEW: An overview of the environmental risks posed by neonicotinoid insecticides. *Journal of Applied Ecology*. **50**: pp. 977–987. (2013).
- Goulson, D., Nicholls, E., Botías, C. & Rotheray, E. L. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science*. **34**: 6229. (2015).
- Rao, S., Strange, J.P. Bumble Bee (Hymenoptera: Apidae) Foraging Distance and Colony Density Associated With a Late-Season Mass Flowering Crop. *Environmental Entomology*, **41**: pp. 905–915. (2012).
- Simpson, S.J. & Raubenheimer, D. The Nature of Nutrition. *Princeton University Press*. (2012).
- Stanley, D. A., Russell, A. L., Morrison, S. J., Rogers, C., Raine, N. E. Investigating the impacts of field-realistic exposure to a neonicotinoid pesticide on bumblebee foraging, homing ability and colony growth. *Journal of Applied Ecology*. **53**: pp. 1440–1449. (2016).
- Tyler, E.R., Adams, S., Mallon, E.B. An immune response in the bumblebee, *Bombus terrestris* leads to increased food consumption. *BMC Physiology* **6**: doi:10.1186/1472-6793-6-6. (2006).

Woodard, S.H. Bumble bee ecophysiology: integrating the changing environment and the organism. *Current Opinion in Insect Science* **22**: pp. 101-108. (2017).

Chapter 1

Impacts of neonicotinoid insecticides on bumble bee energy metabolism are revealed under starvation conditions.

1. Introduction

Relationships between flowering plants and their pollinators are strongly influenced by energetic exchanges. Most insect pollinators rely on flowering plants for nectar and pollen, their primary nutrient sources (Roulston and Cane, 2000; Heinrich, 2004). Nectar provides insect pollinators with the majority of their dietary sugars (including sucrose, fructose, and glucose), which are converted to energy through sugar metabolic processes (Heinrich, 1975; Heinrich, 2004; Willmer, 2011; McCallum et al., 2013). Ultimately, these sugars fuel energetically demanding behaviors associated with pollination (Woodard and Jha, 2017), such as flight (Sacktor, 1970; Kammer and Heinrich, 1978; Rothe and Nachtigall, 1989). Many plants, in turn, rely on the movements of pollinators for efficient cross-pollination (Ollerton et al., 2011). To attract pollinators, plants can produce abundant nectar or nectar with higher sugar concentrations, which can promote repeated visitation to plant species (Heinrich, 1976; Willmer, 2011). Energetic exchanges between pollinators and plants, and pollinator energy metabolism, are thus fundamental to the performance and allocation of pollinator foraging and, ultimately, plant pollination and ecosystem health.

Bumble bees (genus *Bombus*, family Apidae) are a group of generalist pollinators that visit many plant species in natural and agricultural ecosystems (Corbet et al., 1991; Rao and Strange, 2012) and are also commercially managed for crop pollination (Velthuis and van Doorn, 2006). A key finding to emerge from bumble bee research is that the neonicotinoid-type insecticides, which are used widely in agriculture (Jeschke et al., 2011; Casida and Durkin, 2013; Wood and Goulson, 2017), have broad adverse effects on bumble bees, including at sublethal, field-realistic exposure levels (reviewed in Goulson, 2013; Van der Sluijs et al., 2013; Wood and Goulson, 2017). Neonicotinoids act by binding to nicotinic acetylcholine receptors (nAChRs) present in the insect nervous system (Casida and Durkin, 2013; Moffat et al., 2016). Exposure at sublethal levels can reduce bumble bee foraging efficiency (Feltham et al., 2014; Stanley et al., 2016) due in part to the negative effects of exposure on cognition, navigation, and foraging behaviors that are critical components of bumble bee pollination (Blacquière et al., 2012; Goulson, 2013; Lundin et al., 2015). These impairments have also been detected in other wild bee groups outside of bumble bees (Stuligross et al., 2023). However, the full breadth of effects of neonicotinoids on bumble bees and other pollinators is still being characterized. In particular, the influence of neonicotinoids on underlying physiological processes, such as energy metabolism, is largely unknown (Alkassab and Kirchner, 2017; but see Cook, 2019).

Neonicotinoids may negatively impact energy metabolism in part by disrupting some of the canonical mechanisms that regulate energetic status. Exposure to pesticides within

this class results in mitochondrial dysfunction and neurodegeneration within the insect brain, including in bumble bees (Nicodemo et al., 2014; Moffat et al., 2015; Martelli et al., 2020; Wei et al., 2020). In honey bees (genus *Apis*, family Apidae), sublethal concentrations of imidacloprid reduce thoracic mitochondria respiration and ATP levels (Nicodemo et al., 2014). Further, field-realistic neonicotinoid exposure also induces mitochondrial dysfunction in other insects, such as *Drosophila melanogaster* (Martelli et al., 2020) and *Chironomus dilutus*, an aquatic midge species (Wei et al., 2020). Given the fundamental role of mitochondria in ATP production, their dysfunction likely precipitates downstream effects on overall physiology in insects (Martelli et al., 2020).

In addition to harmful effects of neonicotinoids and other pesticide classes, another key driver of pollinator declines is the widespread loss of foraging habitat. Limited or insufficient nutrient resources is a stressor that can have broad adverse effects on wild bees, including bumble bees (reviewed in Goulson et al., 2015; Wang et al., 2019). Nectar and pollen availability for bumble bees can be limited in many landscapes (Pamminger et al., 2019) and during particular parts of the season, including late summer (Timberlake et al., 2019; Timberlake et al., 2021). Nutritional stress alone can alter the bumble bee metabolome, specifically pathways involved in energy metabolism (Wang et al., 2019). Further, nutritional stress can influence immunity (Tyler et al., 2006), reproduction (Vaudo et al., 2018), and responses to other stressors in the environment (Brown et al., 2003; Goulson et al., 2015; Costa et al., 2022). Nutritional status can also modulate the susceptibility or resilience to pesticides, including neonicotinoids (Goulson

et al., 2015; Costa et al., 2022). The interaction between these two stressors at the physiological level remains relatively unexplored (reviewed in Holmstrup et al., 2010 and Orr et al., 2020), particularly at the metabolic level.

In this study, we used a metabolomic approach to examine how sublethal, field-realistic exposure (5 ppb) to the neonicotinoid insecticide imidacloprid, on its own and alongside starvation stress, influences energy metabolism in the common eastern bumble bee *Bombus impatiens*. We exposed *B. impatiens* workers to imidacloprid according to one of three exposure paradigms that were designed to explore how sustained versus more limited (either early-on and followed by a recovery period, or more recent) pesticide exposure influences bee energetic states. We then used metabolomic analyses targeting central carbon metabolism to assess how metabolites that are key components of sugar energy metabolism are influenced by pesticide exposure and the timing upon which it occurs. Based on the knowledge that neonicotinoids cause disruptions in mitochondrial function and ATP production (Nicodemo et al., 2014; Moffat et al., 2015; Martelli et al., 2020), we explored the hypothesis that pesticide exposure negatively influences bee energetic states by disrupting fundamental components of energy metabolism.

Specifically, we predicted that pesticide-treated bees would exhibit reduced levels of sugars and metabolites involved in sugar metabolism processes (e.g., glycolysis and the tricarboxylic acid cycle), with more sustained exposure having a particularly strong influence. Additionally, we used an experimental paradigm that allowed us to explore

how starvation influences bee energetic states on its own, and also in combination with pesticide exposure.

2. Material & Methods

2.1. Bee rearing

Mature, queenright bumble bee colonies ($n = 3$) were provided by Koppert Biological Systems (Howell, MI) and kept in the Entomology Building at the University of California, Riverside. Colonies were maintained at ~ 24 °C and $\sim 60\%$ RH under dim red light, which is not visible to bees. They were fed *ad libitum* mixed-source, honey bee collected pollen (Brushy Mountain Bee Farm) and a nectar substitute supplied by Koppert Biological Systems. Individual bees used in the experiment were removed from their natal colonies, placed in individual small cages (W7.5 x D7.5 x H4.5 cm), and maintained in an incubator (Drosophila Invictus) at 25 °C and 70% RH and under constant darkness. All bees remained in this incubator for the duration of the experiment until they were collected. The ages of bees used in the experiment were unknown, but no bees that showed obvious patterns of senescence (*e.g.*, wing wear) were included in the experiment.

Across the experiment, bees were inspected every 1-2 days for mortality and the date of any deaths was recorded. Liquid feeders (described below) were replaced every 2-3 days to prevent mold growth. All bees in the study were deprived of pollen after they were removed from their natal colonies. Our rationale for doing this was to avoid introducing

variation into our metabolomic analysis that could be attributed to uncontrollable differences in pollen consumption, as pollen contains amino acids, lipids, carbohydrates, and other secondary metabolites, some of which would be detected in our targeted analysis (Roulston and Cane, 2000; Nicolson, 2011; Irwin et al., 2014; Vaudo et al., 2016).

2.2. Preparation of imidacloprid treatments

A 5 ppb (micrograms/L) imidacloprid solution was prepared by adding dilutions of an imidacloprid stock solution (Pestanal analytical standard, Sigma-Aldrich) to 1000 mL of 50% sucrose solution (w/v; hereafter, “artificial nectar”). This concentration of imidacloprid represents a field-realistic exposure level for bumble bees based on a survey of the available literature on neonicotinoid levels used in lab and field experiments or estimated from environmental screenings (Goulson, 2013; Stewart et al., 2014; Feltham et al., 2014; Leza et al., 2018). The stock pesticide solution was prepared by dissolving 1 mg of imidacloprid into 100 mL of distilled water to produce a 10 ppm imidacloprid stock. Individual aliquots of 0.5 mL of the 10 ppm imidacloprid stock were added to 99.5 mL of distilled water to achieve 100 mL imidacloprid solution, which was then added to the 50% sucrose solution. Concentrations of all imidacloprid-treated artificial nectar solutions were validated using EnviroLogix Quantiplate Kits (#EP006), and all fell within 5-5.5 ppb. A fresh imidacloprid-treated artificial nectar solution was produced for each of the three trials in the experiment (see below).

2.3. Experimental design

Individual worker bees (n = 195) were assigned to one of four treatment groups: 1) *sustained*, 2) *limited-early*, 3) *limited-late*, and 4) *untreated* (control) (n = 11-13 bees per group). To account for the influence of natal colony on experimental responses, we included individuals from three different natal colonies, with multiple individuals from each colony represented within each treatment group (Appendix 1, Table A1.1). The experiment was run in three trials with an equal number of bees from the three natal colonies in each treatment (n= 3-4 bees/colony/treatment). After removal from their natal colony, all bees first underwent a seven-day exposure period wherein they were provided with either treated (*sustained*, *limited-early*, *limited-late*) or untreated sucrose solution (*untreated*; control group) according to their treatment group (Figure 1.1). *Sustained* bees were exposed to pesticide-treated sucrose solution for the full seven-day period. *Limited-early* bees were given a pesticide-treated sucrose solution for the first two days of the seven-day feeding period, then were switched to the untreated sucrose solution for the remaining five days. *Limited-late* bees were given an untreated sucrose solution for the first five days and then switched to a pesticide-treated sucrose solution for the last two days of the feeding period. *Untreated* bees were fed untreated sucrose solution for the full seven days. All *untreated* bees were fed an untreated 50% sucrose solution (w/v), whereas all treated bee groups (*sustained*, *limited-early*, and *limited-late*) were fed a 50% sucrose solution (w/v) with 5 ppb of imidacloprid (as described above). All bees were held individually in isolation during the entirety of the experiment, from when they were removed from their natal colonies until they were collected for metabolomic analysis.

After the seven-day exposure period, all bees (in all treatment groups) underwent a brief starvation period of four hours wherein their liquid feeders were removed, followed by a brief refeeding period of one hour where they were all given untreated sucrose solution (Figure 1.2). This refeeding process was performed to allow bees to consume sugar solution, presumably to satiety, to equalize (across bees) the amount of ingested sugars at the point that the experimental starvation stress period began (Mayack and Naug, 2010). After the one-hour refeeding period, the sucrose solution was removed. The liquid feeders were weighed before and after the one-hour refeeding period to validate that all bees consumed sucrose solution during this time and all bees were confirmed to have consumed a minimum of 0.05 g of sucrose solution. Following the refeeding period, one set of bees was immediately collected (0-hour group) in order to determine how imidacloprid exposure alone impacts bee metabolite levels in the absence of starvation. We then subsequently collected bees at one of three additional starvation time points, separated by eight-hour intervals, at 8, 16, or 24 hours following the refeeding period (Figure 1.2) to examine how both imidacloprid exposure and starvation impact metabolite levels.

2.4. Tissue preparation

Bees were collected into liquid nitrogen to prevent metabolite degradation (Álvarez-Sánchez et al., 2010). We used whole thoraces for metabolomic analysis because the thorax contains muscles involved in flight and other important behaviors (Heinrich, 1972;

Heinrich, 1975; McCallum et al., 2013) and thus has high metabolic demands (Heinrich, 1975). Within thoracic tissue, energy metabolism maintains levels of ATP, the unit of energy needed for muscle contraction (McCallum et al., 2013). Thoraces were separated from the rest of the bodies over dry ice and then stored at -80°C until they were used for analysis at the UC Riverside Metabolomics Core Facility (n= 175, Appendix 1, Table A1.2).

2.5. Metabolomic Analyses

Metabolomic analyses were performed by the Metabolomics Core Facility at the University of California, Riverside. We used targeted analysis of polar metabolites, as described previously in Vliet et al. (2019), designed to detect ~100 constituents of central carbon metabolism. This component of metabolism encompasses sugar phosphates (involved in glycolysis), organic acids (involved in the TCA cycle), amino acids, purines, and pyrimidines. This targeted approach also detects some neutral sugars but does not differentiate between many sugar types (*e.g.*, does not differentiate between types of hexoses).

Before analysis, thorax tissue samples were weighed and freeze dried. We used a bead mill to reduce the thorax to a fine powder, then an extraction solvent was added (with the amount normalized to fresh weight). Samples were then vortexed and centrifuged to extract metabolites. Metabolites from each sample were separated using a TQ-XS triple quadrupole mass spectrometer (Waters) coupled to an I-class Ultra-Performance Liquid

Chromatography (UPLC) system (Waters). Separated metabolites were measured and results were displayed on a mass spectrum plot, from which metabolites were identified. Data processing was performed using the Progenesis Qi software (Nonlinear Dynamics). Additional details about methods used for targeted metabolomics are available in (Vliet et al., 2019) and Appendix 1.2. In total, we generated metabolomic data for 172 samples of our original set of 195 that entered the experiment; nineteen bees died during the experiment, and an additional three samples were not used for metabolomic analysis due to quality control issues.

2.6. Statistical analyses

All statistical analyses were carried out in R (version 4.1.1), and all values were considered significant at $P < 0.05$ unless otherwise noted. Survival analysis (function `coxph` in package “survival”; Therneau and Grambsch, 2000; Therneau, 2015) was used to assess whether our pesticide treatment or duration of starvation influenced mortality during the assay. We then used the `omu` package (Tiffany and Bäumlner, 2019) to run an analysis of variance (ANOVA) to explore the influence of our primary factors of interest (pesticide treatment and duration of starvation), natal colony (*i.e.*, the colony that a worker originated from), and the interaction between these factors, on individual metabolite levels. A total of three separate ANOVAs were run to assess the impact of each combination of factors on metabolite levels. ANOVA is one of the most commonly used statistical approaches in metabolomic studies because it can reduce a large set of measured metabolites to those that show a strong response associated with treatment or

experimental conditions (Bartel et al., 2013; Saccenti et al., 2014). First, we asked how metabolite levels are influenced by each experimental factor. We then used the online interface Metaboanalyst (Pang et al., 2021) to perform overrepresentation analysis (ORA) to identify KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways overrepresented within the lists of metabolites that were significantly impacted by either pesticide exposure or duration of starvation. Although Metaboanalyst uses human metabolic pathways, the basic metabolic pathways among different species are largely the same, making this analysis relatively universal (Horgan et al. 2009). We constrained our ORA to significant metabolites detected within our metabolomic analysis ($n = 52$) by using the list of all metabolites detected in our study ($n = 78$), across all samples, as our background list (Wieder et al., 2021). In addition to our primary ANOVA, we also looked within each starvation duration to ask how pesticide exposure influenced metabolite levels within these treatment groups. We also did the converse, where we looked within each pesticide treatment group to explore how the duration of starvation influenced metabolite levels within these groups. All metabolites and overrepresented pathways were reported as significant at $p\text{-adj} < 0.05$.

We also further examined a subset of metabolites with known associations with neonicotinoid effects across a variety of taxa. To examine these metabolites, we used a linear plot to visualize the change in metabolites through time under starvation as well as boxplots at specific starvation timepoints to compare the treatment groups after starvation had begun. The production of the metabolite nicotinamide adenine dinucleotide (NAD) is

negatively impacted by imidacloprid in honey bee larvae after acute, sublethal exposure (Paten et al., 2022). NAD is a crucial metabolite involved in many oxidation-reduction reactions for energy metabolism, including glycolysis, the TCA cycle and the electron transport chain which generates ATP. Another set of metabolites we explored in greater detail include the purines and pyrimidines, the nitrogenous bases of nucleic acids that also function to regulate metabolism and transfer energy. Purine and pyrimidine metabolic pathways can be disrupted by neonicotinoid exposure (Zhang et al., 2022; Zhou et al., 2023). The purine adenosine is an important compound involved in energy release and is the precursor for energy metabolites, such as adenosine triphosphate (ATP). Furthermore, across a variety of taxa, neuromodulatory metabolites, such as gamma-aminobutyric acid (GABA) and its precursors can also be impacted by neonicotinoid exposure (Abd-Elhakim et al., 2018; Shi et al., 2018; Paten et al., 2022; Zhou et al., 2023). GABA functions as an inhibitory neurotransmitter and neonicotinoids act as a partial antagonist to GABA receptors which can partially block GABA-induced currents (Déglise et al., 2002; Taylor-Wells et al., 2015). Lastly, there is existing evidence that glycerophosphocholine, a precursor to acetylcholine, and glycerophospholipid metabolic pathways are impacted by neonicotinoid exposure (Shi et al., 2018; Olguin-Jacobson et al., 2021; Zhou et al., 2023). Neonicotinoids act by binding to nicotinic acetylcholine receptors (nAChRs) in the central nervous system (Casida and Durkin, 2013; Moffat et al., 2016), and likely influence the abundance and production of acetylcholine. These metabolites are all crucial to the central carbon metabolism and have the most evidence of being impacted by neonicotinoids across multiple taxa.

3. Results

3.1. Influence of treatment on bee survival

10% of bees (n = 19 of 195) died during the experiment. Imidacloprid exposure did not influence bee mortality during the seven-day exposure period, when bees had unlimited nectar (Pr ($>|z|$) values > 0.05 , Appendix 1, Table A1.3). However, imidacloprid exposure and natal colony (Pr ($>|z|$) values < 0.05 , Appendix 1, Table A1.4) both significantly increased worker mortality by the end of the entire experiment, after 24 hours of starvation. No untreated bees died during the 24-hour starvation period.

3.2. Metabolomic analysis

We detected a total of 78 metabolites in our study, across all samples, out of a total of 195 possible metabolites targeted with our analysis. The greatest number of metabolites were impacted by the duration of starvation (n = 39 metabolites influenced by this factor at a q-value < 0.05 , Appendix 1, Table A1.5), followed by worker natal colony (n = 18 metabolites; q-value < 0.05). Pesticide exposure influenced levels of 10 metabolites (q-value < 0.05 ; Tables 1.1 and 1.2), with only small numbers of metabolites (two and three, respectively) specifically associated with *early-limited* and *late-limited* exposure. Levels of an additional six metabolites were associated with the interaction between pesticide exposure and duration of starvation (q-value < 0.05 ; Tables 1.3 and 1.4). Our analysis of the effect of pesticide exposure across different durations of starvation revealed that there was no impact of pesticide exposure prior to the starvation period; it was only when bees were starved for some duration that the effect of the pesticide was observed (Treatment*

0-hour timepoint q -value > 0.05). Complete lists of metabolites and their associated pathways are provided in Tables 1.1 and 1.3, and Table A1.5. Overrepresentation analysis (ORA) of our pesticide and duration of starvation metabolite lists revealed that no particular types of processes were overrepresented with these metabolite lists.

3.3 Key metabolites

Average abundances of nicotinamide adenine dinucleotide (NAD), adenosine, cytidine, gamma-aminobutyric acid, and glycerophosphocholine were impacted by either imidacloprid alone, or the interaction between imidacloprid and starvation (adjusted p -values < 0.05). Significant differences in these metabolites did not occur until after starvation had begun. NAD was impacted by the interaction between pesticide exposure and duration of starvation (Figures 1.3 A-B) after 8 hours of starvation. Adenosine, a purine, was also impacted by the interaction between pesticide exposure and duration of starvation at the 8-hour starvation timepoint (Figures 1.3 C-D). Cytidine, glycerophosphocholine, and gamma-aminobutyric acid (GABA) were all impacted by pesticide alone. The strongest difference in levels of GABA were between the *untreated* and *sustained* treatment groups (Figures 1.3 E-F). The figures for cytidine and glycerophosphocholine can be found in Appendix 1, Figure A1.1.

4. Discussion

This was the first study to use metabolomics to examine the effects of neonicotinoids in bumble bees, which complements other studies that examine the effects of pesticides at

the metabolic level in other insects (Wei et al., 2020; Paten et al., 2022). We hypothesized that sublethal exposure to the neonicotinoid imidacloprid disrupts bumble bee energy metabolism, with the prediction that levels of sugars and other key metabolites involved in energy metabolism would be reduced in bees exposed to this pesticide. We tested this idea by administering imidacloprid to worker bumble bees in either a sustained or more limited exposure paradigms, then examining metabolite levels through time under starvation conditions. We found that imidacloprid exposure in the absence of nectar starvation had little impact on metabolite levels. Instead, the strongest effects were observed when pesticide-treated bees were also exposed to nectar starvation for some duration of time. These results suggest that some of the previously reported, combinatorial influences of neonicotinoid-type insecticides and nutritional stress might be mediated in part by the influence of these stressors on energy metabolism.

