NATURAL AND ANTHROPOGENIC DRIVERS OF VARIATION IN BUMBLE BEE GUT MICROBIOTAS

MICHELLE HOTCHKISS

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Department of Biology
Faculty of Science
University of Ottawa

Abstract

Bumble bees are key pollinators in natural and agricultural ecosystems. Like all social bees, their health is supported by a core gut microbiota consisting of a few, specialized bacterial phylotypes. The composition and community structure of bumble bee gut microbiotas can vary in response to natural and anthropogenic factors, which may impact its ability to support host health and performance. My thesis characterizes variation in bumble bee gut microbiotas caused by anthropogenic drivers, like pesticide exposure, and natural factors, like diapause and host species. In Chapter 2, I qualitatively and quantitatively synthesized literature on pesticide-induced disturbances in bee gut microbiotas. I found that core phylotypes vary in their susceptibility to pesticide-induced disturbance, and that pesticide concentration, exposure duration, and concurrent stressors all influence whether and how bee gut microbiotas are disturbed. I also identified gaps in our knowledge of pesticide-bee gut microbiota interactions, some of which I addressed in Chapter 3. There, I found that exposure to the fungicide chlorothalonil for a short, field-realistic duration did not alter bumble bee fecal microbiota community structure regardless of concentration, and that fecal microbiota structure did not accurately reflect gut microbiota structure. In Chapter 4, I characterized shifts in queen bumble bee gut microbiotas during diapause, an important stage in the bumble bee colony life cycle. I found that during diapause microbial abundance fell by an order of magnitude and gut microbial communities underwent dramatic restructuring, with some core phylotypes becoming undetectable past diapause onset. Finally, in Chapter 5 I examined how bumble bee gut microbiota composition and function vary with host species and origin (i.e., whether bees come from wild or commercial populations). I found that gut microbiota community structure varied between bumble bee species, between commercial and wild populations within the same species, and between populations from different commercial suppliers. However, despite this variation in community structure, gut microbiota metabolic potential was largely consistent across all hosts, including for metabolic capabilities related to host performance. Altogether, my thesis makes significant contributions to understanding variation in the gut microbiotas of a key, native pollinator and provides a strong foundation for future studies on this fascinating symbiosis.

Résumé

Les bourdons sont des pollinisateurs clés dans les écosystèmes naturels et agricoles. Comme toutes les abeilles sociales, leur santé est soutenue par un microbiote intestinal central composé de quelques phylotypes bactériens spécialisés. Chez les bourdons, la composition et la structure de la communauté de leur microbiote intestinal peuvent varier en réponse à des facteurs naturels et anthropiques, ce qui peut avoir un impact sur sa capacité à soutenir la santé et la performance de l'hôte. Ma thèse caractérise la variation du microbiote intestinal des bourdons causée par des facteurs anthropiques, comme l'exposition aux pesticides, et des facteurs naturels, comme la diapause et les espèces hôtes. Dans le chapitre 2, j'ai synthétisé qualitativement et quantitativement la littérature sur les perturbations induites par les pesticides dans les microbiotes intestinaux des abeilles. J'ai découvert que les phylotypes centraux varient dans leur sensibilité aux perturbations induites par les pesticides, et que la concentration de pesticides, la durée d'exposition et les facteurs de stress concomitants influencent tous si et comment les microbiotes intestinaux des abeilles sont perturbés. J'ai également identifié des lacunes dans nos connaissances sur les interactions entre les pesticides et le microbiote intestinal des abeilles, dont certaines ont été abordées au chapitre 3. Là, j'ai constaté que l'exposition au fongicide chlorothalonil pendant une courte durée, réaliste aux expositions sur le terrain, ne modifiait pas la structure de la communauté du microbiote fécal des bourdons, quelle que soit la concentration, et que la structure du microbiote fécal ne reflétait pas avec précision la structure du microbiote intestinal. Dans le chapitre 4, j'ai caractérisé les changements dans les microbiotes intestinaux des reines bourdons pendant la diapause, une étape importante du cycle de vie des colonies de bourdons. J'ai constaté que pendant la diapause, l'abondance microbienne diminue d'un ordre de grandeur et que les communautés microbiennes intestinales subissent une restructuration dramatique, certains phylotypes centraux devenant indétectables après le début de la diapause. Enfin, dans le chapitre 5, j'ai examiné comment la composition et la fonction du microbiote intestinal des bourdons varient selon l'espèce hôte et l'origine (c'est-à-dire si les abeilles proviennent de populations sauvages ou commerciales). J'ai constaté que la structure de la communauté du microbiote intestinal variait d'une espèce de bourdon à l'autre, d'une population commerciale à une population sauvage au sein d'une même espèce, et d'une population à l'autre de différents fournisseurs commerciaux. Cependant, malgré cette variation dans la structure de la communauté, le potentiel métabolique du microbiote intestinal était largement constant chez tous les hôtes, y compris pour les capacités métaboliques liées à la performance de l'hôte. Dans l'ensemble, ma thèse apporte des contributions significatives à la compréhension de la variation des microbiotes intestinaux d'un pollinisateur natif clé et fournit une base solide pour de futures études sur cette symbiose fascinante.