The strongest impacts of imidacloprid in our study were revealed when bees experienced nectar starvation for some duration of time. We detected almost no pesticide effects on metabolite levels immediately following imidacloprid exposure but prior to exposure to nutritional stress. It was only when bees were nectar starved for extended periods of time, specifically between eight and sixteen hours, that differences in metabolite levels across the treatment groups become apparent. These results suggest that when bees are well fed, specifically with nectar, the harmful effects of imidacloprid can be somewhat buffered at the metabolic level. However, within eight hours of starvation stress, imidacloprid can greatly impact metabolite levels that are essential for energy metabolism, such as

nicotinamide adenine dinucleotide (NAD) and adenosine. According to these results, bees exposed to imidacloprid do not respond the same to nutritional stress at the metabolic level as bees that have not been exposed to imidacloprid. Any alteration in energy metabolite levels can potentially disrupt energy metabolism, which can have many downstream effects on overall physiology and behavioral performance.

We also detected a recovery of metabolite levels following longer durations of nectar starvation. At the 24-hour starvation time point, levels of nearly all metabolites that were impacted by imidacloprid or nectar starvation, on their own or in combination at earlier time points in the experiment had stabilized and no longer differed across all treatment groups. This suggests that with respect to the impacts of the stressors on bumble bee energy metabolism, there is a stabilization period after which metabolite levels return to homeostasis. Organismal stress responses have been characterized as having three molecular phases, with the first phase representing the baseline level of molecules involved in the stress response, the second phase is a spike in the molecules, and the third phase is the decay of the molecules (Taborsky et al., 2021). The decay phase is necessary because if the molecules involved in stress are constantly elevated, damage can occur at the cellular, tissue, and organismal level. Our results align with the concept that there is a decay period in order to preserve energy metabolites and prevent detrimental damage to cells and tissues. However, importantly, this does not mean that the pattern of stress response halts entirely. This pattern of peak and decay in molecules would likely continue while the bees experience the stressors.

Our more sustained pesticide exposure paradigm (where bees were exposed for the entire seven-day duration) likely more closely mimics real-world conditions in areas of intensive agriculture. Imidacloprid and other neonicotinoids are omnipresent in soil and waterways (reviewed in Bonmatin et al., 2005; reviewed in Morrissey et al., 2015; Hladik and Kolpin, 2016), particularly in or adjacent to agricultural areas where these insecticides are primarily applied as a seed treatment to crops (reviewed in Morrissey et al., 2015). Moreover, the level of exposure administered in our study (5 ppb) was far lower than neonicotinoid levels that have been detected in pollen and nectar collected from crop plants and wildflowers in field margins (reviewed in Wood and Goulson, 2017). Our limited exposure groups were designed to mimic short-term exposure to neonicotinoids, in systems where bees may encounter these insecticides for a brief period but are not consistently exposed. Our goals were to determine whether limited exposure is sufficient to impact metabolite levels (*limited-late*) and whether a history of exposure has persistent impacts on metabolite levels even after exposure has ceased (*limited-early* group). For some metabolites in our study, the limited-late and sustained treatment groups followed similar patterns in changes in metabolite levels (such as NAD), and the limited-early and untreated groups follow similar patterns in changes. For other metabolites, the limited exposure groups did not have a significant effect on metabolite levels. For example, GABA was impacted only by sustained pesticide exposure. Further, some metabolites were impacted by all pesticide exposure groups, such as adenosine, where all exposure groups had a relatively stable level of adenosine through time, whereas the untreated group had a significant spike at the 8-hour timepoint. Adenosine is

essentially a precursor to adenosine triphosphate (ATP) and can also have a neuromodulatory role in insects (Magazanik and Fedorova, 2003; Dolezelova et al, 2007). Evidence has shown that adenosine can inhibit synaptic transmission during metabolic stress or excessive activity. The spike in adenosine in *untreated* bees may be attributed to a physiological response to nutritional, or metabolic stress. If this is the case, then these results suggest that bees exposed to imidacloprid respond differently to this nutritional stress, at least at the physiological level.

Starvation, unsurprisingly, had the strongest influence on central carbon metabolism in our study. This finding is consistent with multiple studies on bumble bee nutrition. Starvation has documented effects on bumble bee nutrient and metabolite levels (Stabler et al., 2015; Wang et al., 2019). Our study allowed us to further explore how starvation impacts specific metabolites that are important for energy metabolism (Appendix 1, Table A1.5). Specifically, we found that amino acids were the class of metabolites that were most affected by starvation stress, which aligns with previous evidence showing that nutritionally stressed bees respond with increased protein catabolism (Wang et al., 2019). Some caution is warranted in that all bees in our study were also starved of pollen for the duration of our experiment (seven days). Adult bumble bee workers do not consume significant quantities of pollen (Stabler et al., 2015), and pollen starvation has negligible effects on bumble bee survival across the timescale examined in our study (Smeets and Duchateau, 2003). Honey bees can compensate for the lack of particular

nutrients in their diet by shifting between metabolizing different nutrients (Wang et al., 2016), and, likely, bumble bees can too (Woodard et al., 2019).

Although we did not investigate this effect as closely, natal colony also had a relatively strong impact on metabolite levels. Colony effects may be genetic, as all bees from a colony share the same mother and probably the same father, given the low mating frequency observed in bumble bees (Schmid-Hempel & Schmid-Hempel, 2000). They may also reflect differences in social conditions between colonies. In this study, natal colony impacted the relative abundance of 18 metabolites. However, the patterns of change in these metabolites through time under starvation were similar across the pesticide exposure groups irrespective of the natal colony. One possible limitation of this study is that we did not account for age, which can be a potential factor that impacts energy metabolism (Collatz, 1996). The three colonies used in this study were around the same life-cycle stage (young, worker-producing). All workers that were used in the experiment were pulled from colonies across the span of 20 days. Since workers are constantly being produced in a young bumble bee (*B. impatiens*) colony for around four weeks, we were able to pull workers who appeared to be relatively young across the 20-day experiment. Accounting for age was an additional factor that would have made it difficult to reach the sample size that we were aiming for, and we would not have been able to start all bees on the imidacloprid treatment at the same time in a given trial.

The results from our study show that diet and nutrition can help buffer bees against some of the harmful effects of pesticides at the metabolic level. Our results support the findings in a recent study that found that pollen diet can help buffer the harmful effects of pesticides in bumble bee queens (Costa et al., 2022). Having a buffer against the strong effects of pesticides is promising for bumble bee resilience in certain environmental systems. However, having a buffer, such as a high quality diet, may not always be available in real-world conditions. Evidence has shown that bees are likely to encounter more than one stressor in their environment, and it is important to understand how these stressors interact and impact bees at the physiological level. If researchers and pesticide companies are only looking at the impact of pesticide alone when running risk assessments, they could be underestimating the harmful effects on bees. Pesticide companies should include multiple stressors in their testing protocols in order to obtain more accurate results that align with real-world conditions. Our study supports more regulation when it comes to pesticide use due to the harmful effects at the metabolic level.

5. Conclusion

We investigated the effects of imidacloprid and starvation, two common stressors, on bumble bee energy metabolism. This is a novel study in which we used metabolomic techniques to explore how imidacloprid and starvation interact at the metabolic level. The interactive effects of imidacloprid and nutritional stress were shown to impact essential metabolites involved in energy metabolism, which can lead to a disruption in energy

metabolism. All organisms depend on a properly functioning energy metabolism in order to survive and carry out essential tasks, and any disruption can have detrimental effects at the cellular, tissue, and organismal level. We suggest that pesticide risk assessments, as well as additional research studies, should include multiple stressors to more closely reflect real-world environments.

Tables and Figures

Figure 1.1. Exposure period

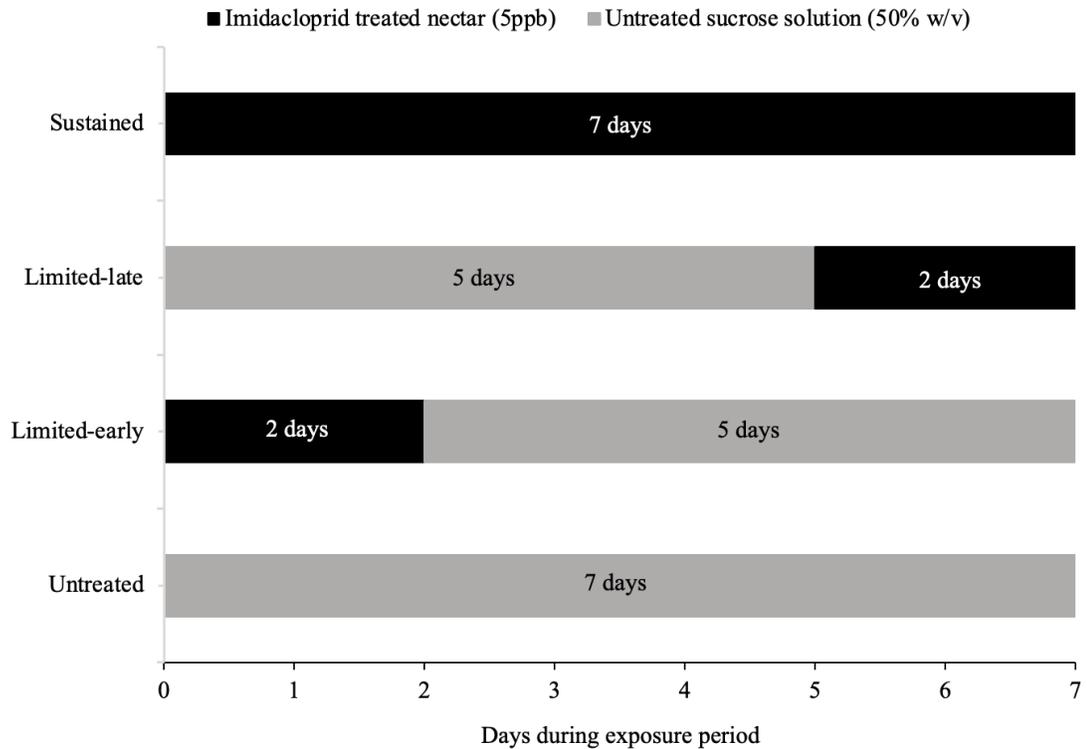


Figure 1.1. Schedule for imidacloprid exposure according to treatment group (y-axis) across a seven-day exposure period (x-axis). The *untreated* group served as a control and only received 50% sucrose solution. The *limited* exposure groups were exposed to imidacloprid for two days either early (*limited-early*) or late (*limited-late*) during the exposure period. The *sustained* group was exposed to imidacloprid for the full seven days.

Figure 1.2. Starvation period

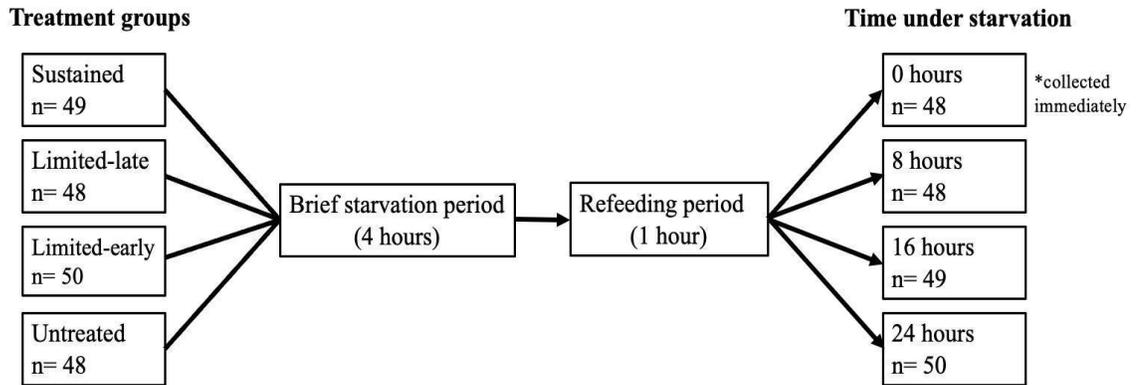


Figure 1.2. Flow diagram of starvation period. Following the exposure period, bees from all treatment groups underwent a brief starvation period (4 hours) followed by a refeeding period (1 hour), in which all bees were given an untreated sucrose solution to feed on. After the refeeding period, bees from all treatment groups were randomly assigned to a starvation group, wherein they were starved for either 0, 8, 16, or 24 hours. Bees assigned to the 0-hour starvation group were collected immediately after the refeeding period.

Table 1.1. Significant metabolites by pesticide treatment and their pathways.

<i>Significant Metabolites by Imidacloprid Treatment</i>			
Metabolite	Class	KEGG ID	Pathways
N,N-dimethylglycine	Methylation	C01026	Glycine, serine and threonine metabolism
N-Acetylputrescine	Polyamine	C00624	Arginine and proline metabolism
3-Ureidopropioninc acid	Pyrimidine	C02642	Pyrimidine metabolism; beta-Alanine metabolism; Pantothenate and CoA biosynthesis
Betaine	Methylation	C00719	Glycine, serine and threonine metabolism
Cysteinesulfinic acid	Amino acid	C00606	Cysteine and methionine metabolism; Taurine and hypotaurine metabolism
Cytidine	Pyrimidine	C00475	Pyrimidine metabolism
Gamma-Aminobutyric acid (GABA)	Amino acid	C00334	Alanine, aspartate and glutamate metabolism; arginine and proline metabolism; beta-alanine metabolism; butanoate metabolism; nicotinate and nicotinamide metabolism
Glycerophosphocholine	Choline	C00670	Glycerophospholipid metabolism
Isoleucine	Amino acid	C00407	Valine, leucine, isoleucine degradation and biosynthesis; aminoacyl-tRNA biosynthesis
Valine	Amino acid	C00183	Valine, leucine, isoleucine degradation and biosynthesis; Pantothenate and CoA biosynthesis; aminoacyl-tRNA biosynthesis

Table 1.2. ANOVA tests and adjusted p-values (q-values) for each metabolite impacted by imidacloprid treatment alone.

<i>Significant Metabolites by Imidacloprid Treatment- ANOVA</i>		
Metabolite	ANOVA Treatment	q-values
N,N-dimethylglycine	Untreated*Limited-early	3.31e-05
	Untreated*Sustained	1.43e-05
N-Acetylputrescine	Untreated*Limited-late	0.040
3-Ureidopropioninc acid	Sustained*Limited-early	0.002
Betaine	Sustained*Limited-early	0.014
Cysteinesulfinic acid	Limited-late*Limited-early	0.001
	Sustained*Limited-early	0.003
	Untreated*Limited-late	0.011
	Untreated*Sustained	2.71e-02
Cytidine	Sustained*Limited-early	0.048
	Untreated*Sustained	2.74e-02
Gamma-Aminobutyric acid (GABA)	Untreated*Sustained	3.82e-02
Glycerophosphocholine	Untreated*Sustained	4.01e-03
Isoleucine	Sustained*Limited-late	0.014
Valine	Sustained*Limited-late	0.015

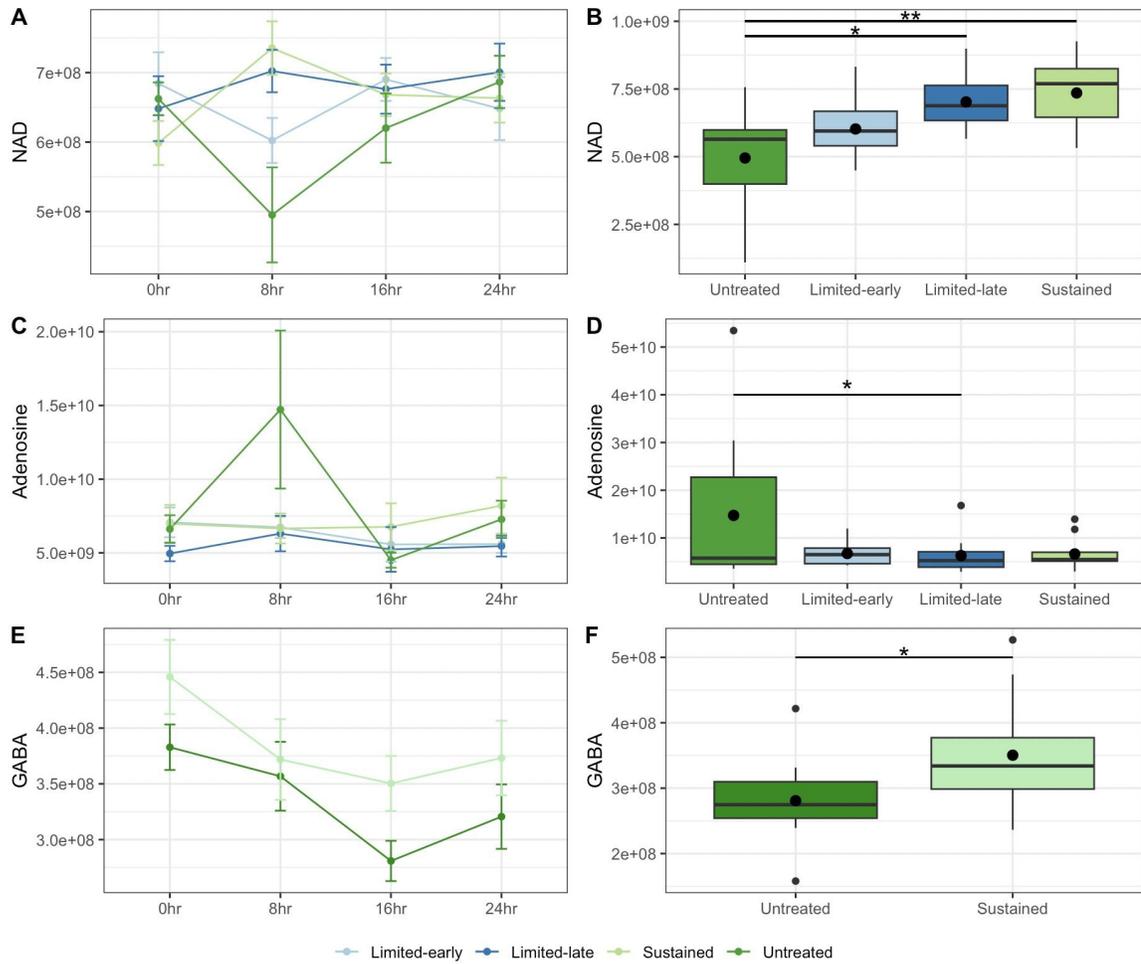
Table 1.3. Significant metabolites by Treatment*Starvation and their pathways.

<i>Significant Metabolites by Treatment * Starvation</i>			
Metabolite	Class	KEGG ID	Pathways
N-Acetylputrescine	Polyamine	C00624	Arginine and proline metabolism
3-Ureidopropioninc acid	Pyrimidine	C02642	Pyrimidine metabolism, beta-Alanine metabolism, Pantothenate and CoA biosynthesis
Adenosine	Purine	C00212	Purine metabolism; nucleotide metabolism; cGMP-PKG signaling pathway; cAMP signaling pathway; neuroactive ligand-receptor interaction; vascular smooth muscle contraction
Cysteinesulfinic acid	Amino acid	C00606	Cysteine and methionine metabolism; Taurine and hypotaurine metabolism
Kynurenine	Amino acid	C00328	Tryptophan metabolism
NAD	Electron carrier	C00003	Oxidative phosphorylation; Thiamine metabolism; nicotinate and nicotinamide metabolism

Table 1.4. ANOVA tests and adjusted p-values (q-value) for each metabolite impacted by the interaction between imidacloprid treatment and starvation duration.

<i>Significant Metabolites by Treatment * Starvation- ANOVA</i>		
Metabolite	ANOVA Treatment*Time	q-values
N-Acetylputrescine	Untreated-8hr*Limited-early-8hr	0.041
	Untreated-8hr*Limited-late-8hr	0.023
3-Ureidopropioninc acid	Sustained-16hr*Limited-early-16hr	0.037
Adenosine	Untreated-8hr*Limited-late-8hr	0.042
Cysteinesulfinic acid	Untreated-16hr*Limited-late-16hr	0.029
	Untreated-16hr*Sustained-16hr	0.041
Kynurenine	Untreated-16hr*Limited-early-16hr	0.027
NAD	Untreated-8hr*Limited-late-8hr	0.037
	Untreated-8hr*Sustained-8hr	0.005

Figures 1.3. A-F



Figures 1.3. A-F: Key metabolites of interest that were impacted after bees were nutritionally stressed (after either 8 hours or 16 hours of starvation). The plots in the left column show the changes in metabolites through time under starvation and the plots in the right column show boxplots for those metabolites specifically at the 8- hour or 16- hour starvation timepoint. Black dots inside the boxplot represent the average; **** = p value \leq 0.005 and * = p value $<$ 0.05** (A) Relative abundance of nicotinamide adenine dinucleotide (NAD, y-axis) through time under starvation (x-axis). Relative abundance of NAD differed significantly after 8 hours of starvation. (B) Average abundance of NAD at the 8-hour starvation timepoint for each treatment group. NAD levels in the untreated group differed significantly from both the *sustained* and *limited-late* groups. (C) Relative abundance of adenosine through time under starvation. Levels of adenosine differ between *untreated* bees and all pesticide treated bees, particularly at the 8-hour starvation timepoint. (D) Average abundance of adenosine at the 8-hour starvation timepoint. (E) Relative abundance of gamma-aminobutyric acid (GABA) through time under starvation for *untreated* and *sustained* treatment groups. Bees within the *sustained* treatment group have higher relative abundance of GABA, particularly at the 16-hour timepoint. (F) Average abundance of GABA at the 16-hour starvation timepoint.

References

- Abd-Elhakim, Y.M., Mohammed, H.H., Mohamed, W.A.M. Imidacloprid Impacts on Neurobehavioral Performance, Oxidative Stress, and Apoptotic Events in the Brain of Adolescent and Adult Rats. *Journal of Agricultural and Food Chemistry* **66**: pp. 13513-13524. (2018).
- Alkassab, A. and Kirchner, W. Sublethal exposure to neonicotinoids and related side effects on insect pollinators: honeybees, bumblebees, and solitary bees. *Journal of Plant Diseases and Protection*, **124**: pp. 1-30. (2016).
- Álvarez-Sánchez, B., Priego-Capote, F., Luque de Castro, M. D. Metabolomics analysis I. Selection of biological samples and practical aspects preceding sample preparation. *TrAC - Trends in Analytical Chemistry* **29**: pp. 111–119. (2010).
- Bartel, J., Krumsiek, J., Theis, F. Statistical methods for the analysis of high-throughput metabolomics data. *Computational and Structural Biotechnology Journal*, **4**: e20130100. (2013).
- Blacquièrè, T., Smagghe, G., van Gestel, C. A. M., Mommaerts, V. Neonicotinoids in bees: a review on concentrations, side-effects and risk assessment. *Ecotoxicology* **21**: pp. 973–992. (2012).
- Blatt, J. and Roces, F. Haemolymph sugar levels in foraging honeybees (*Apis mellifera* Carnica): Dependence on metabolic rate and in vivo measurement of maximal rates of trehalose synthesis. *Journal of Experimental Biology* **204**: pp. 2709–2716. (2001).
- Bonmatin, J-M., Moineau, I., Charvet, R., Colin, M.E., Fleche, C., Bengsch, E.R. Behaviour of imidacloprid in fields. Toxicity for honey bees In: Lichtfouse, E., Schwarzbauer, J., Robert, D. (eds) Environmental chemistry. Springer, Berlin, pp. 483–494. (2005).
- Botías, C., David, A., Horwood, J., Abdul-Sada, A., Nicholls, E., Hill, E., Goulson, D. Neonicotinoid Residues in Wildflowers, a Potential Route of Chronic Exposure for Bees. *Environmental Science and Technology* **49**: pp. 12731–12740. (2015).
- Brittain, C. and Potts, S. G. The potential impacts of insecticides on the life-history traits of bees and the consequences for pollination. *Basic and Applied Ecology* **12**: pp. 321–331. (2011).
- Brown, M.J.F., Loosli, R., Schmid-Hempel, P. Condition-dependent expression of virulence in a trypanosome infecting bumblebees. *Oikos Advancing Ecology*. **91**: pp. 421-427. (2003).

- Cameron, S., Lozier, J., Strange, J., Koch, J., Cordes, N., Solter, L., Griswold, T. Patterns of widespread decline in North American bumble bees. *Proceedings of the National Academy of Science U. S. A.* **108**: pp. 662–667. (2011).
- Casida, J.E., Durkin, K.A. Neuroactive insecticides: targets, selectivity, resistance and secondary effects. *Annual Review in Entomology* **58**: pp. 99–117. (2013).
- Collatz, K.G. Fifteen years of *Phormia* on the value of an insect for the study of aging. *Archives of Gerontology and Geriatrics* **25**: pp. 83-90. (1996).
- Corbet, S.A., Williams, I.H., Osborne, J.L. Bees and the pollination of crops and wild flowers in the European community. *Bee World.* **72**: pp. 47–59. (1991).
- Costa, C.P., Leza, M., Duennes, M.A., Fisher, K., Vollaro, A., Hur, M., Kirkwood, J.S., Woodard, S.H., 2022. Pollen diet mediates how pesticide exposure impacts brain gene expression in nest-founding bumble bee queens. *Science of the Total Environment* **833**: 155216. (2022).
- Cook, S.C. Compound and dose-dependent effects of two neonicotinoid pesticides on honey bee (*Apis mellifera*) metabolic physiology. *Insects.* **10**:18. <https://doi.org/10.3390/insects10010018>. (2019).
- Cresswell, J., Page, C., Uygun, M., Holmbergh, M., Li, Y., Wheeler, J., Laycock, I. Pool, C., de Ibarra, N., Smirnoff, N., Tyler, C. Differential sensitivity of honey bees and bumble bees to a dietary insecticide (imidacloprid). *Zoology* **115**: pp. 365–371. (2012).
- David, A., Botias, C., Abdul-Sada, A., Nicholls, E., Rotheray, E., Hill, E., Goulson, D. Widespread contamination of wildflower and bee-collected pollen with complex mixtures of neonicotinoids and fungicides commonly applied to crops. *Environment International* **88**: pp. 169–178. (2016).
- Déglise, P., Grünewald, B., Gauthier, M. The insecticide imidacloprid is a partial agonist of the nicotinic receptor of honeybee Kenyon cells. *Neuroscience letters*, **321**: pp. 13-16. (2002).
- Dolezelova E., Nothacker, H., Civelli, O, Bryant, P.J., Zurovec, M. A *Drosophila* adenosine receptor activates cAMP and calcium signaling. *Insect Biochemistry and Molecular Biology*, **37**: pp. 318-329. (2007).
- Erban, T., Sopko, B., Talacko, P., Harant, K., Kadlikova, K., Halesova, T., Riddellova, K., Pekas, A. Chronic exposure of bumblebees to neonicotinoid imidacloprid

- suppresses the entire mevalonate pathway and fatty acid synthesis. *Journal of Proteomics* **196**: pp. 69–80. (2019).
- Feltham, H., Park, K. & Goulson, D. Field realistic doses of pesticide imidacloprid reduce bumblebee pollen foraging efficiency. *Ecotoxicology* **23**: pp. 317–323. (2014).
- Gill, R. J. and Raine, N. E. Chronic impairment of bumblebee natural foraging behaviour induced by sublethal pesticide exposure. *Functional Ecology* **28**: pp. 1459–1471. (2014).
- Goulson, D. REVIEW: An overview of the environmental risks posed by neonicotinoid insecticides. *Journal of Applied Ecology*. **50**: pp. 977–987. (2013).
- Goulson, D., Nicholls, E., Botías, C. & Rotheray, E. L. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science*. **34**: 6229. (2015).
- Heinrich, B. Energetics of temperature regulation and foraging in a bumblebee, *Bombus terrestris* kirby. *Journal of Comparative Physiology*. **77**: pp. 49–64. (1972).
- Heinrich, B. Energetics of Pollination. *Annual Review of Ecological Systems*. **6**: pp. 139–170. (1975).
- Heinrich, B. The foraging specializations of individual bumble bees. *Ecological Monographs*. **46**: pp. 105-128. (1976).
- Heinrich, B. Bumblebee Economics. *Harvard University Press*. (2004).
- Hladik, M.L., Kolpin, D.W. First national-scale reconnaissance of neonicotinoid insecticides in streams across the USA. *Environmental Chemistry* **13**:12–20. (2016).
- Holmstrup, M., Bindesbøl, A., Oostingh, G., Duschl, A., Scheil, V., Köhler, H., Loureiro, S., Soares, A., Ferreira, A., Kienle, C., Gerhardt, A., Laskowski, R., Kramarz, P., Bayley, M., Svendsen, C., Spurgeon, D. Interactions between effects of environmental chemicals and natural stressors: A review. *Science of the Total Environment*, **408**: 3746-3762. (2010).
- Horgan, R. P., Clancy, O. H., Myers, J. E. & Baker, P. N. An overview of proteomic and metabolomic technologies and their application to pregnancy research. *BJOG* **116**: pp. 173–181. (2009).

- Irwin, R., Cook, D., Richardson, L., Manson, J., Gardner, D. Secondary compounds in floral rewards of toxic rangeland plants: Impacts on pollinators. *Journal of Agricultural and Food Chemistry* **62**: pp. 7335-7344. (2014).
- Jacobson, M. M., Tucker, E. M., Mathiasson, M. E. & Rehan, S. M. Decline of bumble bees in northeastern North America, with special focus on *Bombus terricola*. *Biological Conservation* **217**: pp. 437–445. (2018).
- Jeschke P, Nauen R, Schindler M, Elbert A. Overview of the status and global strategy for neonicotinoids. *Journal of Agricultural Food Chemistry* **59**: pp. 2897–2908. (2011).
- Kammer, A. E. and Heinrich, B. Insect Flight Metabolism. *Advances In Insect Physiology* **13**: pp. 133–228. (1978).
- Knee, W. J. and Medler, J. T. The Seasonal Size Increase of Bumblebee Workers (Hymenoptera: *Bombus*). *The Canadian Entomologist*. **97**: pp. 1149–1155. (1965).
- Kosior, A., Celary, W., Olejniczak, P., Fijal, J., Król, W., Solarz, W., Plonka, P. The decline of the bumble bees and cuckoo bees (Hymenoptera: Apidae: Bombini) of Western and Central Europe. *ORYX* **41**: pp. 79–88. (2007).
- Lämsä, J., Kuusela, E., Tuomi, J., Juntunen, S., Watts, P. C. Low dose of neonicotinoid insecticide reduces foraging motivation of bumblebees. *Proceedings of the Royal Society B Biological Sciences*. **285**: 20180506. (2018).
- Leza, M., Watrous, K. M., Bratu, J., Woodard, S. H. Effects of neonicotinoid insecticide exposure and monofloral diet on nest-founding bumblebee queens. *Proceedings of the Royal Society B Biological Sciences*. **285**: 20180761. (2018).
- Lundin, O., Rundlöf, M., Smith, H. G., Fries, I., Bommarco, R. Neonicotinoid Insecticides and Their Impacts on Bees: A Systematic Review of Research Approaches and Identification of Knowledge Gaps. *PLoS One*. **10**: e0136928. (2015).
- MacLean, B., Tomazela, D.M., Shulman, N., Chambers, M., Finney, G.L., Frewen, B., Kern, R., Tabb, D.L., Liebler, D.C., MacCoss, M.J. Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics* **26**: pp. 966-968. (2010).
- Magazanik, L.G. and Fedorova, I.M. Modulatory role of adenosine receptors in insect motor nerve terminals. *Neurochemical Research* **28**: pp. 617-624. (2003).

- Manjon, C., Troczka, B., Zaworra, M., *et al.* Unravelling the Molecular Determinants of Bee Sensitivity to Neonicotinoid Insecticides. *Current Biology* **28**: pp. 1137-1143.e5 (2018).
- Martelli, F., Zhongyuan, Z., Wang, J., *et al.* Low doses of the neonicotinoid insecticide imidacloprid induce ROS triggering neurological and metabolic impairments in *Drosophila*. *PNAS* **117**(41): pp. 25840-25850 (2020).
- Mayack, C., Naug, D. Parasitic infection leads to decline in hemolymph sugar levels in honeybee foragers. *Journal of Insect Physiology*, **56**(11): pp. 1572-1575. (2010).
- McCallum, K. P., McDougall, F. O., Seymour, R. S. A review of the energetics of pollination biology. *Journal of Comparative Physiology B*. **183**: pp. 867–876. (2013).
- Medler, J. T. Morphometric Studies on Bumble Bees. *Annals of the Entomological Society of America*. **55**: pp. 212–218. (1962).
- Moffat, C., Pacheco, J.G., Sharp, S., Samson, A.J., Bollan, K.A., Huang, J., Buckland S.T., Connolly, C.N. Chronic exposure to neonicotinoids increases neuronal vulnerability to mitochondrial dysfunction in the bumblebee (*Bombus terrestris*). *FASEB J*. **29**: pp. 2112–2119. (2015).
- Moffat, C., Buckland, S.T., Samson, A.J., McArthur, R., Chamosa Pino, V., Bollan, K.A., Huang, J., Connolly, C.N. Neonicotinoids target distinct nicotinic acetylcholine receptors and neurons, leading to differential risks to bumblebees. *Scientific Reports* **6**: 24764. (2016).
- Morrissey, C.A., Mineau, P., Devries, J.H., Sánchez-Bayo, F., Liess, M., Cavallaro, M.C., Liber, K. Neonicotinoid contamination of global surfacewaters and associated risk to aquatic invertebrates: a review. *Environ Int* **74**: pp. 291–303. (2015).
- Nicodemo, D., Maoili, M.A., Medeiros, H.C.D, Guelfi, M., Baleira, K.V.B., De Jong, D., Mingatto, F.E. Fipronil and imidacloprid reduce honeybee mitochondrial activity. *Environmental Toxicology and Chemistry*. **33**: pp. 2070–2075 (2014).
- Nicolson, S. Bee food: the chemistry and nutritional value of nectar, pollen and mixtures of the two. *African Zoology* **46:2**, pp. 197-204. (2011).
- Ollerton, J., Winfree, R., Tarrant, S. How many flowering plants are pollinated by animals. *Oikos*, **120**(3): pp. 321-326. (2011).

- Olguin-Jacobson, C., Pitt, K.A., Carroll, A.R., Melvin, S.D. Chronic pesticide exposure elicits a subtle carry-over effect on the metabolome of *Aurelia coerulea* ephyrae. *Environmental Pollution*, **275**: 116641. (2021).
- Orr, J., Vinebrooke, R.D., Jackson, M.C., Kroeker, K.J., Kordas, R.L., Mantyka-Pringle, C., Van den Brink, P.J., De Laender, F., Stoks, R., Holmstrup, M., Matthaei, C.D., Monk, W.A., Penk, M.R., Leuzinger, S., Schäfer, R.B. and Piggott, J.J. Towards a unified study of multiple stressors: divisions and common goals across research disciplines. *ProcB*, **287**: 20200421. (2020).
- Owen, R. E. Body size variation and optimal body size of bumble bee queens (hymenoptera: Apidae). *The Canadian Entomologist*. **120**: pp. 19–27. (1988).
- Pamminger, T., Becker, R., Himmelreich, S., Schneider, C.W. and Bergtold, M. The nectar report: Quantitative review of nectar sugar concentrations offered by bee visited flowers in agricultural and non-agricultural landscapes. *PeerJ*, **7**: p.e6329. (2019).
- Pang, Z., Chong, J., Zhou, G., *et al.* MetaboAnalyst 5.0: narrowing the gap between raw spectra and functional insights. *Nucleic Acids Research*, **49**: W388–W396. (2021).
- Paten, A.M., Taylor, M.C., Williams, M.R., Warden, A.C., Kumar, A. Acute sublethal exposure to a neonicotinoid pesticide triggers a short-term metabolic response in honey bee larvae. In: Beale, D.J., Hillyer, K.E., Warden, A.C., Jones, O.A.H. (eds) *Applied Environmental Metabolomics*, Academic Press, pp. 359-376. (2022).
- Pereboom, J. The composition of larval food and the significance of exocrine secretions in the bumblebee *Bombus terrestris*. *Insectes Sociaux* **47**: pp. 11-20. (2000).
- Rao, S., Strange, J.P. Bumble Bee (Hymenoptera: Apidae) Foraging Distance and Colony Density Associated With a Late-Season Mass Flowering Crop. *Environmental Entomology*, **41**: pp. 905–915. (2012).
- Rothe, U. and Nachtigall, W. Flight of the honey bee IV. Respiratory quotients and metabolic rates during sitting, walking and flying. *Journal of Comparative Physiology B* **158**: pp. 739-749. (1989).
- Roulston, T.H., Cane, J.H. Pollen nutritional content and digestibility for animals. *Plant Systematics and Evolution* **222**: pp. 187-209. (2000).
- Saccetti, E., Hoefsloot, H., Smilde, A., Westerhuis, J., Hendriks, M. Reflections on univariate and multivariate analysis of metabolomics data. *Metabolomics*, **10**: pp. 361-374. (2014).

- Sacktor, B. Regulation of Intermediary Metabolism, with Special Reference to the Control Mechanisms in Insect Flight Muscle. *Advances In Insect Physiology* **7**: pp. 267–347. (1970).
- Schmid-Hempel, R. and Schmid-Hempel, P. Female mating frequencies in *Bombus* spp. From central Europe. *Insectes Sociaux* **47**: pp. 36-41. (2000).
- Sheets, L. P. Imidacloprid: A Neonicotinoid Insecticide. in *Hayes' Handbook of Pesticide Toxicology* 2055–2064 (Elsevier Inc.). doi:10.1016/B978-0-12-374367-1.00095-1. (2010).
- Shi, T., Burton, S., Wang, Y., Xu, S., Zhang, W., Yu, L. Metabolomic analysis of honey bee, *Apis mellifera* L. response to thiacloprid. *Pesticide Biochemistry and Physiology* **152**: pp.17-23. (2018).
- Shpigler, H., Tamarkin, M., Gruber, Y., Poleg, M., Siegel, A.J., Bloch, G. Social influences on body size and developmental time in the bumblebee *Bombus terrestris*. *Behavioral Ecology and Sociobiology*. **67**: pp. 1601–1612. (2013).
- Smeets, P., Duchateau, J.M. Longevity of *Bombus terrestris* workers (Hymenoptera: Apidae) in relation to pollen availability, in the absence of foraging. *Apidologie* **34**: pp. 333-337. (2003).
- Stabler, D., Paoli, P.P., Nicolson, S.W., Wright, G.A. Nutrient balancing of the adult worker bumblebee (*Bombus terrestris*) depends on the dietary source of essential amino acids. *The Journal of Experimental Biology*, **218**: pp. 793-802. (2015).
- Stanley, D. A., Russell, A. L., Morrison, S. J., Rogers, C., Raine, N. E. Investigating the impacts of field-realistic exposure to a neonicotinoid pesticide on bumblebee foraging, homing ability and colony growth. *Journal of Applied Ecology*. **53**: pp. 1440–1449. (2016).
- Stewart, S. D., Lorenz, G.M., Catchot, A.L., *et al.* Potential exposure of pollinators to neonicotinoid insecticides from the use of insecticide seed treatments in the mid-southern United States. *Environmental Science and Technology*. **48**: pp. 9762–9769. (2014).
- Stuligross, C., Melone, G.G, Wang, L., Williams, N.M. Sublethal behavioral impacts of resource limitation and insecticide exposure reinforce negative fitness outcomes for a solitary bee. *Science of the Total Environment* **867**: 161392. (2023).
- Sur, R. and Stork, A. Uptake, translocation and metabolism of imidacloprid in plants. *Bulletin of Insectology* **56**: pp. 35–40. (2003).

- Taborsky, B., English, S., Fawcett, T.W., Kuijper, B., Leimar, O., McNamara, J.M., Ruuskanen, S., Sandi, C. Towards an Evolutionary Theory of Stress Responses. *Trends in Ecology and Evolution* **36**:39-48. (2021).
- Taylor-Wells, J., Brooke, B.D., Bermudez, I., Jones, A.K. The neonicotinoid imidacloprid, and the pyrethroid deltamethrin, are antagonists of the insect Rdl GABA receptor. *Journal of Neurochemistry*, **135**: pp. 705-713. (2015).
- Therneau, T.M. and Grambsch, P.M. The cox model. In *Modeling survival data: extending the Cox model* (pp. 39-77). Springer, New York, NY. (2000).
- Therneau, T. Mixed effects Cox models. *CRAN repository*. (2015).
- Tiffany, C.R. and Bäumler, A.J. omu, a Metabolomics Count Data Analysis Tool for Intuitive Figures and Convenient Metadata Collection. *Microbiology Resource Announcements* **8**, e00129-19. (2019).
- Timberlake, T.P., Vaughan, I.P., Baude, M. and Memmott, J. Bumblebee colony density on farmland is influenced by late-summer nectar supply and garden cover. *Journal of Applied Ecology*, **58**(5): pp. 1006-1016. (2021).
- Timberlake, T.P., Vaughan, I.P. and Memmott, J. Phenology of farmland floral resources reveals seasonal gaps in nectar availability for bumblebees. *Journal of Applied Ecology*, **56**(7): pp. 1585-1596. (2019).
- Tosi, S., Nieh, J. C., Sgolastra, F., Cabbri, R., Medrzycki, P. Neonicotinoid pesticides and nutritional stress synergistically reduce survival in honey bees. *Proceedings B Biological Sciences* **284**: 20171711. (2017).
- Tyler, E.R., Adams, S., Mallon, E.B. An immune response in the bumblebee, *Bombus terrestris* leads to increased food consumption. *BMC Physiology* **6**: doi:10.1186/1472-6793-6-6. (2006).
- Van der Sluijs, J. P., Simon-Delso, N., Goulson, D., Maxim, L., Bonmatin, J-M., Belzunces, L.P. Neonicotinoids, bee disorders and the sustainability of pollinator services. *Current Opinion in Environmental Sustainability* **5**: pp. 293–305 (2013).
- Vaudo, A., Patch, H., Mortensen, D., Tooker, J., Grozinger, C. Macronutrient ratios in pollen shape bumble bee (*Bombus impatiens*) foraging strategies and floral preferences. *PNAS* **113**(28) E4035-E4042. (2016).

- Vaudo, A., Farrell, L., Patch, H., Grozinger, C., Tooker, J. Consistent pollen nutritional intake drives bumble bee (*Bombus impatiens*) colony growth and reproduction across different habitats. *Ecology and Evolution* **8**: pp. 5765-5776. (2018).
- Velthuis, H. and van Doorn, A. A century of advances in bumblebee domestication and the economic and environmental aspects of its commercialization for pollination. *Apidologie* **37**: pp. 421-451. (2006).
- Vliet, S.M.F., Dasgupta, S., Sparks, N.R.L., Kirkwood, J.S., Vollaro, A., Hur, M., Zur Nieden, N.I., & Volz, D.C. Maternal-to-zygotic transition as a potential target for niclosamide during early embryogenesis. *Toxicology and Applied Pharmacology*, **380**: 114699. (2019).
- Wang, Y., Campbell, J.B, Kaftanoglu, O., Page, R.E, Amdam, G., Harrison, J.F. Larval starvation improves metabolic response to adult starvation in honey bees (*Apis mellifera* L.). *Journal of Experimental Biology*, **219**: pp. 960-968. (2016).
- Wang, L., Meeus, I., Rombouts, C., Meulebroek, L.V., Vanhaecke, L., Smagghe, G. Metabolomics-based biomarker discovery for bee health monitoring: A proof of concept study concerning nutritional stress in *Bombus terrestris*. *Scientific Reports* **9**: 11423 (2019).
- Wei, F., Wang, D., Li, H., Xia, P., Ran, Y., You, J. Toxicogenomics provides insights to toxicity pathways of neonicotinoids to aquatic insect, *Chironomus dilutus*. *Environmental Pollution*, **260**: 114011. (2020).
- Wieder, C., Frainay, C., Poupin, N., Rodríguez-Mier, P., Vinson, F., Cooke, J., Lai, R.P., Bundy, J.G., Jourdan, F. and Ebbels, T. Pathway analysis in metabolomics: Recommendations for the use of over-representation analysis. *PLoS computational biology*, **17**(9): p.e1009105. (2021).
- Willmer, P. Pollination and Floral Ecology. *Princeton University Press*. (2011).
- Wolschin, F. and Amdam, G. V. Comparative proteomics reveal characteristics of life-history transitions in a social insect. *Proteome Science* **5**: DOI: 10.1186/1477-5956-5-10. (2007).
- Wood, T.J. and Goulson, D. The environmental risks of neonicotinoid pesticides: a review of the evidence post 2013. *Environmental Science and Pollution Research* **24**: pp. 17285–17325. (2017).
- Woodard, S.H. and Jha, S. Wild bee nutritional ecology: predicting pollinator population dynamics, movement, and services from floral resources. *Current Opinion in Insect Science* **21**: pp. 83-90. (2017).

- Woodard, S.H., Duennes, M.A., Watrous, K.M., Jha, S. Diet and nutritional status during early adult life have immediate and persistent effects on queen bumble bees. *Conservation Physiology* **7**: <https://doi.org/10.1093/conphys/coz048>. (2019).
- Yerushalmi, S., Bodenheimer, S., Bloch, G. Developmentally determined attenuation in circadian rhythms links chronobiology to social organization in bees. *Journal of Experimental Biology*. **209**: pp. 1044–1051. (2006).
- Zhang, Y., Chen, D., Xu, Y., Ma, L., Du, M., Li, P., Yin, Z., Xu, H., Wu, X. Stereoselective toxicity mechanism of neonicotinoid dinotefuran in honeybees: New perspective from a spatial metabolomics study. *Science of the Total Environment*, **809**: 151116. (2022).
- Zhou, X., Ming, R., Guo, M., Jiao, H., Cui, H., Hu, D., Lu, P. Characterization of imidacloprid-induced hepatotoxicity and its mechanisms based on a metabolomic approach in *Xenopus laevis*. *Science of the Total Environment*, **869**: 161794. (2023).
- Zhu, Y. C., Yao, J., Adamczyk, J., Luttrell, R. Feeding toxicity and impact of imidacloprid formulation and mixtures with six representative pesticides at residue concentrations on honey bee physiology (*Apis mellifera*). *PLoS One* **12**: e01784221. (2017).

Chapter 2

Exploring the molecular mechanisms associated with bumble bee foraging behavioral states.

1. Introduction

Biologists have long been fascinated by the phenomenon of individual animals exhibiting consistency in their behavior. Behavior is often highly plastic, idiosyncratic, and responsive to the environmental conditions in which it is expressed. Yet, some behaviors are highly stereotypical or consistent, such as courtship rituals and foraging strategies. In ethological research, behavioral consistency that is exhibited for an appreciable duration of time is referred to as a behavioral state, whereas more instantaneous behaviors are referred to as events (Altmann, 1974). Behavioral states are typically examined across longer timescales, on the order of hours, days, or even longer periods.

In the context of pollination ecology, individual pollinators can exhibit behavioral consistency in their foraging behavior in multiple ways. One of these ways is tactic constancy, which refers to when pollinators persistently perform a single resource collection tactic over prolonged periods of time (Bronstein et al., 2017). Tactic constancy is a relatively recent concept to emerge to help describe behavioral consistency in pollinators, and it has been studied most extensively in the context of nectar robbing by bumble bees. When bumble bees collect nectar, they can either forage legitimately or nectar rob. Legitimate foraging occurs when bees visit the natural openings of the flower

and contact the flower's reproductive parts when collecting nectar (Bronstein et al., 2017). Nectar robbing is a tactic wherein bees bypass the flower's natural opening to feed on nectar through perforations that have been made on other parts of the flower (Bronstein et al., 2017). Bumble bees have been observed consistently performing nectar robbing behavior within a single foraging bout, and also across foraging bouts over the span of days (Lichtenberg et al., 2020a; Lichtenberg et al., 2020b). Since bumble bees exhibit constancy to this tactic for an appreciable duration of time (days), nectar robbing can be considered a behavioral state.

The ecological drivers of nectar robbing behavior have been explored over recent years (Newman and Thomson, 2005; Irwin et al., 2010; Bronstein et al., 2017; Lichtenberg et al., 2018; Lichtenberg et al., 2020a). Environmental context, sensory ecology, floral visitor communities, flower morphology, and physical restraints (reviewed in Irwin et al., 2010; Richman et al., 2021) can all shape nectar robbing behavior. While the ecological perspective is informative and important for examining the significance of nectar robbing behavior, the internal or physiological drivers remain almost entirely unknown.

Identifying the underlying molecular mechanisms regulating nectar robbing can help fill the gap in our understanding as to why a bee employs or remains constant to this specific tactic (Irwin et al., 2010; Bronstein et al., 2017). It has been hypothesized that nectar robbing is used to conserve energy (Bronstein et al., 2017; Lichtenberg et al., 2018; Lichtenberg et al., 2020b). One mechanistic prediction based on this hypothesis is that nectar robbing is driven by or associated with differences in energy metabolism among

foraging bees. If nectar robbing does facilitate energy conservation, then this might be reflected at the molecular level, particularly in the canonical pathways involved in energy metabolism, such as ATP production. This line of thinking complements the ecological work that frames nectar robbing around optimal foraging theory and examines foraging efficiencies associated with nectar robbing behavior (Lichtenberg et al., 2018, Lichtenberg et al., 2020b).

Analysis of brain gene expression can be used to understand the molecular basis of behavioral states and has been used successfully to understand the underlying differences in feeding- and foraging-related behavioral differences among animals. For example, in the fruit fly *Drosophila melanogaster*, larvae can be classified as rovers or sitters by their foraging behaviors, which is attributed to allelic variance of the *foraging* (*for*) gene (de Belle et al., 1989). An increase in *for* gene expression in the mushroom bodies can alter the behavior of sitters to be more exploratory, like the rovers (Burns et al., 2012). In honey bees, this same gene also regulates the behavioral transition from nurse to forager (Heylen et al., 2008). Further, in distinct brain regions, *foraging* gene expression can influence sucrose responsiveness and division of labor in honey bees (Thamm and Scheiner, 2014). The *foraging* gene is one of many genes that are associated with the transition from nurse to forager behavioral states in honey bees (Liu et al., 2019). Thus, examining brain gene expression in bumble bee foragers can bring insight into some of the molecular mechanisms associated with tactic constancy and nectar robbing behavior.

In this study, we used analysis of brain gene expression in foraging workers of the bumble bee *Bombus bifarius* to explore whether there are molecular mechanisms associated with specific foraging behavioral states, with a focus on nectar robbing behavior. We collected foraging workers exhibiting the foraging behaviors of interest in a natural field setting. Specifically, we collected foraging workers that were consistently observed exhibiting either legitimate foraging or nectar robbing, or alternated between nectar robbing and legitimate foraging within a single foraging bout (hereafter referred to as “switching”). After collecting bees in the field, we then used RNA sequencing (RNA-seq) to examine brain gene expression patterns in each of these behavioral categories to identify genes with patterns of expression associated with foraging tactic. This represents the first analysis to explore the molecular basis of nectar robbing behavior. Using our gene expression data, we explored our hypothesis that there is a molecular basis for nectar robbing behavior, with the prediction that nectar robbers versus legitimate foragers would exhibit differential expression of genes associated with energy metabolism within the brain. We also explored a second hypothesis focused on foragers that switched between those two tactics. Foragers that switch between tactics exhibit flexibility in transitioning between these two states. Thus, we hypothesized that gene expression patterns might reflect an intermediate state between the tactic constant nectar robbers and legitimate foragers.

2. Methods

2.1. Study system

Bumble bee (*Bombus bifarius*) workers were collected across two summer seasons (July through August 2020-2021) in or near the Rocky Mountain Biological Laboratory (RMBL), located in Gunnison County, Colorado. The RMBL is an ideal habitat for the study of bumble bees because it has relatively minimal anthropogenic impacts and low prevalence of bumble bee pathogens, thus reducing the impact of these variables, which have known impacts on brain gene expression in bumble bees (Doublet et al., 2016; Bebane et al., 2019; Fisher et al., 2022). Bees were collected across four field sites, all approximately 16-23 kilometers apart, with the exception of two of the field sites which were < 0.8 kilometers apart. Based on the known foraging ranges and nesting densities of bumble bees (Osborne et al., 1999; Knight et al., 2005; Greenleaf et al., 2007; Osborne et al. 2008), bees from these two sites may have originated from the same colonies but likely did not.

B. bifarius is one of the most abundant bumble bee species at the RMBL (Pleasants 1981). This relatively short-tongued species, with a proboscis < 9 mm in length (Pyke 1982), has been observed to forage legitimately and also rob nectar from several plant species at the RMBL (Mayfield et al., 2001; Newman and Thomson, 2005; Pyke et al., 2012; Lichtenberg et al., 2020a; Lichtenberg et al., 2020b). *B. bifarius* cannot perform primary nectar robbing, meaning they cannot make perforations to rob from, but they can

secondarily rob flowers that have perforations created by primary robbers, such as the bumble bee *B. occidentalis*.

We examined the behavior of *B. bifarius* on *Linaria vulgaris* (Plantaginaceae), commonly known as “butter-and-eggs” toadflax. *L. vulgaris* is a self-incompatible perennial with yellow flowers that are snapdragon-like with a lanceolate upper and lower lobe. The bottom lobe covers the floral opening and must be pulled down to access the nectar legitimately. The nectar spur is 15-20 mm in length (Irwin and Maloof, 2002). Bumble bees are among the visitors strong enough to push past the flower’s lobe and access the floral rewards. *L. vulgaris* experiences primary robbing from *B. occidentalis* (Irwin and Maloof, 2002) and secondary robbing from *B. bifarius*. However, *B. bifarius* can also forage legitimately for both nectar and pollen from *L. vulgaris* (Arnold, 1982; Pyke, 1982; Knight et al., 2005; Lichtenberg et al., 2020a; Lichtenberg et al., 2020b).

2.2. Field methods

Individual *Bombus bifarius* workers collected for the study (n = 121) were observed foraging on *Linaria vulgaris* exclusively for a total of 10 consecutive minutes. This duration was selected based on previous studies in this system, wherein bees were followed for longer durations of time and found to remain tactic constant throughout the entirety of observation times (Irwin personal comm). We also observed a subset of *Bombus bifarius* workers (n = 88) for longer durations (anywhere between 15-60 minutes) to validate that ten minutes is sufficient time to detect tactic constancy; 100% of

these bees continued to forage on *L. vulgaris* beyond the ten minute time period, either until they flew out of sight or until approximately 60 minutes of observation had been completed. After 10 minutes of observation, focal bees in the study were collected into a falcon tube on dry ice then subsequently stored at -80°C.

2.3. Sample preparation and transcriptomic analysis

Bumble bee heads were separated from the rest of the body and brains were dissected over dry ice. Brain tissue was homogenized in 100 uL of TRizol reagent with metal beads at maximum frequency for two minutes using a TissueLyser II (QIAGEN). RNA was isolated from homogenized tissue using the RNeasy Mini Kit (QIAGEN) according to the manufacturer's protocol. RNA quality and quantity was validated using an Agilent 2100 Bioanalyzer or Advanced Analytics Fragment Analyzer at the UC Riverside Institute for Integrative Genome Biology Core Facility. Library preparation and sequencing was performed by Novogene for a total of 94 samples. For library preparation, mRNA was purified from total RNA and fragmented. First strand cDNA was synthesized using hexamer primer, followed by second strand cDNA synthesis using DNA polymers I and RnaseH. After terminal repair, adenylation, and ligation, cDNA fragments were purified, and PCR was performed. PCR products were purified and the quality of the resulting 370-420 bp libraries were assessed using the Agilent Bioanalyzer 2100 system. Libraries were sequenced on an Illumina Novaseq platform and 150 bp paired-end reads were generated.

Raw data were processed by removing reads that contained an adapter, poly-N, or any low-quality reads. At the same time, Q20, Q30, and GC content from the cleaned data were calculated. Reads were mapped to the *Bombus impatiens* reference genome (Sadd et al., 2015). Gene expression was quantified using featureCounts v1.5.0-p3, which counts the number of reads mapped to each gene and then calculates the Fragments per Kilobase of transcript per Millions base pairs sequenced (FPKM) of each gene.

2.4. Statistical analyses

Data were analyzed in Rstudio (version 4.1.1) using the DEseq2 package (Love et al., 2014). Genes with 10 or fewer reads across all samples were removed before differential expression was analyzed. DEseq2 provides a statistical pipeline for determining differentially expressed genes (DEGs) by running pairwise comparisons using Wald tests. DEseq2 performs differential expression analysis of two groups, and the resulting p-values are adjusted using the Benjamini-Hochberg's approach to control for false discovery rate (FDR). Genes with an adjusted p-value (q-value) < 0.05 are then assigned as differentially expressed for our primary analysis, but we also examine differential expression using a more relaxed, unadjusted p-value (< 0.05). This field study consists of three behavioral groups: nectar robbers (referred to as "Robbers" in dataset); legitimate foragers (referred to as "Legitimate"); and switchers. We also considered expression differences related to the site ($n = 4$) that a bee was collected from. Differential expression analyses were performed for each pairwise comparison between behavioral groups to determine the DEGs between each group. Additionally, differential expression

analyses were performed for each pairwise comparison between sites. Following DEG analysis, Gene Ontology (GO) enrichment analysis was performed using the topGO package (Colgan et al., 2019) in Rstudio. Following the procedure used in Costa et al. (2022), we explored gene function from GO annotations that were identified using the *Drosophila melanogaster* gene orthologs from Ensembl Metazoa Biomart (Kinsella et al., 2011). We used this analysis to investigate gene functions that were overrepresented in the behavioral DEG lists. GO analysis uses p-values from the DEG lists instead of q-values because of edge effects associated with q-value (Costa et al., 2022). Despite this relaxed stringency, GO analysis itself builds stringency in that there must be enough gene representation to find processes that are impacted.

3. Results

3.1. Behavior: Ten-minute observations are sufficient to characterize tactic constancy

A total of 88 *Bombus bifarius* workers were observed between 10-60 minutes across four different field sites in or surrounding the RMBL. Bees that switched between tactics usually switched within two to six minutes of observation (Figure 2.1). Longer observations of the switcher bees revealed that they continued to switch for extended periods of time (up to 50 minutes). Furthermore, longer observations revealed that 100% of bees that had exhibited tactic constancy within the first 10 minutes remained tactic constant throughout the 30-60 minutes of observation (Figure 2.1). Thus, 10-minute observations can be sufficient to determine whether a bee is exhibiting short-term tactic

constancy in this study system. The number of bees that exhibited tactic constancy to either “legitimate” (n= 35) or “nectar robbing” (n= 35) behavior were seen at equal levels, whereas “switching” between nectar robbing and legitimate foraging was seen less often during observation times (n= 18; Figure 2.2).

3.2. Differential gene expression

Of the 12,912 genes present in our transcriptome data set, which represents 98% of the genes in *B. impatiens* genome (Sadd et al., 2015), no significant differences in gene expression patterns were detected among the three groups based on q-values (q-value >0.05). When we relaxed our threshold for significance and used non-corrected p-values, we detected a total of 3,442 differentially expressed genes across the pairwise comparisons of the behavioral groups (p-value < 0.05). A total of 1307 genes were differentially expressed between nectar robbers and legitimate foragers; 1225 genes were differentially expressed between nectar robbers and bees that switched between legitimate foraging and nectar robbing; and 910 genes were differentially expressed between bees that exhibited legitimate foraging and bees that switched between legitimate foraging and nectar robbing (Figure 2.3). Furthermore, there were no significant differences in gene expression patterns associated with field site that foraging workers were collected from, when using the q-value. When we relaxed the threshold of significance for field sites, using non-corrected p-values, we found that there were differences in gene expression patterns associated with field site. Anywhere between 540-801 genes were differentially expressed between site comparisons.

Using the non-corrected p-values from the DEG list, the Gene Ontology enrichment analysis identified a total of 14 statistically significant GO terms using q-values (q-value < 0.05) across three GO category types (biological processes, cellular components, and molecular functions). Specifically, between nectar robbers and legitimate foragers, we found a total of four significant GO terms (q-value < 0.05). Examination of significantly enriched GO biological process terms (n = 3) revealed enrichment of the organic substance metabolic process, cell development, and cellular response to DNA damage. Between nectar robbers and bees that switched between tactics, we found a total of 10 significant GO terms (q-value < 0.05). The GO biological process terms (n = 2) that were enriched between nectar robbers and bees that switched tactics were translation and mitochondrial gene expression. There were no GO terms enriched in the differentially expressed gene list for legitimate foragers versus switchers. All significant GO terms using q-value across all GO categories can be found in Table 2.1.

When we relaxed our threshold for significance using the non-corrected p-values for GO terms, we identified 48 statistically significant terms (p-value < 0.05) across the three GO categories. Between nectar robbers and legitimate foragers, we found 17 significant GO terms (p-value < 0.05). Examining enriched GO biological process terms (n = 11) revealed significant terms associated with protein metabolism, particularly protein breakdown, and muscle cell differentiation, as well as organic substance metabolic process, cell development, and cellular response to DNA damage. Between nectar

robbers and bees that switched between tactics, we found a total of 26 significant GO terms ($p\text{-value} < 0.05$). The GO biological process terms ($n = 14$) that were the most significant were translation and mitochondrial gene expression, followed by terms associated with protein metabolism, cell signaling, and carbohydrate metabolism. Interestingly, when examining the GO cellular component terms for nectar robbers and switcher foragers, the terms that were most significant using both p -value and q -value were associated with mitochondrial gene expression and structure. Between legitimate foragers and bees that switched tactics, we found a total of five significant GO terms ($p\text{-value} < 0.05$). The GO biological process terms that were enriched ($n = 4$) were mitochondrial gene expression, translation, and ion transport (Table 2.2). All significant GO terms using p -value for the other pairwise comparisons can be found in Appendix 2, Table A2.1.

4. Discussion

This is the first study to explore the molecular basis for nectar robbing behavior in bumble bees. Here, we tested the hypothesis that differential gene expression patterns within the brain would be associated with behavioral differences between nectar robbing behavior when compared to legitimate foraging. We specifically predicted that nectar robbing behavior would be associated with differential expression of genes related to energy metabolism, predicated on the hypothesis that nectar robbing is a strategy used to conserve energy. To test our hypothesis, we observed and collected wild *Bombus bifarius* foraging workers in a natural field setting that were either nectar robbing, legitimately

foraging, or were observed switching between these two behaviors. We then used RNA sequencing to measure gene expression levels in the brains of the foraging workers.

When using the adjusted p-value (q-value), which is the standard in transcriptomic studies, we did not detect any differences in gene expression patterns among the three behavioral groups that were examined. This suggests that there are not large scale, robust patterns of gene regulation in the brain that mediate these behavioral differences.

Although we observed bees foraging for only 10-60 minutes to validate their behavioral tactic, previous studies have shown that these states are more persistent through time, on the order of days (Lichtenberg et al., 2020a; Lichtenberg et al., 2020b), which aligns with the definition of a behavioral state (Altmann, 1974). However, despite the endurance of behavioral states, bees can quickly shift into a new state in response to environmental cues (reviewed in Zayed and Robinson, 2012). Based on this idea, foraging tactic behavioral states could be driven by external cues only or nutritional feedback, rather than large-scale, stable differences in brain gene regulation.

Additionally, there are technical or methodological explanations for why our results ultimately do not support our hypothesis. We know through studies across social insects that there is a close relationship between brain gene expression and behavioral states (reviewed in Zayed and Robinson, 2012). However, behavioral states may also be influenced or mediated by other processes, such as posttranslational modifications, neuronal development, neurophysiology, and neurochemistry (reviewed in Zayed and

Robinson, 2012), which are not necessarily reflected by gene expression patterns. Moreover, the behavioral states examined in our study might be associated with low levels of gene expression, which are harder to detect (Łabaj et al., 2011). Further, our analysis was performed at the level of the whole brain, which has been used in previous studies to detect differential patterns of gene expression patterns in bumble bees (Costa et al. 2020; Costa et al., 2022). However, in the context of nectar robbing behavior, there may be differences in gene expression that are unique to specific brain regions or specific cell types (Burns et al., 2012; Thamm and Scheiner, 2013), which we could not detect. Additionally, this study focused on wild bees in their natural environment. The drivers of gene expression in the surrounding environment could have had a stronger effect than the behaviors themselves, and thus, overshadowed associations between gene expression and foraging behavior. However, we did not find significant differences in gene expression patterns associated with site when using q-values, which suggests that the immediate surrounding environment in each site did not elicit any large-scale differences in gene expression patterns. Although, when we relaxed our threshold for significance, we saw that there were site-level effects on gene expression patterns, which could be explored in a more controlled study. Additional factors that have a strong effect on bumble bee gene expression are natal colony (Costa et al., 2020), and age, which we could not account for in this study. While we chose sites that were far enough from each other to ensure that bees across sites would not be from the same colony, there was no way to know if bees originated from the same colony within a given site, or how old they were.

Given that we did find some differences in gene expression associated with behavioral states when we relaxed our threshold for significance, we may have detected some patterns that could be better characterized with greater and more controlled sampling. The processes that were most strongly impacted according to the GO analysis were associated with translation, as well as mitochondrial gene expression and structure, specifically in the contrast between nectar robbers and switchers. Mitochondrial gene expression is involved in maintaining mitochondrial function and producing protein subunits that act as enzymes involved in oxidative phosphorylation (Kotrys and Szczesny, 2019), the process where large amounts of ATP are synthesized. The structures of the mitochondria that were impacted included the mitochondrial matrix and the mitochondrial protein containing complex, both of which are associated with the citric acid cycle and the electron transport chain, respectively. These results suggest that there may be a difference in ATP production, and thus energy metabolism, between nectar robbers and switchers. Interestingly, we did not see these difference between nectar robbers and legitimate foragers, which does not corroborate our original hypothesis. This might suggest that the energetic differences are instead between tactic constancy and switching behavior. In contrast to tactic constancy, switching between behaviors has been hypothesized to result in temporal, cognitive, or energetic costs (Chittka et al., 1999; Goulson, 1999). This is similar to costs associated with switching between types of floral resources in comparison to exhibiting floral constancy (Chittka et al., 1999; Goulson, 1999). Based on this hypothesis, switching between legitimate foraging and nectar robbing may actually be more energetically costly when compared to tactic constancy.

Our observational data validated that there are distinct foraging behavioral states exhibited by *B.bifarius* foraging workers. We found that switching between two foraging tactics occurs within the first ten minutes of observations, and that tactic constant bees will use one tactic consistently for at least 60 minutes during a single foraging bout. This aligns with evidence from previous studies that measured the duration of tactic constancy within foraging bouts and among separate foraging bouts (Lichtenberg et al., 2020b). All of this evidence taken together suggests that these foraging behaviors are in fact behavioral states based on the definition described previously by Altmann (1974). Furthermore, our observational results align with previous studies, finding a similar proportion of bees performing each behavior of interest (Bronstein et al. 2017; Lichtenberg et al., 2020a; Lichtenberg et al., 2020b).

5. Conclusion

Ultimately, in this study we found that there are no large-scale patterns of gene regulation in the brain that are associated with behavioral differences among nectar robbers, legitimate foragers, and foragers that switched between these two tactics in this system. However, there were some limitations that may have contributed to these findings. This study was conducted in the field with little control over many factors that can contribute to gene expression. We suggest that a more controlled study should be conducted to explore some of the patterns that we detected when we relaxed the threshold for significance using non-adjusted p-values for differentially expressed. Future directions can also include examining nutritional states to determine if individual nutrient levels,

which more closely reflect energetic status, are associated with foraging decisions and behavioral states. Additionally, future directions may include examining neural mechanisms that are associated with these behavioral states that might not be reflected at the level of gene expression. Lastly, through our GO enrichment analysis we found that gene expression differences may be associated with tactic constancy and switching behavior, rather than the specific behaviors themselves, which would align more closely with hypotheses associated with floral constancy.

Tables and Figures

Figure 2.1.

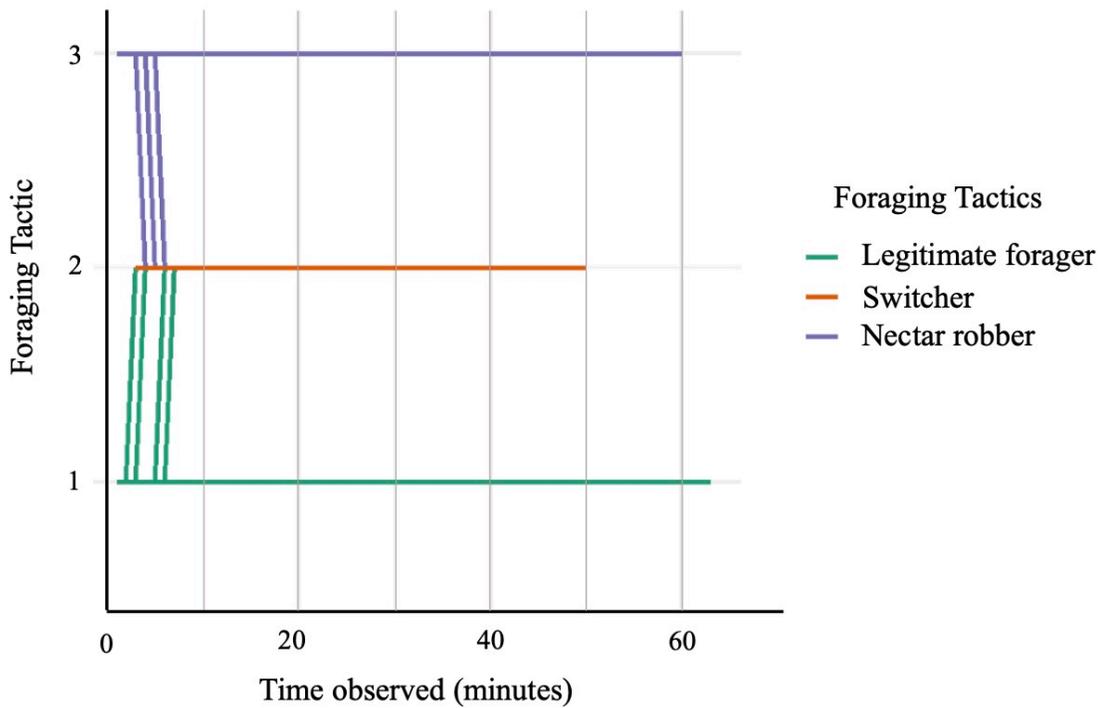


Figure 2.1. Spaghetti plot showing observation times for each tactic or switching behavior ranging from 10-60 minutes. The x-axis shows the time observed in minutes and the y-axis shows the behaviors of interest. (1) Represents legitimate foragers ($n = 35$). (2) Represents foraging workers that switched between tactics ($n = 18$); these foragers either started as legitimate foragers or nectar robbers, and the time that they switched to the other tactic was noted. (3) Represents nectar robbers ($n = 35$). Switchers would change tactics within the first two-six minutes of observation times, represented by the vertical lines meeting at the orange horizontal line in the middle. Tactic constant bees (either legitimate foragers or nectar robbers) consistently performed the same tactic for up to 60 minutes.

Figure 2.2.

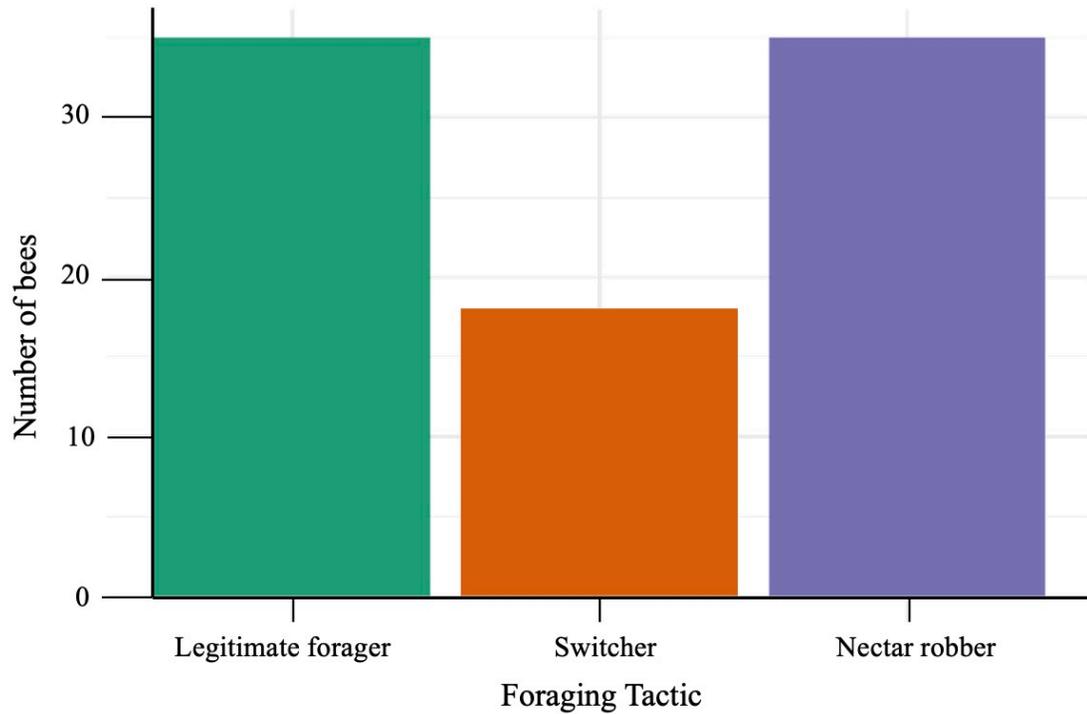


Figure 2.2. Bar plot showing proportion of foraging workers per foraging tactic or behavior. A total of 88 foraging workers were observed. Equal proportions of legitimate foragers ($n = 35$) and nectar robbers ($n = 35$) were observed during observations. Switching between both tactics (i.e., “Switching”, $n = 18$) was seen less often during observations.

Figure 2.3.

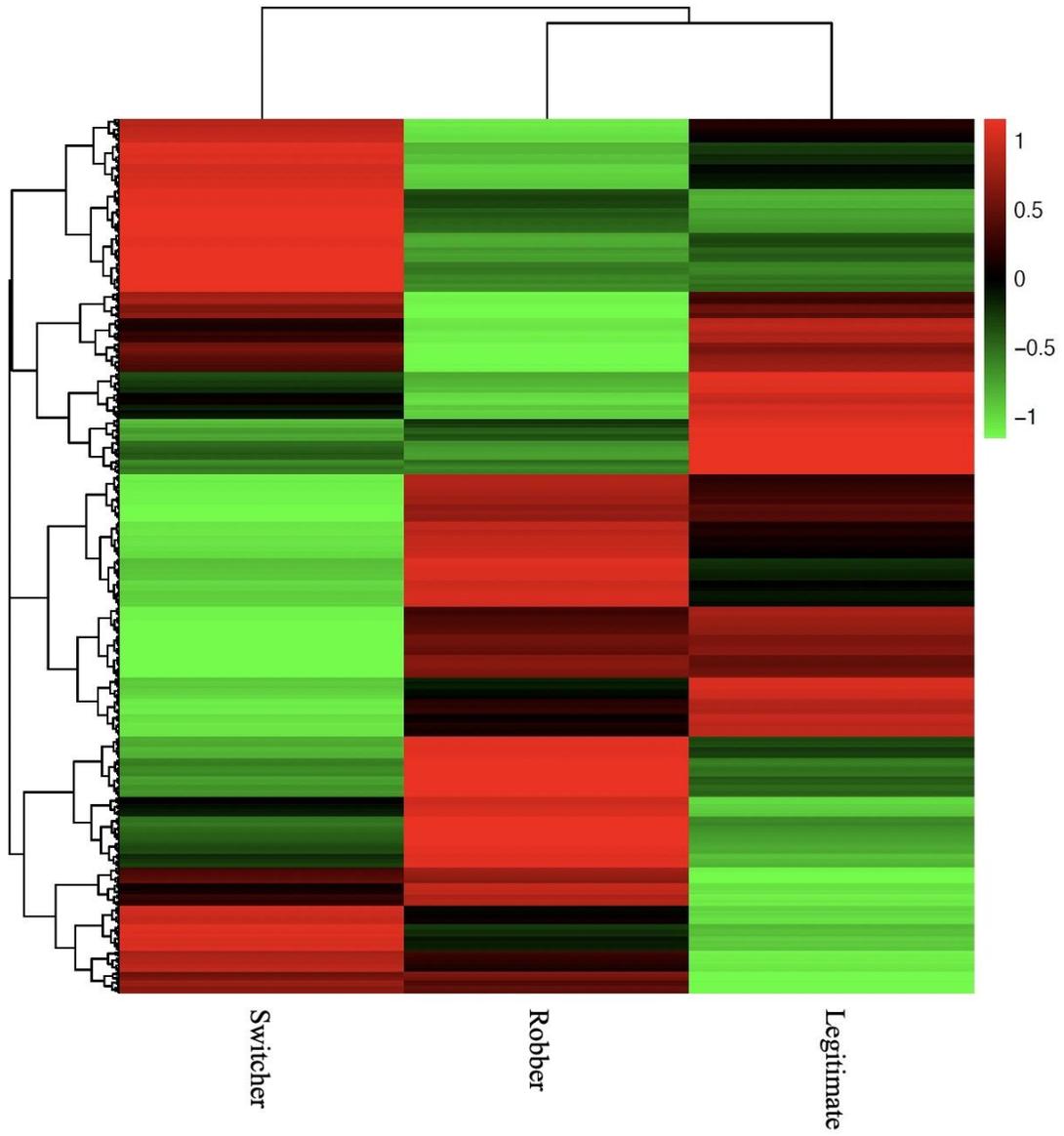


Figure 2.3. Heatmap showing relative levels of differentially expressed genes between the foraging behavioral states when using the non-adjusted p-values (p -value < 0.05).

Table 2.1. Gene ontology enrichment analysis results, showing the significant terms using GO q-values for each behavioral pairwise comparison. The comparison between legitimate foragers and switchers yielded no significant terms using q-value. A total of 14 terms were identified using q-value. GO categories: BP = Biological processes; CC = Cellular Component; MF = Molecular Function.

Significant GO terms by q-value				
Nectar robber vs. Legitimate forager	GO term	GO ID	q-value	GO category
Nectar robber vs. Legitimate forager	Cellular response to DNA damage stimulus	GO:0006974	0.04333	BP
	Organic substance metabolic process	GO:0071704	0.05158	BP
	Cell development	GO:0048468	0.04333	BP
	Supramolecular fiber	GO:0099512	0.04386	CC
Nectar robber vs Switcher	Translation	GO:0006412	5.26E-07	BP
	Mitochondrial gene expression	GO:0140053	3.03E-06	BP
	Mitochondrial protein-containing complex	GO:0098798	3.77E-08	CC
	Mitochondrial matrix	GO:0005759	2.75E-06	CC
	Ribosomal subunit	GO:0044391	4.76E-06	CC
	Catalytic complex	GO:1902494	0.046628	CC
	Intracellular protein-containing complex	GO:0140535	0.046628	CC
	Ribonucleoprotein complex	GO:1990904	0.046628	CC
	Nucleolus	GO:0005730	0.02805	CC
	Structural constituent of ribosome	GO:0003735	4.47E-06	MF

Table 2.2. Gene Ontology enrichment analysis results showing significant terms using p-value for comparison between Legitimate Foragers and Switchers only. Significant terms by p-value for the other behavioral pairwise comparisons can be found in Appendix 2, Table A1. GO categories: BP = Biological processes; CC = Cellular Component; MF = Molecular Function.

Significant GO terms by p-value				
Legitimate forager vs Switcher	GO term	GO ID	p-value	GO category
	Mitochondrial gene expression	GO:0140053	0.0379	BP
	Translation	GO:0006412	0.0143	BP
	Regulation of translation	GO:0006417	0.052	BP
	Ion transport	GO:0006811	0.0291	BP
	Golgi membrane	GO:0000139	0.0511	CC

References

- Altmann, J. Observational Study of Behavior: Sampling Methods. *Behaviour*, **49**(3), pp. 227–266. doi:10.1163/156853974x00534. (1974).
- Arnold, R. M. Pollination, Predation and Seed Set in *Linaria vulgaris* (Scrophulariaceae). *American Midland Naturalist*. **107**: pp. 360–369. (1982).
- Bebane, P., Hunt, B., Pegoraro, M., Jones, A., Marshall, H., Rosato, E., Mallon, E. The effects of the neonicotinoid imidacloprid on gene expression and DNA methylation in the buff-tailed bumblebee *Bombus terrestris*. *ProcB*, **286**; 0190718. (2019).
- Bronstein, J. L., Barker, J. L., Lichtenberg, E. M., Richardson, L. L. & Irwin, R. E. The behavioral ecology of nectar robbing: why be tactic constant? *Current Opinion in Insect Science* **21**: pp. 14–18. (2017).
- Burns, J.G., Svetec, N., Rowe, L., Mery, F., Dolan, M.J., Boyce, W.T., Sokolowski, M.B. Gene–environment interplay in *Drosophila melanogaster*: Chronic food deprivation in early life affects adult exploratory and fitness traits. *PNAS*, **109**: pp. 17239-17244. (2012).
- Chittka, L., Thomson, J. D. & Waser, N. M. Flower Constancy, Insect Psychology, and Plant Evolution. *Naturwissenschaften* **86**: pp. 361-377. (1999).
- Colgan, T.J., Fletcher, I.K., Arce, A.N., Gill, R.J., Ramos Rodrigues, A., Stolle, E., Chittka, L., Wurm, Y. Caste-and pesticide-specific effects of neonicotinoid pesticide exposure on gene expression in bumblebees. *Molecular Ecology* **28** (8): pp. 1964-1974. (2019).
- Costa, C., Duennes, M., Fisher, K., Der, J., Watrous, K., Okamoto, N., Yamanaka, N., Woodard, S.H. Transcriptome analysis reveals nutrition- and age-related patterns of gene expression in the fat body of pre-overwintering bumble bee queens. *Molecular Ecology*, **29**; pp. 720-737. (2020).
- Costa, C.P., Leza, M., Duennes, M.A., Fisher, K., Vollaro, A., Hur, M., Kirkwood, J.S., Woodard, S.H. Pollen diet mediates how pesticide exposure impacts brain gene expression in nest-founding bumble bee queens. *Science of the Total Environment* **833**: 155216. (2022).
- De Belle, J., Hilliker, A., Sokolowski, M. Genetic localization of foraging (for): a major gene for larval behavior in *Drosophila melanogaster*. *Genetics*, **123**: pp. 157-163. (1989).

- Doublet, V., Paxton, R., McDonnell, C., Dubois, E., Nidelet, S., Moritz, R., Alaux, C., Le Conte, Y. Brain transcriptomes of honey bees (*Apis mellifera*) experimentally infected by two pathogens: Black queen cell virus and *Nosema ceranae*. *Genomics data*, **10**; pp. 79-82. (2016).
- Fisher, K., Duennes, M.A., Woodard, S.H. Mountains as refugia: evidence from bumble bee brain transcriptomes. *bioRxiv*. (2022).
- Goulson, D. Foraging strategies of insects for gathering nectar and pollen, and implications for plant ecology and evolution. *Perspectives in Plant Ecology, Evolution, and Systematics* **2**: pp. 185-209. (1999).
- Greenleaf, S. S., Williams, N. M., Winfree, R. & Kremen, C. Bee foraging ranges and their relationship to body size. *Oecologia* **153**: pp. 589–596 (2007).
- Heylen, K., Gobin, B., Billen, J., Hu, T., Arckens, L., Huybrechts, R. Amfor expression in the honeybee brain: A trigger mechanism for nurse–forager transition. *Journal of Insect Physiology*, **54**: pp 1400-1403. (2008).
- Irwin, R. E. & Maloof, J. E. Variation in nectar robbing over time, space, and species. *Oecologia* **133**: pp. 525–533 (2002).
- Irwin, R.E., Bronstein, J.L., Manson, J.S., Richardson, L. Nectar Robbing: Ecological and Evolutionary Perspectives. *The Annual Review of Ecology, Evolution and Systematics*, **41**: pp. 271-292. (2010).
- Kinsella, R.J., Kähäri, A., Haider, S., Zamora, J., Proctor, G., Spudich, G., Almeida-King, J., Staines, D., Derwent, P., Kerhornou, A., Kersey, P., Flicek, P. Ensembl BioMarts: a hub for data retrieval across taxonomic space. Database (Oxford). (2011).
- Knight, M. E., Martin, A., Bishop, S., Osborne, J., Hale, R.J., Sanderson, R.A., Goulson, D. An interspecific comparison of foraging range and nest density of four bumblebee (*Bombus*) species. *Molecular Ecology* **14**: pp. 1811–1820 (2005).
- Kotrys, A.V. and Szczesny, R.J. Mitochondrial Gene Expression and Beyond—Novel Aspects of Cellular Physiology. *Cells*, **9**; <https://doi.org/10.3390/cells9010017>. (2019).
- Łabaj, P.P., Leparc, G.G., Linggi, B.E., Markillie, L.M., Wiley, H.S., Kreil, D.P. Characterization and improvement of RNA-Seq precision in quantitative transcript expression profiling. *Bioinformatics*, **27**: pp. I383–i391. (2011).

- Lichtenberg, E., Irwin, R.E., Bronstein, J.L. Costs and benefits of alternative food handling tactics help explain facultative exploitation of pollination mutualisms. *Ecology*, **99**: pp. 1815-1824. (2018)
- Lichtenberg, E., Richman, S., Irwin, R., Bronstein, J. Competition for nectar resources does not affect bee foraging tactic constancy. *Ecological Entomology* **45**: pp. 904-909. (2020a).
- Lichtenberg, E., Irwin, R., Bronstein, J. Bumble bees are constant to nectar-robbing behavior despite low switching costs. *Animal Behavior* **170**: pp. 177-188. (2020b).
- Liu, F., Shi, T., Qi, L., Su, X., Wang, D., Dong, J., Huang Z.Y. lncRNA profile of *Apis mellifera* and its possible role in behavioural transition from nurses to foragers. *BMC Genomics*, **20**: 393. (2019).
- Love, M.I., Huber, W., Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology* **15** (12): 550. (2014).
- Mayfield, M. M., Waser, N. M. & Price, M. V. Exploring the ‘most effective pollinator principle’ with complex flowers: Bumblebees and *Ipomopsis aggregata*. *Annals of Botany*. **88**: pp. 591–596 (2001).
- Newman, D. A. & Thomson, J. D. Effects of nectar robbing on nectar dynamics and bumblebee foraging strategies in *Linaria vulgaris* (*Scrophulariaceae*). *Oikos* **110**: pp. 309-320. (2005).
- Osborne, J. L. *et al.* A landscape-scale study of bumble bee foraging range and constancy, using harmonic radar. *Journal of Applied Ecology* **36**: pp. 519–533 (1999).
- Osborne, J. L. *et al.* Bumblebee flight distances in relation to the forage landscape. *Journal of Animal Ecology* **77**: pp. 406–415 (2008).
- Pleasants, J. M. Bumblebee Response to Variation in Nectar Availability. *Ecology* **62**: pp. 1648-1661. (1981).
- Pyke, G. H. Local geographic distributions of bumblebees, near Crested Butte, Colorado: competition and community structure. *Ecology* **63**: pp. 555–573 (1982).
- Pyke, G. H., Inouye, D. W. & Thomson, J. D. Local Geographic Distributions of Bumble Bees Near Crested Butte, Colorado: Competition and Community Structure Revisited. *Environmental Entomology* **41**: pp. 1332–1349. (2012).

Richman, S.K., Barker, J.L., Baek., M. Papaj, D.R., Irwin, R.E., Bronstein, J.L. The Sensory and Cognitive Ecology of Nectar Robbing. *Ecology and Evolution*, **9**:698137. (2021).

Sadd, B.M., et al. The genomes of two key bumblebee species with primitive eusocial organization. *Genome Biology*, **16**, DOI 10.1186/s13059-015-0623-3. (2015).

Thamm, M., Scheiner, R. PKG in honey bees: Spatial expression, Amfor gene expression, sucrose responsiveness, and division of labor. *Journal of Comparative Neurology*, **522**: pp. 1786-1799. (2014).

Zayed, A., Robinson, G. Understanding the Relationship Between Brain Gene Expression and Social Behavior: Lessons from the Honey Bee. *The Annual Review of Genetics*, **46**: pp. 591-615.

Chapter 3

Care-giver identity enforces physiological mechanisms associated with offspring survival resistance.

1. Introduction

Across the Animal Kingdom, developmental history and early-life social experiences can have lasting effects on offspring physiology, behavior, and overall fitness (Møller, 1997; Lindström, 1999; Hensch, 2004; Uller, 2008). Variable or unique processes and experiences, such as parental care (Clutton-Brock, 1991; Hunt and Simmons, 1999; Uller, 2008) and nutritional status (Hunt and Simmons, 1999) during development, can result in phenotypic variation in offspring. Phenotypic variation as a result of developmental history can improve, or in some cases hinder, offspring quality and fitness (Nettle and Bateson, 2015). For example, across a wide array of taxa, parental effects and nutrition during development can result in offspring phenotypes that promote survival and resistance to nutritional stress as adults (Clutton-Brock, 1991; Cadby et al., 2011; Hibshman et al., 2016; Wang et al., 2016a; Wang et al., 2016b). Studying the ways in which developmental history impacts adult offspring is important to understand variation in offspring phenotypes and can provide insight into the mechanisms by which those variations occur (Nettle and Bateson, 2015).

In social insects, the processes and social interactions experienced by developing brood in the nest have major consequences for offspring and nest development. Processes

occurring during developmental history play an important role in caste differentiation (Ribeiro et al., 1999; Leimar et al., 2012; Jandt et al., 2017), offspring body size and shape (Plowright & Jay, 1977; Shpigler et al., 2013; Wang et al., 2014), and behavioral performance (Rittschof et al., 2015; Wang et al., 2016). Many of these influences on development are shaped by the behaviors of adults in the nest who feed larvae via progressive provisioning, a behavior where adults in the nest continuously feed larvae until they reach the pupal stage (Michener, 1969). This close interaction between adults and developing brood creates opportunities for control or influence over development. Nutritional manipulation of brood diet, wherein adult care-givers control the amount and quality of food administered, is associated with differences in offspring development and phenotypes, including size, caste, and other traits (Plowright & Jay, 1977; Pendrel & Plowright, 1981; Ribeiro et al., 1999; Hunt et al., 2007; Shpigler et al., 2013; Jandt et al., 2017). Further, care-giver identity, such as parents or siblings, may influence offspring in various ways through nutritional manipulation. For example, carpenter bee mothers manipulate the quality and quantity of dietary provisions to produce dwarf eldest daughters that remain in the nest and help raise their siblings (Lawson et al., 2016). Further, maternal and sibling-care can differ in the number and rates of feeding events (Hunt and Amdam, 2005; Jandt et al., 2017), as well as possible differences in dietary additives, such as endogenously produced proteins (LeBoeuf et al., 2016).

Bumble bees (genus *Bombus*, family Apidae) are a group of eusocial bees in which nest-founding queens care for their brood only at the onset of colony development. After the

first brood emerge as adults, brood care is taken over by adult workers. Thus, bumble bee nests undergo a developmental transition from maternal to sibling care (Shpigler et al., 2013; Woodard et al., 2013). As a result of this transition in care-giver identity, there are differences between queen-reared and worker-reared offspring, in terms of body size and survival under starvation conditions (Shpigler et al., 2013; Costa et al., 2021; Barie and Amsalem, 2022). For example, queen-reared offspring are more resistant to starvation than worker-reared offspring (Costa et al., 2021). Further, care-giver identity influences body size, in that queen-reared offspring are smaller than worker-reared offspring (Costa et al., 2021). Given that nutrition is a key driver of body size in insects (Bakker, 1959; Davidowitz et al., 2003; Boggs and Freeman, 2005), this suggests that queen bumble bees may nutritionally manipulate larval diets in ways that simultaneously result in smaller body sizes and enforce metabolic mechanisms that contribute to starvation resilience. However, the mechanisms underlying these differences in bumble bee adult offspring have not been examined. Of particular interest are the mechanisms underlying differences in starvation resilience, as bumble bees in the wild face widespread loss of foraging habitat or insufficient nutrient resources. It is likely that these mechanisms are metabolic or associated with nutritional status. In honey bees, starvation resilience can be attributed to the ability to better maintain hemolymph sugar levels as well as the ability to switch between fuels more readily during starvation as adults (Wang et al., 2016a; Wang et al., 2016b). Yet no studies to date have explored these mechanisms in bumble bees that are reared by the queen.

In this study, we explored how care-giver identity impacts adult offspring nutritional status and survival under starvation conditions. We hypothesized that the differences in bumble bee adult offspring found in previous studies (Costa et al., 2021) can be attributed to differences in nutrient metabolism that are enforced by care-giver identity during development. We performed an experiment wherein we manipulated young bumble bee nests so that the brood were reared either by the queen alone, or by a cohort of five workers. We then examined how various types and durations of starvation impacted offspring hemolymph sugar levels as adults. Measuring hemolymph sugar levels allows us to examine the maintenance of sugar levels as well as shifts in the metabolism of particular nutrient classes. We predicted that resistance to starvation in queen-reared offspring, as seen in Costa et al. (2021), can be attributed to their ability to better maintain consistent hemolymph sugar levels than worker-reared offspring, similar to the mechanisms involved in honey bee starvation resistance (Wang et al., 2016a).

2. Methods

2.1. Bumble bee colony rearing and queen mating

10 bumble bee colonies from Koppert Biological Systems were used in this experiment. Colonies were maintained at 21°C and uncontrolled humidity (but > 40% RH) and were fed mixed-source, honey bee collected pollen (Brushy Mountain Bee Farm). Artificial nectar was supplied by Koppert Biological Systems. Callow queens were pulled from their natal colonies and placed in small cages with pollen and artificial nectar (Boyle et al., 2018). On day three, queens were placed in mating cages with males from different source colonies than the queens originated from. Mating cages were observed every 30

minutes from 9:00 AM- 5:00 PM to verify mating status. Bees in mating cages were provisioned with pollen and strange nectar, an artificial nectar solution that contains citric acid to prevent mold growth. Queens that mated (i.e., were observed copulating) were removed from the mating cage and placed temporarily in individual cages (W7.5 x D7.5 x H4.5 cm), which were kept in an incubator maintained at 25°C and 75% relative humidity (RH). If a queen did not mate by age 8-10 days, she was excluded from the experiment.

2.2. Nest initiation and creation of treatment groups

At ages 12 and 13 days, mated queens (n = 227) were gassed with carbon dioxide for two consecutive days for 30 minutes/day (Roseler, 1985). After gassing, queens were transferred to a nest box (approximately W15 x D15 x H10 cm) and placed in a room in the Insectary and Quarantine Facility at UC Riverside, maintained at 26°C and 75% RH under dim red lights. Mated queens were fed the same pollen and strange nectar described above. Nest boxes were checked twice daily (morning and afternoon) to determine the exact day that eggs were laid. Queens that did not lay eggs within 30 days were excluded (n = 55). Queens were either left in their cage with the eggs for our queen-reared nests (n = 98) or were removed from the cage 3-5 days after eggs were first observed. When queens were removed, five callow workers were added in her place to create our worker-reared nests (n = 74). All the workers added to a given nest came from the same source colony, which was different from the source colony that the queen originated from.

2.3. Offspring collection and starvation treatment groups

Nests were then allowed to develop until offspring emergence. Female offspring were removed from nests within 24 hours after eclosion and placed individually in cages (W7.5 x D7.5 x H4.5 cm) with pollen and a 60% sucrose solution, which was replaced every other day. Cages were kept in an incubator maintained at 25°C and 70% humidity. Any male offspring were also taken out of the nest within 24 hours but were not used in the experiment. Hereafter, any offspring mentioned in the methods and results refers to female offspring only. Offspring were assigned to one of four starvation treatment groups, either at two days old or seven days old (Figure 3.1). These age groups were chosen based on a previous study with honey bees, showing that there are differences in nutritional shifts between newly-emerged versus older offspring (Wang et al., 2016a; Wang et al., 2016b). The starvation treatment groups were as follows: *control*, *pollen starved*, *nectar starved*, and *starved*. The control group was given 60% sucrose solution and pollen mixed with sucrose solution *ad libitum*. The *pollen starved* group was only given a 60% sucrose solution. The *nectar starved* group was given only pollen that had been mixed with water, and no sucrose solution. The *starved* group was not given any pollen or sucrose solution. Offspring were placed back in the incubator and maintained on their respective starvation treatment for either six hours or twelve hours. After this, their hemolymph was collected immediately (methods described below). Total sample sizes collected for each group can be found in Table 3.1, and total number of hemolymph samples used for nutritional analyses can be found in Table 3.2.

2.4. Survival treatment groups

To examine the effects of care-giver identity on offspring starvation resistance we conducted survival assays, wherein a subset of offspring was placed on the previously mentioned starvation treatments and their mortality was measured. In our previous study (Costa et al., 2021), queen-reared offspring survived longer under starvation than worker-reared offspring. However, we wanted to test how queen-reared and worker-reared offspring survival differs under different types of starvation, such as nectar-starvation and pollen-starvation, rather than just looking at total starvation alone. The protocol for survival treatments was the same as was described above for the starvation assay, with the exception that, here, offspring were starved until mortality, rather than for six hours or twelve hours. Offspring were placed on their respective starvation treatments at either two days old or seven days old. Bees were inspected every twelve hours after they started treatment to note any mortality. Any nectar or pollen present was changed out every two days to prevent mold growth.

2.5. Hemolymph collection

Bees were anesthetized by placing them in a freezer at -20°C for about 2-4 minutes and mounted in a Stender dish. An incision was made dorsally between the second and third abdominal segments. At least 2 μL of hemolymph was collected using a micropipette. The hemolymph was immediately placed on dry ice and stored at -80°C . The bodies of the bees were also stored at -80°C for body size measurements (methods below; following protocol in Costa et al., 2021).

2.6. Glucose and trehalose assays

Glucose and trehalose are the primary sugars in bee hemolymph (Wyatt and Kalf, 1957; Wyatt, 1967) and are required in metabolic processes that generate adenosine triphosphate (ATP), the standard unit of energy that maintains energy balance. Glucose is a simple sugar and trehalose is a disaccharide that consists of two glucose molecules bonded together. To measure hemolymph glucose concentrations, we performed glucose assays using the Glucose (HK) Assay Kit from Sigma Aldrich (GAHK-20). The kit contains a glucose standard and an enzyme mix that reacts with the glucose in the samples leading to a series of chemical reactions resulting in an equimolar amount of NAD⁺ being reduced to NADH. This reduction was detected spectrophotometrically as an increase in absorbance at 340 nm, which is directly proportional to the glucose concentration in the samples. Absorbance was read using a Varioskan Lux microplate reader. Following glucose assays, the enzyme trehalase from porcine kidney (Sigma Aldrich, T-8778) was added to each of the microplate wells to break down trehalose into glucose. Microplates were covered and incubated at 37°C overnight for 18-20 hours, and the absorbance was read at 340 nm. The first glucose reading was subtracted from the second reading to calculate the concentration of trehalose in the samples. All assays followed the protocol in Hartfelder et al. (2013).

2.7. Body size measurements

For all offspring, we measured the length of the marginal cell, which is a proxy for body size (Knee and Medler, 1965). All bees used for hemolymph sugar quantification were

included in body size measurements, along with bees that were not used for hemolymph sugar quantification. We collected a total of 456 queen-reared offspring (from 99 nests) and 370 worker-reared offspring (from 74 nests) for body size measurements.

2.8. Statistical analyses

Statistical analyses were performed using Rstudio (version 4.1.1). We used generalized linear mixed models (GLMMs) for all tests. GLMMs were performed using the lme4 package (Bates et al., 2014). The best-fit model for each GLMM was determined based on Akaike's Information Criterion (AIC), and post-hoc t-tests were conducted using Tukey's multiple comparisons of means. All models included queen source colony and individual nest as random factors. First, we used a GLMM to examine the effects of rearing history on body size. Then we used a GLMM to examine how care-giver identity, body size, age, and starvation treatment contributed to hemolymph sugar levels and survival. We used a GLMM that included all starvation treatment groups, ages, and durations of starvation to examine the effects on glucose and trehalose titers. The fixed effects included care-giver identity, body size, age, starvation treatment, duration of starvation, and their interactions. Then, we performed GLMMs within each starvation treatment group for each age group (either 2 days or 7 days) and each starvation duration (6 hours and 12 hours). The fixed effects included care-giver identity, body size, and the interaction between the two. For starvation resistance, also referred to as our survival assay, we ran a GLMM within each survival treatment for both age groups with care-giver identity and body size as fixed effects.

3. Results

3.1. Body sizes of queen-reared and worker-reared offspring

The best fit model associated with body size included care-giver identity as a fixed effect (p-value < 2e-16). We found that, on average, queen-reared offspring were significantly smaller than worker-reared offspring (mean \pm s.d. marginal cell length of 2.22 ± 0.27 mm versus 2.66 ± 0.28 mm, respectively; Figure 3.2).

3.2. Survival under starvation conditions

For both *nectar-starved* and *starved* groups, both care-giver identity and body size were included in the best fit model and thus, contributed to significant differences in survival under starvation conditions (Tukey's post-hoc for care-giver identity within each starvation treatment: *nectar-starved*, two-day old p-value = 4.85e-07; *nectar-starved*, seven-day old p-value = 0.00132; *starved*, two-day old p-value = 0.00468), with the exception of the *starved* seven-day old offspring. Queen-reared offspring survived significantly longer than worker-reared offspring for both age groups, except for *starved* offspring at seven days old (Figure 3.3). The seven-day old offspring in the *starved* treatment group did not show a significant difference between queen-reared or worker-reared offspring. For the *pollen-starved* two-day old group, the best fit model included the interaction between care-giver identity and body size. The best fit model for the *pollen-starved* seven-day old group only included body size. However, both queen-reared and worker-reared offspring in both age groups survived for long periods of time under pollen starvation, up to a month or longer.

3.3. Glucose levels in queen-reared and worker-reared offspring

Glucose levels were only significantly impacted by care-giver identity in offspring that were two days old and nectar-starved for six hours (*nectar-starved*, for six hours at two days old p-value = 0.029). Specifically, queen-reared offspring in this treatment group had significantly higher levels of glucose compared to the worker-reared offspring (mean \pm s.d. glucose concentration of 6.41 ± 3.75 ug/mL versus 1.16 ± 1.23 ug/mL, respectively; Figure 3.4). Care-giver identity was not included in the best fit models for glucose levels in bees across the other starvation treatment groups, starvation durations, or age groups. Glucose levels significantly differed between the starvation treatment groups irrespective of care-giver identity (p-values for each starvation treatment comparison < 0.05). However, glucose levels in pollen-starved bees did not significantly differ from the control bees in both age groups and starvation durations (p-value = 0.997).

3.4. Trehalose levels in queen-reared and worker-reared offspring

Trehalose levels were not significantly impacted by care-giver identity across all groups (p-value > 0.05). The best-fit models included body size, but not care-giver identity. Trehalose levels remained relatively stable across all starvation treatments within each age group and starvation duration (Figure 3.5).

4. Discussion

We hypothesized that differences in bumble bee adult offspring starvation resistance are related to differences in nutritional status that are shaped by care-giver identity during

early development. Based on this, we predicted that queen-reared offspring would maintain more consistent hemolymph sugar levels than worker-reared offspring under starvation conditions. We tested this hypothesis by manipulating bumble bee brood to be reared by the queen alone or by a cohort of workers, then introduced the adult offspring to different types and durations of starvation. We found that care-giver identity impacted young offspring hemolymph glucose levels under starvation conditions specifically under a short time period (six hours). Longer durations of starvation (twelve hours) deplete hemolymph sugar levels equally, irrespective of care-giver identity during development. We also found that queen-reared offspring were smaller on average and more resistant to starvation than worker-reared offspring, which is consistent with our previous study (Costa et al., 2021). Additionally, both queen-reared and worker-reared offspring were able to survive pollen-starvation for long periods of time (up to 30 days), as long as artificial nectar was present.

Care-giver identity impacted hemolymph glucose levels only under nectar starvation in relatively young bees for a short-time period (six hours). Specifically, two-day old queen-reared offspring were better able to maintain consistent hemolymph glucose levels under nectar starvation in comparison to worker-reared offspring. Additionally, queen-reared offspring survived longer than the worker-reared offspring under both nectar starvation and total (pollen and nectar) starvation. This supports our hypothesis, in part, that queens enforce a metabolic mechanism during early development that contributes to offspring starvation resistance during adulthood. Worker-reared offspring appear to metabolize

hemolymph glucose levels faster than the queen-reared offspring under nectar starvation. This suggests that they are forced to switch to alternative nutrient stores, and likely use those nutritional reserves faster than the queen-reared offspring. This could explain why the worker-reared offspring cannot survive as long as the queen-reared offspring under nectar starvation and total (pollen and nectar) starvation. However, longer durations of nectar starvation depleted glucose to equal levels irrespective of care-giver identity during development. Thus, under persistent starvation, glucose levels are used until almost complete depletion, irrespective of care-giver history, but worker-reared offspring likely reach depletion before the queen-reared offspring.

Pollen starvation alone did not have the same effect on glucose levels as nectar starvation in our experiment. Under pollen starvation, queen-reared and worker-reared offspring had similar levels of glucose in the hemolymph across both age groups and starvation durations. This indicates that as long as bees have access to nectar, they are able to maintain hemolymph sugar levels, regardless of care-giver identity during early development. Additionally, pollen-starved offspring were able to survive for long periods of time, approximately 30 days or more, regardless of care-giver identity or offspring age upon entering starvation. This aligns with the idea that the primary nutrient resource for survival in adult bumble bees is nectar, whereas pollen constitutes a small part of the adult diet (Stabler et al., 2015). Although amino acids can play an important role in energy metabolism, as well as other important metabolic processes, including in bumble bees (Stabler et al., 2015), sugars are more fundamentally required for energy

metabolism. Therefore, as long as bumble bees have access to nectar, they can maintain their core metabolic functions, irrespective of care-giver identity.

We did not see the same effects of rearing history on hemolymph glucose levels across the two age groups. The seven-day old offspring under nectar starvation had equal levels of glucose, irrespective of care-giver identity. This could suggest that the seven-day old offspring had more time to sequester nutrients before they faced starvation conditions, and thus had more nutrient stores to help maintain hemolymph sugar levels. Interestingly, our results are similar to a study in honey bees, which found that young honey bees (< 24 hours old) that underwent starvation as larvae had significantly higher glucose levels when they experience starvation as adults (Wang et al., 2016a). This study also detected an age effect, where seven-day old bees that underwent larval starvation also did not have any differences in hemolymph sugar levels under starvation conditions as adults; the effect was only detected in only 1-day old adult bees (Wang et al., 2016a).

The relative stability of trehalose levels across all treatment groups could be due to the fact that trehalose is the primary energy source in the hemolymph (Elbein et al., 2003) and can also act as a stress protectant during extreme environmental conditions (Elbein et al., 2003; Liebl et al., 2009; Mayack and Naug, 2010; Wang et al., 2019). If nectar is present, then trehalose levels naturally remain stable due to the persistent ingestion of sugar. If nectar is absent but pollen is present, it is possible that the amino acids from pollen are used in the process gluconeogenesis to generate more glucose, which then

binds together to form trehalose (Wang et al., 2019). However, we also found that completely starved bees (i.e., without nectar or pollen) still had stable trehalose levels. This might suggest that any nutrients used from storage, such as sugar from glycogen or endogenous amino acids from protein, are primarily used to maintain trehalose levels. One caveat to these results is that in our study, bees that underwent starvation were relatively inactive. It is likely that under starvation, trehalose levels would decrease if bees were active, such as during flight or foraging behavior (Blatt and Roces, 2001). Additionally, since trehalose is more stabilized, it may take longer than twelve hours for levels to exhibit depletion, as is seen in honey bees (Mayack and Naug, 2010).

Based on our findings, we propose that bumble bee queens influence offspring in early development by changing their nutritional status in ways that persist into adulthood. Moreover, we specifically posit that queens create starvation-like conditions during larval development, irrespective of food availability, that shape metabolic conditions during adulthood. This hypothesis is predicated on two sets of findings. First, there is similarity in our results, related to care-giver identity, and previous work in honey bees, which examined the impacts of starvation during early development (Wang et al. 2016a). Second, the bumble bee queen influence on body size has been demonstrated in several previous studies (Shpigler et al. 2013, Costa et al. 2021, Chole et al. 2022, Barie and Amsalem, 2022), with queens consistently producing smaller-bodied offspring than when larvae are reared by workers. In animals, body size determination and nutritional status are inextricably linked (Bakker, 1959; Davidowitz et al., 2003; Boggs and Freeman,

2005), with smaller-bodied individuals often being produced under more food-limited conditions. The underlying mechanisms mediating the queen effect on offspring development are currently unknown. Bumble bee queens may restrict food quantity in some way when feeding larvae, or some other, non-nutritional cue may ultimately act on nutritional mechanisms.

This study contributes to our understanding of how factors during development, such as care-giver identity and nutritional history, impact bumble bee starvation resistance. It is important to better understand bumble bee starvation resistance and the mechanisms that enhance this resilience because bees increasingly face nutritional stress in their environment (reviewed in Goulson et al., 2015; Wang et al., 2019). In addition, the nutritional status and mechanisms underlying starvation resistance may also impact the trajectory of offspring foraging behaviors (Wang et al 2016a; Hendriksma et al., 2019). Knowing how nutrients are used during starvation, as well as the impacts on foraging behavior, can help inform how to best support bees in environments where there are limited nutrient resources.

5. Conclusion

We explored the effects of bumble bee care-giver identity on the nutritional status and sugar metabolism of adult offspring. The results from our study align with a previous study (Costa et al., 2021), wherein bumble bee offspring that are reared by the queen alone are smaller and survive longer under starvation compared to offspring that are

reared by a cohort of workers. We found that queen-reared offspring are better able to maintain consistent hemolymph sugar levels under short-term nectar starvation. Further, our hemolymph sugar results are similar to those found in honey bees that were starved as larvae (Wang et al., 2016a). Based on this evidence, we suggest that bumble bee queens restrict food quantity in some way when feeding larvae, or otherwise provide a cue that acts on the nutritional status of developing offspring. Future directions for this research include analysis of more starvation duration timepoints, to explore the timescale of hemolymph sugar depletion and to examine how longer durations impact trehalose levels. Future studies could also explore these effects on active bees, such as during flight or foraging, to examine how nutrient levels differ between queen-reared and worker-reared offspring under more realistic conditions.

Tables and Figures

Figure 3.1. Flow diagram of treatment groups.

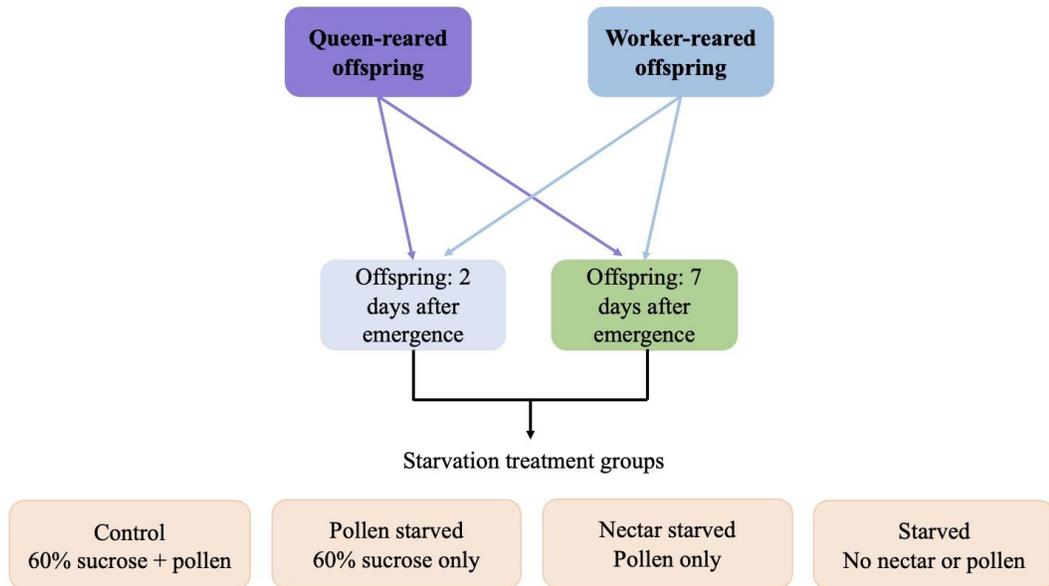


Figure 3.1. Queen-reared and worker-reared offspring were removed from their nests as soon as they emerged and kept in an incubator for either two or seven days, after which they were assigned to a starvation treatment group. The starvation treatment groups are as follows: *control*, *pollen starved*, *nectar starved*, and *starved*. Below, each of the starvation treatments describes what was administered to bees during the starvation period. Bees were kept on the starvation treatment for either six hours or twelve hours.

Table 3.1. Total number of hemolymph samples collected across all treatment groups.

Hemolymph samples used for analysis				
	Starvation- 6 hours		Starvation- 12 hours	
	Queen-reared	Worker-reared	Queen-reared	Worker-reared
Control. 2d	19	12	16	9
Pollen starved. 2d	21	14	13	10
Nectar starved. 2d	20	13	11	10
Starved. 2d	20	13	14	9
Control. 7d	17	18	14	15
Pollen starved. 7d	17	17	15	14
Nectar starved. 7d	19	18	14	15
Starved. 7d	15	18	13	12
Total	148	123	110	94
	Total samples overall = 475			

Table 3.2. Total number of hemolymph samples used for glucose and trehalose analysis.

Hemolymph samples used for analysis				
	Starvation- 6 hours		Starvation- 12 hours	
	Queen-reared	Worker-reared	Queen-reared	Worker-reared
Control. 2d	9	9	10	9
Pollen starved. 2d	10	8	11	10
Nectar starved. 2d	10	9	10	10
Starved. 2d	7	10	10	9
Control. 7d	9	10	11	10
Pollen starved. 7d	10	10	10	9
Nectar starved. 7d	10	10	11	12
Starved. 7d	9	11	7	10
Total	74	77	80	79
	Total samples overall = 310			

Figure 3.2

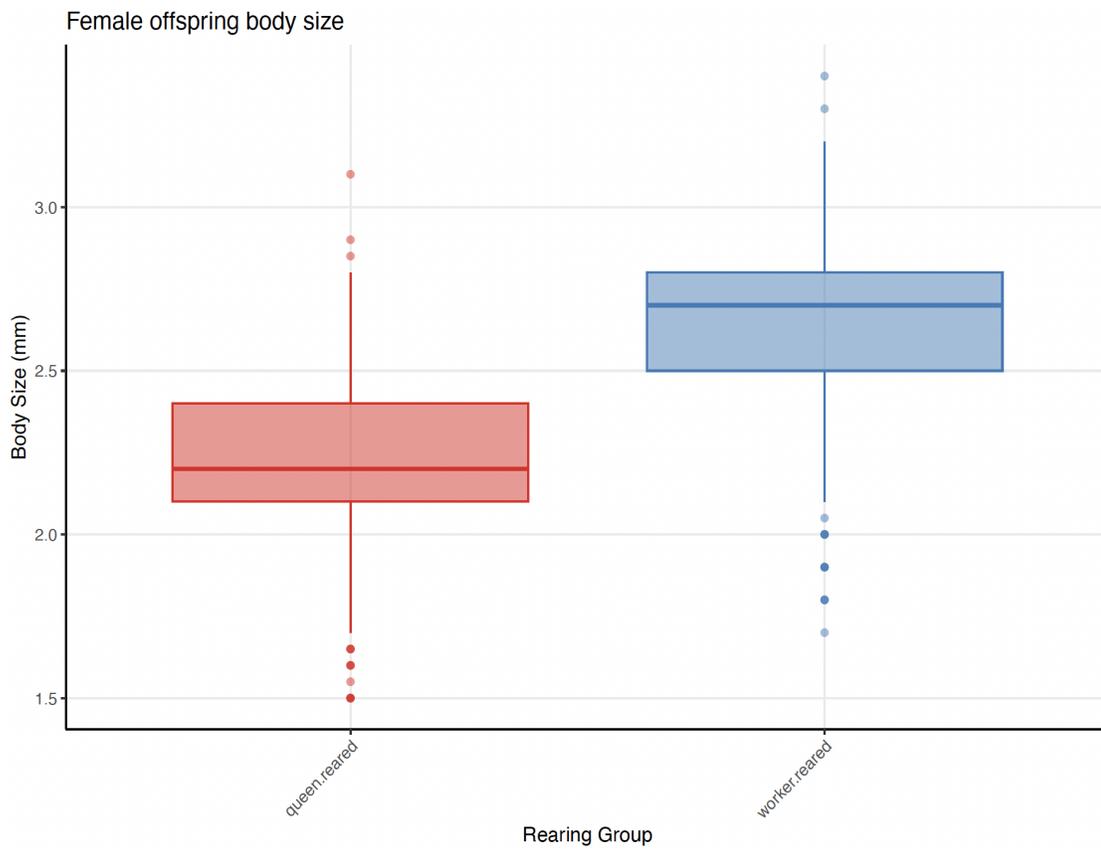


Figure 3.2. Boxplot representing queen-reared (red) and worker-reared (blue) offspring body size. Care-giver identity significantly impacted body size (p -value $< 2e-16$), with queen-reared offspring significantly smaller than worker-reared offspring.

Figure 3.3

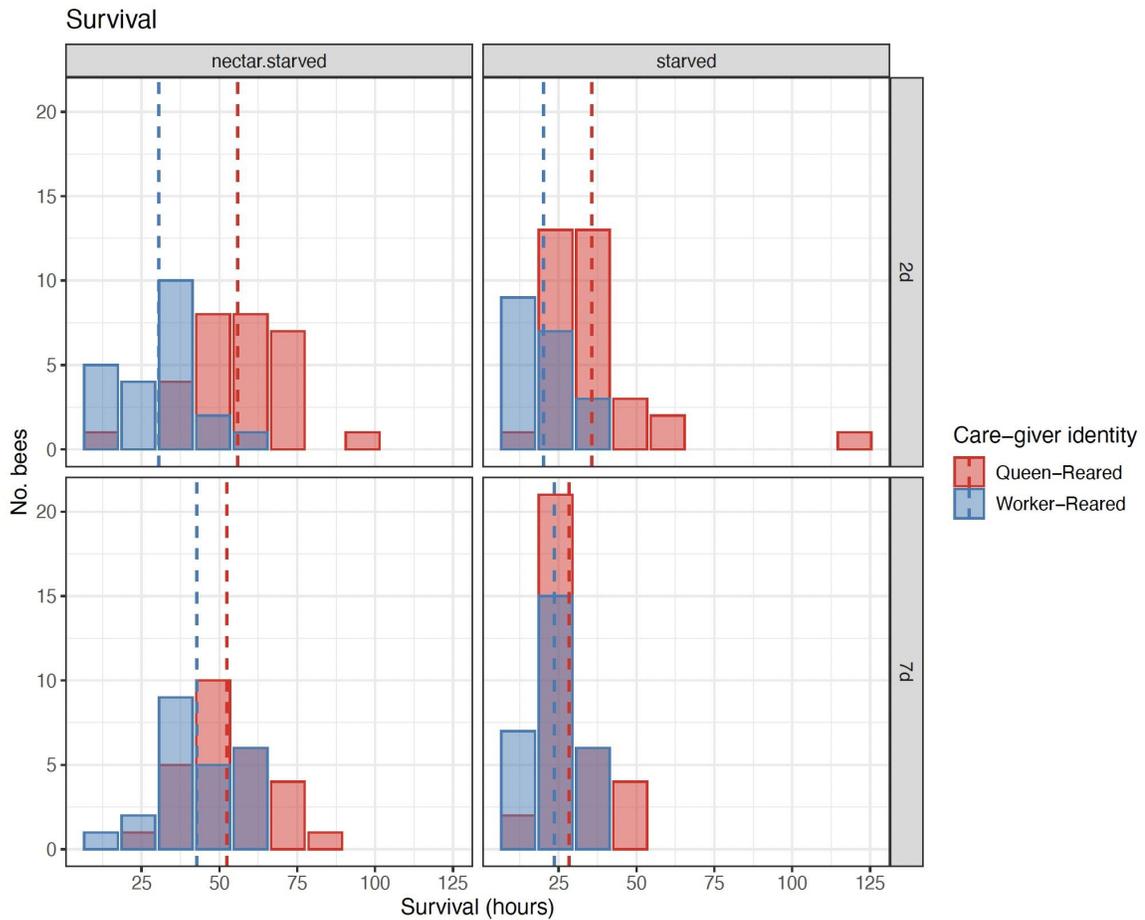


Figure 3.3. Histogram showing how long queen-reared (red) and worker-reared (blue) offspring survived under starvation treatments (excluding the *pollen-starved* treatment). The left column of plots represent offspring in the *nectar-starved* treatment and the right column represents offspring in the *starved* treatment. The top row of plots corresponds to two-day old offspring, and the bottom row of plots corresponds to seven-day old offspring. All queen-reared offspring survived significantly longer than worker-reared offspring (p -values < 0.01), except in the *starved*, seven-day old treatment group. The *pollen-starved* treatment group was excluded because all offspring survived for a month or longer irrespective of care-giver identity. Dashed lines represent the average survival time (hours) for the queen-reared and worker-reared offspring.

Figure 3.4

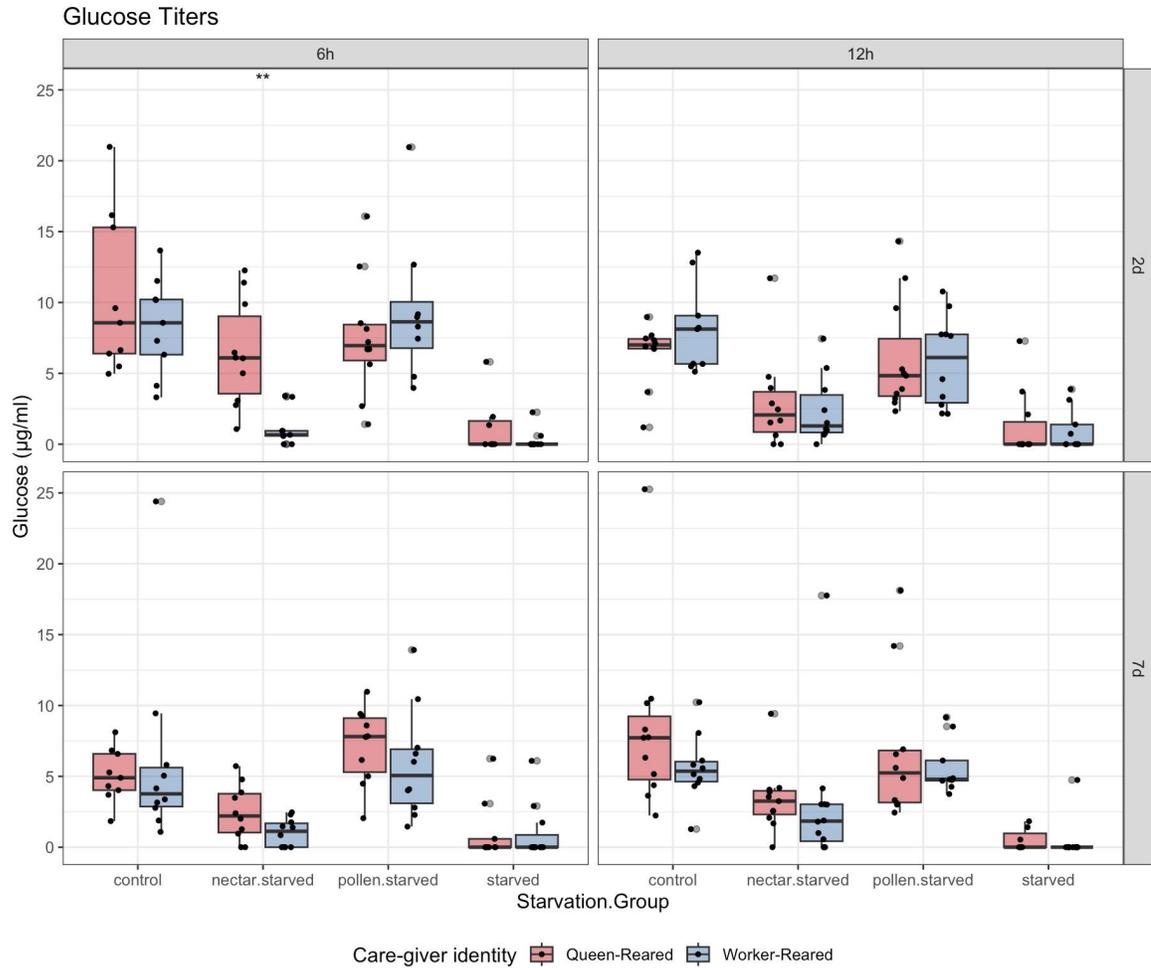


Figure 3.4. Glucose titers for queen-reared (red) and worker-reared (blue) offspring across all starvation treatment groups. The left column of plots represents bees that underwent six hours of starvation, and the right column represents bees that underwent twelve hours of starvation. The top row of plots represents bees that entered starvation treatment at two days old, and the bottom row of plots represents bees that entered starvation at seven days old. Glucose titers significantly differed between queen- and worker-reared offspring, but only when they were nectar starved for six hours at two days old (p -value = 0.029; denoted by asterisks).

Figure 3.5

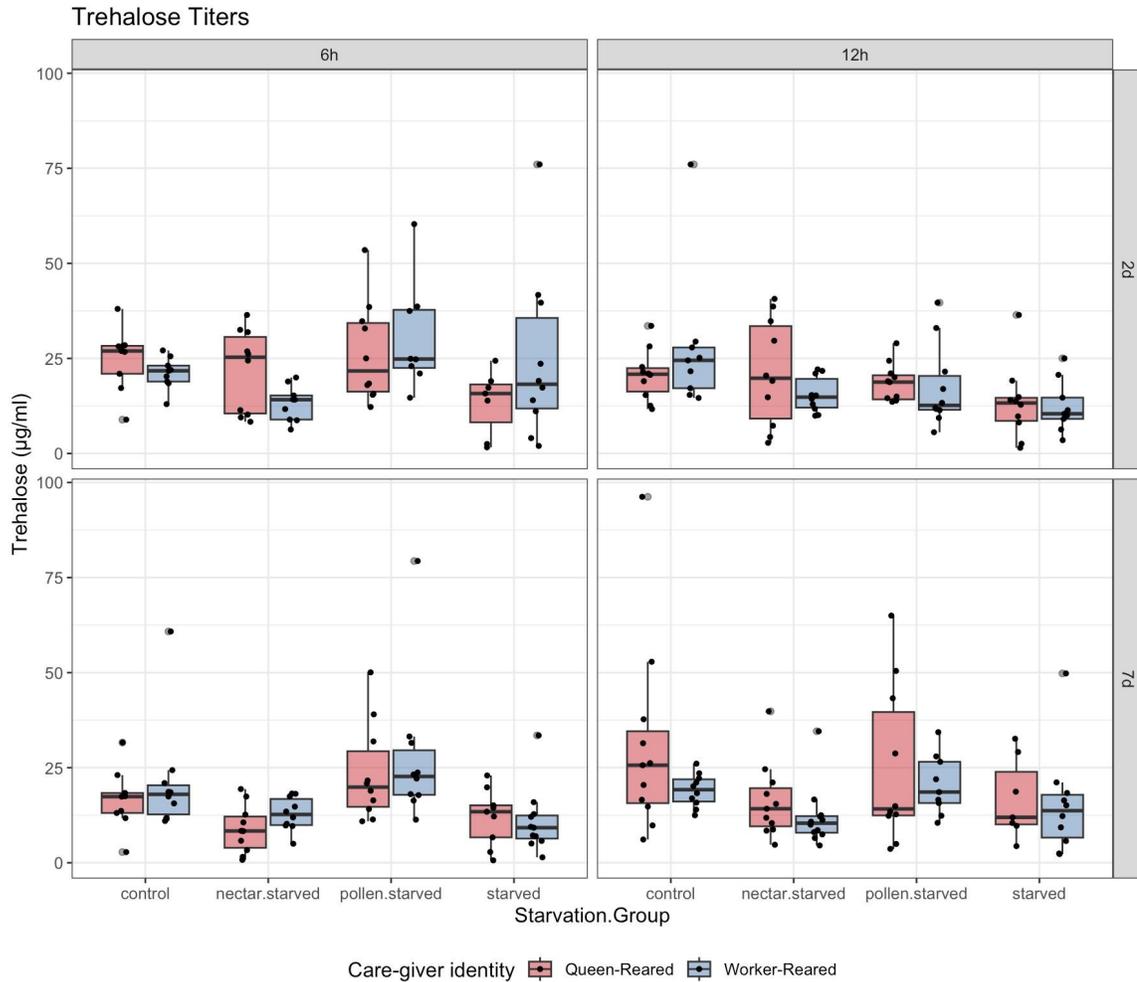


Figure 3.5. Trehalose titers for queen-reared (red) and worker-reared (blue) offspring across all starvation treatment groups. The left column of plots represents the bees that underwent six hours of starvation, and the right column represents bees that underwent twelve hours of starvation. The top row of plots represents bees that entered starvation treatment at two days old, and the bottom row of plots represents bees that entered starvation at seven days old. Trehalose titers were relatively stable in both queen- and worker-reared offspring across all treatment groups (Tukey’s post-hoc comparison for care-giver identity across all treatments, p -values > 0.05).

References

- Bakker K. Feeding period, growth, and pupation in larvae of *Drosophila melanogaster*. *Entomol Exp Appl.*; **2**: pp. 171–86. <https://doi.org/10.1111/j.1570-7458.1959.tb00432.x>. (1959).
- Barie, K., and Amsalem, E. The influence of the social environment on larval development and resulting caste in *Bombus impatiens*. bioRxiv. (2022).
- Bates D, Mächler M, Bolker B, Walker S. Fitting Linear Mixed-Effects Models using lme4. *J Stat Softw.*, **67**: pp. 1–48. Available from: <http://arxiv.org/abs/1406.5823>. (2014).
- Blatt, J. and Roces, F. Haemolymph sugar levels in foraging honeybees (*Apis mellifera Carnica*): Dependence on metabolic rate and in vivo measurement of maximal rates of trehalose synthesis. *Journal of Experimental Biology*, **204**: pp. 2709-2716. (2001).
- Boggs CL, Freeman KD. Larval food limitation in butterflies: effects on adult resource allocation and fitness. *Oecologia.*, **144**: pp. 353–61. <https://doi.org/10.1007/s00442-005-0076-6>. (2005).
- Boyle NK, Tripodi AD, Machtley SA, Strange JP, Pitts-Singer TL, Hagler JR. A Nonlethal Method to examine non-*Apis* bees for mark-capture research. *J Insect Sci.*, **18**:3. <https://doi.org/10.1093/jises a/iey043>. (2018).
- Cadby, C.D, Jones, S.M., Wapstra, E. Potentially adaptive effects of maternal nutrition during gestation on offspring phenotype of a viviparous reptile. *Journal of Experimental Biology*, **214**:24, pp. 4234–4239. (2011).
- Chole, H., de Guinea, M., Woodard, S. H., & Bloch, G. Field-realistic concentrations of a neonicotinoid insecticide influence socially regulated brood development in a bumblebee. *Proceedings of the Royal Society B*, **289**: 20220253. (2022).
- Clutton-Brock, T. H. The evolution of parental care. Princeton Univ. Press, Princeton, NJ. (1991).
- Costa, C.P., Fisher, K., Guillén, B.M., Yamanaka, N., Bloch, G., Woodard, S.H. Care-giver identity impacts offspring development and performance in an annually social bumble bee. *BMC Ecology and Evolution*, **21**:20 (2021).
- Davidowitz G, D’Amico LJ, Nijhout HF. Critical weight in the development of insect body size. *Evol Dev.*, **5**: pp. 188–97. (2003).

- Elbein, A., Pan, Y., Pastuszak, I., Carroll, D. New insights on trehalose: a multifunctional molecule. *Glycobiology*, **13**: pp. 17R-27R. (2003).
- Goulson, D., Nicholls, E., Botías, C. & Rotheray, E. L. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science* **347**: 6229. (2015).
- Hartfelder, K., Bitondi, M.M.G., Brent, C.S., Guidugli-Lazzarin, K.R., Simões, Z.L.P., Stabentheiner, A., Tanaka, É.D., Wang, Y. Standard methods for physiology and biochemistry research in *Apis mellifera*. *Journal of Apicultural Research*, **52**: <http://dx.doi.org/10.3896/IBRA.1.52.1.06>. (2013).
- Hendriksma, H. P., Toth, A. L. & Shafir, S. Individual and Colony Level Foraging Decisions of Bumble Bees and Honey Bees in Relation to Balancing of Nutrient Needs. *Front. Ecol. Evol.* **7**: 177. (2019).
- Hensch, T. K. CRITICAL PERIOD REGULATION. *Annu. Rev. Neurosci* **27**: pp 549–79 (2004).
- Hibshman, J.D., Hung, A., Baugh, L.R. Maternal Diet and Insulin-Like Signaling Control Intergenerational Plasticity of Progeny Size and Starvation Resistance. *PLOS Genetics*, **14**(8): e1007639. (2016).
- Hunt, J., and Simmons, L.W. Maternal and Paternal effects on offspring phenotype in the Dung beetle *Onthophagus taurus*. *Evolution*, **54**; pp. 936-941. (2000).
- Hunt, J.H. and Amdam, G.V. Bivoltinism as an Antecedent to Eusociality in the Paper Wasp Genus *Polistes*. *Science*, **308**: pp. 264-267. (2005).
- Hunt, J. H. *et al.* A diapause pathway underlies the gyne phenotype in *Polistes* wasps, revealing an evolutionary route to caste-containing insect societies. *Proc. Natl. Acad. Sci. U. S. A.* **104**: pp. 14020–14025. (2007).
- Jandt, J. M., Suryanarayanan, S., Hermanson, J. C., Jeanne, R. L. & Toth, A. L. Maternal and nourishment factors interact to influence offspring developmental trajectories in social wasps. *Proc. R. Soc. B Biol. Sci.* **284**: 0170651. (2017).
- Knee, W. J. & Medler, J. T. The Seasonal Size Increase of Bumblebee Workers (Hymenoptera: *Bombus*). *Can. Entomol.* **97**: pp. 1149–1155 (1965).
- Lawson, S.P., Ciaccio, K.N., Rehan, S.M. Maternal manipulation of pollen provisions affects worker production in a small carpenter bee. *Behavioral Ecology and Sociobiology* **70**: pp. 1891-1900. (2016).

- LeBoeuf, A.C., Waridel, P., Brent, C.S., Gonçalves, A.N., Menin, L., Ortiz, D., Riba-Grognuz, O., Koto, A., Soares, Z.G., Privman, E., Miska, E.A., Benton, R., Keller, L. Oral transfer of chemical cues, growth proteins and hormones in social insects. *eLife* **5**: e20375. (2016).
- Leimar, O., Hartfelder, K., Laubichler, M. D. & Page, R. E. Development and evolution of caste dimorphism in honeybees - a modeling approach. *Ecol. Evol.* **2**: pp. 3098–3109 (2012).
- Liebl, M., Nelius, V., Kamp, G., Ando, O., Wegener, G. Fate and effects of the trehalase inhibitor trehazolin in the migratory locust (*Locusta migratoria*). *Journal of Insect Physiology* **56**: pp. 567-574. (2010).
- Lindström, J. Early development and fitness in birds and mammals. *Trends in Ecology and Evolution* **14**: pp. 343–348 (1999).
- Mayack, C., Naug, D. Parasitic infection leads to decline in hemolymph sugar levels in honeybee foragers. *Journal of Insect Physiology*, **56**(11): pp. 1572-1575. (2010).
- Michener CD. Comparative social behaviour of bees. *Annu Rev Entomol.*, **14**: pp. 299–342. (1969).
- Møller, A. P. Developmental stability and fitness: A review. *Am. Nat.* **149**: pp. 916–932 (1997).
- Nettle, D. and Bateson, M. Adaptive developmental plasticity: what is it, how can we recognize it and when can it evolve? *Proceedings of the Royal Society B: Biological Sciences*, **282**: 20151005. (2015).
- Pendrel, B. A. & Plowright, R. C. Larval feeding by adult bumble bee workers (Hymenoptera: Apidae). *Behav. Ecol. Sociobiol.* **8**: pp. 71–76 (1981).
- Plowright, R. C. & Jay, S. C. On the size determination of bumble bee castes (Hymenoptera: Apidae). *Can. J. Zool.* **55**: pp. 1133–1138 (1977).
- Ribeiro, M. F., Velthuis, H. H. W., Duchateau, M. J. & Van Der Tweel, I. Feeding frequency and caste differentiation in *Bombus terrestris* larvae. *Insectes Soc.* **46**: pp. 306–314 (1999).
- Rittschof, C. C., Coombs, C. B., Frazier, M., Grozinger, C. M. & Robinson, G. E. Early-life experience affects honey bee aggression and resilience to immune challenge. *Sci. Rep.* **5**: 15572 (2015).

- Röseler P-F. A technique for year-round rearing of *Bombus terrestris*(Apidae, Bombini) colonies in captivity. *Apidologie*. **16**(2): pp. 165–70. (1985).
- Shpigler, H. *et al.* Social influences on body size and developmental time in the bumblebee *Bombus terrestris*. *Behav. Ecol. Sociobiol.* **67**: pp. 1601–1612 (2013).
- Stabler, D., Paoli, P.P., Nicolson, S.W., Wright, G.A. Nutrient balancing of the adult worker bumblebee (*Bombus terrestris*) depends on the dietary source of essential amino acids. *The Journal of Experimental Biology*, **218**: pp. 793-802. (2015).
- Uller, T. Developmental plasticity and the evolution of parental effects. *Trends in Ecology and Evolution*, **23**:8, pp. 432-438. (2008).
- Wang, Y., Kaftanoglu, O., Fondrk, M. K. & Page, R. E. Nurse bee behaviour manipulates worker honeybee (*Apis mellifera* L.) reproductive development. *Animal Behavior* **92**: pp. 253–261 (2014).
- Wang, Y., Kaftanoglu, O., Brent, C. S., Page, R. E. & Amdam, G. V. Starvation stress during larval development facilitates an adaptive response in adult worker honey bees (*Apis mellifera* L.). *Journal of Experimental Biology* **219**: pp. 949–59 (2016a).
- Wang, Y., Campbell, J.B., Kaftanoglu, O., Page, R. E., Amdam, G. V., Harrison, J.F. Larval starvation improves metabolic response to adult starvation in honey bees (*Apis mellifera* L.). *Journal of Experimental Biology*, **219**: pp. 960-968. (2016b).
- Wang, L., Meeus, I., Rombouts, C., Meulebroek, L.V., Vanhaecke, L., Smagghe, G. Metabolomics-based biomarker discovery for bee health monitoring: A proof of concept study concerning nutritional stress in *Bombus terrestris*. *Scientific Reports* **9**: 11423 (2019).
- Woodard, S. H., Bloch, G., Band, M. R. & Robinson, G. E. Social regulation of maternal traits in nest-founding bumble bee (*Bombus terrestris*) queens. *Journal of Experimental Biology* **216**: pp. 3474–3482 (2013).
- Wyatt G. R., Kalf G.F. THE CHEMISTRY OF INSECT HEMOLYMPH II. TREHALOSE AND OTHER CARBOHYDRATES. *The Journal of General Physiology*. **40**: pp. 833-847. (1957).
- Wyatt G.R. The Biochemistry of Sugars and Polysaccharides in Insects. *Advances in Insect Physiology*. **4**: pp. 287-360. (1967).

Conclusion

Bumble bee foraging behaviors are important for the maintenance of our ecosystems as well as for their own survival. The molecular and metabolic biology of individual bumble bees is important to better understand what drives these foraging behaviors and can contribute to our understanding of how and why their foraging behaviors are negatively impacted by environmental stressors. Overall, the research in this dissertation sheds light on bumble bee molecular and metabolic biology, particularly on energy and nutrient metabolic processes. Specifically, this research reveals some of the molecular and metabolic mechanisms that can be influenced by external stressors and includes the first study that examined the molecular mechanisms associated with foraging behavioral states.

Through this research I found that bumble bee nutritional status is important for resilience to environmental stressors. The results from Chapter One revealed that the effects of the neonicotinoid pesticide, imidacloprid, at the metabolic level are stronger when bees have poor nutritional status. The results from Chapter Three conclude that maintaining a relatively high hemolymph glucose level acts as a mechanism that contributes, in part, to bumble bee starvation resistance. Thus, nutrient metabolism and nutritional status can modulate susceptibility or resilience to both pesticide stress and nutritional stress, and likely other stressors as well.

Additionally, the study in Chapter Two revealed that there are not significant gene expression patterns associated with nectar robbing behavior, or the other behaviors examined. However, other molecular mechanisms, such as post-transcriptional and post-translational mechanisms, can still be explored. Furthermore, this study suggests that there could be energetic differences between tactic constant bees and bees that switch between two tactics, which should be explored in more controlled experiments. Chapter Two was the first study to explore any molecular mechanism associated with the behaviors of interest and thoroughly examines different ideas for how this study can be expanded upon in the future. A full comprehension of the internal mechanisms associated with foraging behavioral states is important to better understand how some of these ecologically important behaviors, such as nectar robbing, evolved. Furthermore, it can shed light on how these foraging behaviors might be influenced by external stressors.

Appendix 1

All tables and figures not included in the main text of Chapter 1.

Table A1.1. Sample sizes for the experiment including bees that died.

	0-hour	8 hours	16 hours	24 hours
Chronic	13	12	12	12
Acute early	12	12	13	13
Acute late	12	12	12	12
Untreated	11	12	12	13

N = 195

Table A1.2. Sample sizes sent for Metabolomic Analysis.

	0 hour	8 hours	16 hours	24 hours
Chronic	11	11	12	10
Acute early	12	11	12	11
Acute late	11	11	11	9
Untreated	11	10	12	10

N = 175

Table A1.3. Survival analysis results for exposure period.

Fixed Factors	Loglik	AICc	Delta	Weight
Null	-73.344	146.7	0.00	0.870
Natal colony	-72.829	150.7	4.06	0.114
Treatment	-73.156	154.7	8.02	0.016
Treatment+ Natal colony	-72.635	162.08	16.08	0.000
Treatment*Natal colony	-69.869	293.7	147.05	0.000

Table A1.4. Survival analysis results for starvation period.

Fixed Factors	Loglik	AICc	Delta	Weight
Treatment + Natal colony	-16.287	-17.4	0.00	1
Treatment* Natal colony	-14.355	13.0	30.42	0
Null	-18.803	37.6	55.03	0
Natal colony	-17.978	46.0	63.38	0
Treatment	-17.197	64.4	81.82	0

Table A1.5. Significant Metabolites impacted by Starvation alone.

<i>Significant Metabolites by Starvation</i>			
Metabolite	Class	KEGG ID	Pathways
N-Acetylglutamic acid	Acetyl amino acid	C00624	Arginine biosynthesis
S-Adenosylhomocysteine	Methylation	C00021	Cysteine and methionine metabolism; biosynthesis of amino acids and cofactors
4-Guanidinobutyric acid	Amino acid	C01035	Arginine and proline metabolism
Acetylcarnitine	Carnitine	C02571	Insulin resistance
Adenine	Purine	C00147	Purine metabolism
Adenosine	Purine	C00212	Purine metabolism
ADP	Purine	C00008	Oxidative phosphorylation; purine metabolism
ADP ribose	Purine	C00301	Purine metabolism
Arginine	Amino acid	C00062	Arginine and proline metabolism; amino acid metabolism; aminoacyl-tRNA biosynthesis
Asparagine	Amino acid	C00152	Alanine, aspartate and glutamate metabolism; aminoacyl-tRNA biosynthesis
Aspartic acid	Amino acid	C00049	Arginine biosynthesis; Alanine, aspartate and glutamate metabolism; glycine, serine and threonine metabolism; cysteine and methionine metabolism; lysine biosynthesis; histidine metabolism; nicotinate and nicotinamide metabolism; aminoacyl-tRNA metabolism; amino acid metabolism

Beta-Alanine	Amino acid	C00099	Pyrimidine metabolism; beta-Alanine metabolism; Pantothenate and CoA biosynthesis; propanoate metabolism
CDP-choline	Phospholipid turnover	C00307	Glycerophospholipid metabolism
CDP-ethanolamine	Phospholipid turnover	C00570	Glycerophospholipid metabolism
Choline	Choline	C00114	Glycine, serine and threonine metabolism, Glycerophospholipid metabolism
Cysteinesulfinic acid	Amino acid	C00606	Cysteine and methionine metabolism; Taurine and hypotaurine metabolism
Gamma-aminobutyric acid	Amino acid	C00334	Alanine, aspartate and glutamate metabolism; arginine and proline metabolism; beta-alanine metabolism; butanoate metabolism; nicotinate and nicotinamide metabolism
Glutamine	Amino acid	C00064	Arginine biosynthesis; purine metabolism; alanine, aspartate and glutamate metabolism; amino acid metabolism; nitrogen metabolism; aminoacyl-tRNA biosynthesis
Glutathione (GSH)	Oxidative stress	C00051	Cysteine and methionine metabolism, glutathione metabolism
Glycerophosphocholine	Choline	C00670	Glycerophospholipid metabolism
Guanine	Purine	C00242	Purine metabolism
Hexose	Sugar	NA	Energy metabolism

Hypoxanthine	Purine	C00262	Purine metabolism
Isoleucine	Amino acid	C00407	Valine, leucine, isoleucine degradation and biosynthesis; aminoacyl-tRNA biosynthesis
Kynurenine	Amino acid	C00328	Tryptophan metabolism
Lysine	Amino acid	C00047	Lysine biosynthesis and degradation; amino acid metabolism, biotin metabolism, aminoacyl-tRNA biosynthesis
Malic acid	TCA cycle	C00149	TCA cycle; pyruvate metabolism; glyoxylate and dicarboxylate metabolism
Methionine	Amino acid	C00073	Cysteine and methionine metabolism; amino acid metabolism; glucosinolate metabolism; aminoacyl-tRNA biosynthesis
NADP	Electron carrier	C00006	Glutathione metabolism; nicotinate and nicotinamide metabolism
Nicotinamide mononucleotide	Electron carrier	C00455	Nicotinate and nicotinamide metabolism
Phenylalanine	Amino acid	C00079	Phenylalanine, tyrosine and tryptophan biosynthesis; aminoacyl-tRNA biosynthesis;
Pipecolic acid	Amino acid	C00408	Lysine degradation
Proline	Amino acid	C00148	Arginine and proline metabolism; aminoacyl-tRNA biosynthesis
Thiamine	B vitamin	C00378	Thiamine metabolism; biosynthesis of cofactors
Threonine	Amino acid	C00188	Glycine, serine and threonine metabolism; valine, leucine, and isoleucine biosynthesis;

			aminoacyl-tRNA biosynthesis
Tryptophan	Amino acid	C00078	Glycine, serine and threonine metabolism; Tryptophan metabolism; phenylalanine, tyrosine and tryptophan biosynthesis; aminoacyl-tRNA biosynthesis; 2-Oxocarboxylic acid metabolism; Biosynthesis of cofactors; Axon regeneration
Urea	Urea cycle	C00086	Arginine biosynthesis; purine metabolism; pyrimidine metabolism; arginine and proline metabolism; biotin metabolism
Valine	Amino acid	C00183	Valine, leucine, isoleucine degradation and biosynthesis; Pantothenate and CoA biosynthesis; aminoacyl-tRNA biosynthesis
Xanthosine	Purine	C01762	Purine metabolism; nucleotide metabolism

Figure A1.1

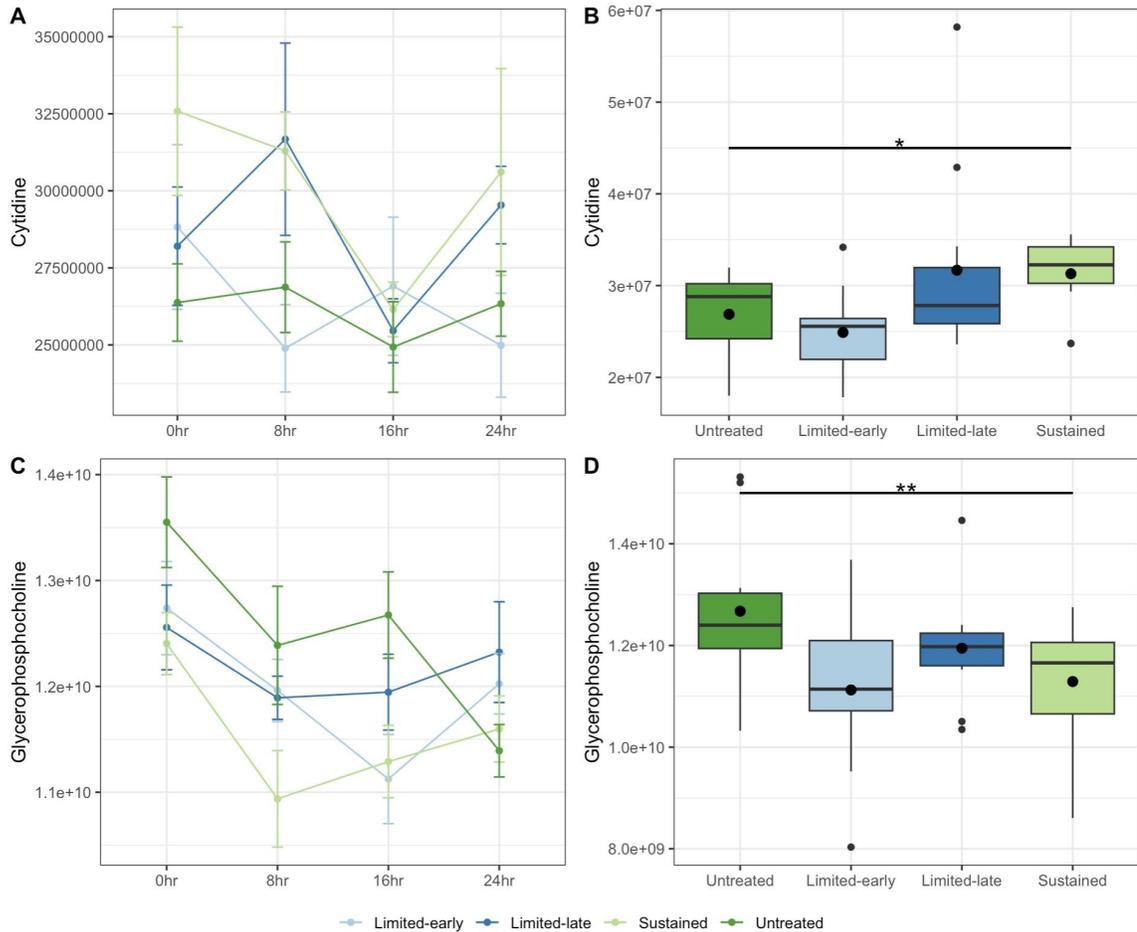


Figure A1.1. Key metabolites of interest that were impacted after bees were nutritionally stressed (after either 8 hours or 16 hours of starvation). The plots in the left column show the changes in metabolites through time under starvation and the plots in the right column show boxplots for those metabolites specifically at the 8-hour or 16-hour starvation timepoint. Black dots inside the boxplot represent the average; ** = p value ≤ 0.005 and * = p value < 0.05 (A) Relative abundance of cytidine through time under starvation. (B) Average abundance of cytidine at the 8-hour starvation timepoint. (C) Relative abundance of glycerophosphocholine through time under starvation. (D) Average abundance of glycerophosphocholine at the 16-hour starvation timepoint.

Appendix 1.2

Specific Metabolomics Methods in Chapter One

Targeted metabolomics was performed on a TQ-XS triple quadrupole mass spectrometer (Waters) coupled to an I-class UPLC system (Waters). Separations were carried out on a ZIC-pHILIC column (2.1 x 150 mm, 5 μ M) (EMD Millipore). The mobile phases were (A) water with 15 mM ammonium bicarbonate adjusted to pH 9.6 with ammonium hydroxide and (B) acetonitrile. The flow rate was 200 μ L/min and the column was held at 50° C. The injection volume was 1 μ L. The gradient was as follows: 0 min, 90% B; 1.5 min, 90% B; 16 min, 20% B; 18 min, 20% B; 20 min, 90% B; 28 min, 90% B. The MS was operated in selected reaction monitoring mode. Source and desolvation temperatures were 150° C and 500° C, respectively. Desolvation gas was set to 1000 L/hr and cone gas to 150 L/hr. Collision gas was set to 0.15 mL/min. All gasses were nitrogen except the collision gas, which was argon. Capillary voltage was 1 kV in positive ion mode and 2 kV in negative ion mode. A quality control sample, generated by pooling equal aliquots of each sample, was analyzed every 3-4 injections to monitor system stability and performance. Samples were analyzed in random order. Targeted data processing was performed with the open-source Skyline software (Maclean et al., 2010).

Appendix 2

Appendix 2 consists of a table that could not fit in the main text of Chapter Two.

Table A2.1. Gene Ontology enrichment analysis results showing significant terms using GO p-values for behavioral pairwise comparisons. A total of 48 significant terms were identified, five of which are shown in Table 2.2 in the main text for the Legitimate foragers vs. Switchers. GO categories: BP = Biological processes; CC = Cellular Component; MF = Molecular Function.

Significant GO terms by p-value				
Nectar robber vs. Legitimate forager	GO term	GO ID	p-value	GO category
	Cellular response to DNA damage stimulus	GO:0006974	0.00011	BP
	Organic substance metabolic process	GO:0071704	0.00025	BP
	Cell development	GO:0048468	0.00014	BP
	Proteasome-mediated ubiquitin dependent ...	GO:0043161	0.00159	BP
	Regulation of proteolysis	GO:0030162	0.01273	BP
	Positive regulation of protein metabolic...	GO:0051247	0.00701	BP
	Cellular component assembly involved in..	GO:0010927	0.00253	BP
	Cell surface receptor signaling pathway	GO:0007166	0.04504	BP
	Non-membrane-bounded organelle assembly	GO:0140694	0.00777	BP
	Supramolecular fiber organization	GO:0097435	0.0035	BP

	Supramolecular fiber	GO:0099512	0.00043	CC
	Mitochondrial protein-containing complex	GO:0098798	0.02436	CC
	Catalytic complex	GO:1902494	0.00286	CC
	Cytoplasm	GO:0005737	0.03121	CC
	Structural molecule activity	GO:0005198	0.0088	MF
	Transcription cis-regulatory region bind...	GO:0000976	0.007	MF
Nectar robber vs Switcher	Translation	GO:0006412	8.5E-10	BP
	Mitochondrial gene expression	GO:0140053	9.8E-09	BP
	rRNA processing	GO:0006364	0.0048	BP
	Cell surface receptor signaling pathway	GO:0007166	0.0109	BP
	Proteasome-mediated ubiquitin-dependent...	GO:0043161	0.0023	BP
	Protein-containing complex assembly	GO:0065003	0.0164	BP
	Circadian rhythm	GO:0007623	0.0245	BP
	Cell-cell signaling by wnt	GO:0198738	0.0013	BP
	Innate immune response	GO:0045087	0.0192	BP
	Regulation of proteolysis	GO:0030162	0.0058	BP

	Neuron projection morphogenesis	GO:0048812	0.0055	BP
	Cellular response to starvation	GO:0009267	0.0027	BP
	Pigmentation	GO:0043473	0.0064	BP
	Regulation of carbohydrate metabolic pro...	GO:0006109	0.0324	BP
	Mitochondrial protein-containing complex	GO:0098798	3.70E-10	CC
	Mitochondrial matrix	GO:0005759	5.40E-08	CC
	Ribosomal subunit	GO:0044391	1.40E-07	CC
	Catalytic complex	GO:1902494	0.0032	CC
	Intracellular protein-containing complex	GO:0140535	0.0026	CC
	Ribonucleoprotein complex	GO:1990904	0.003	CC
	Nucleolus	GO:0005730	0.0011	CC
	Nucleoplasm	GO:0005654	0.021	CC
	Transmembrane transporter complex	GO:1902495	0.0114	CC
	Mitochondrial envelope	GO:0005740	0.0106	CC
	Structural constituent of ribosome	GO:0003735	4.10E-08	MF
	Enzyme binding	GO:0019899	0.037	MF