Statement of Contributions

The analyses, figures, written content, and ideas expressed in the following thesis are my own, although co-authors provided guidance that improved the quality of the work.

Chapter 2 of this thesis is published (Hotchkiss, M. Z., Poulain, A. J., & Forrest, J. R. K. 2022. Pesticide-induced disturbances of bee gut microbiotas. *FEMS Microbiology Reviews*). I reviewed and synthesized the literature and wrote a manuscript draft. Drs. Forrest and Poulain provided comments that shaped the final draft of the manuscript.

Chapter 3 of this thesis is published (Hotchkiss, M. Z., Forrest, J. R. K., & Poulain, A. J. 2024. Exposure to a fungicide for a field-realistic duration does not alter bumble bee fecal microbiota structure. *Applied and Environmental Microbiology*). I conceived of, designed the experiments for, and performed the laboratory work for this chapter. Dr. Jessica Forrest assisted with creating microcolonies and Alexandrea Stewart evaluated microcolony production. Staff at Genome Quebec conducted the DNA sequencing. I performed the statistical analyses and wrote the initial manuscript draft. Drs. Forrest and Poulain provided comments that shaped the final version of the manuscript.

I conceived of and designed the experiment for Chapter 4. I performed all laboratory work and staff at Genome Quebec conducted the DNA sequencing. A metagenomic bioinformatics pipeline by Rui Zhang served as the basis for the development of my own pipeline. I performed the statistical analyses and wrote the initial manuscript draft. Drs. Forrest and Poulain provided comments that shaped the final version of the manuscript. A modified version of Chapter 4 has been submitted to *Insect Molecular Biology* for peer-review and publication.

I conceived of and designed the commercial colony and field survey for Chapter 5. I performed field work with the assistance of Perrin Bryson, Tovah Kashetsky, Noémie Lavoie, and Megan Reich. I performed all laboratory work and staff at Genome Quebec conducted the DNA sequencing. A metagenomic bioinformatics pipeline by Rui Zhang served as the basis for the development of my own pipeline. I performed the statistical analyses and wrote the initial manuscript draft. Drs. Forrest and Poulain provided comments that shaped the final version of the manuscript.

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Chapter 1

Introduction

Bumble bees are key, native pollinators in a diverse array of ecosystems. Not only do they pollinate numerous wild plant species (Goulson, 2009), often more effectively than honey bees (Page et al., 2021; Page & Williams, 2023), but also many agricultural crops (Button & Elle, 2014; Eeraerts et al., 2020; Garibaldi et al., 2013; Lyu et al., 2023; Nayak et al., 2019; Roldán Serrano & Guerra-Sanz, 2006; Willmer et al., 1994), particularly those which require buzz pollination (e.g., tomatoes, blueberries, and peppers), a service honey bees cannot perform. Alarm has been raised in recent decades over declines in bumble bee abundance and diversity, with some species experiencing major reductions in geographic distributions and declines in their relative abundances of up to 96% (Cameron et al., 2011; Cameron & Sadd, 2020). Maintaining the viability of bumble bee populations, and thereby the health and productively of the plant communities they pollinate, depends on obtaining a strong understanding of all components of bumble bee health and performance, including their gut microbial symbionts.

A gut microbiota is a community of microbes which lives in the gastro-intestinal tract of an animal (Carey & Duddleston, 2014). While the gut microbial communities of some animals consist primarily of transient microbes and pathogens (Hammer et al., 2019), other animals have consistent, stable symbioses with specific microbes that confer benefits necessary for host survival (McFall-Ngai et al., 2013; Perlman et al., 2022). Microbial taxa which systematically colonize most or all individuals of a host taxon are said to constitute the "core microbiota", with non-core taxa being those microbes whose presence is more sporadic (Neu et al., 2021; Perlman et al., 2022). In insect gut microbiotas, core microbes have various functions including processing and provisioning nutrients, digesting recalcitrant compounds, and protecting hosts against pathogen infection (Brune, 2014; Brune & Dietrich, 2015; P. Engel & Moran, 2013; Flórez et al., 2015; Shan et al., 2021; X. Zhang et al., 2022). Eusocial, corbiculate bees are included among insect taxa with core gut microbiotas and whilst not dependent on gut microbes for survival (e.g., Leger & McFrederick, 2020; Zheng et al., 2017), their health is nevertheless supported by their symbiont communities (de Paula et al., 2021; Hammer et al., 2021; Kwong & Moran, 2016; Sarton-Lohéac et al., 2023).

Of the roughly 17,000 extant bee species, only around 5% are eusocial, a lifestyle characterized by cohabitating adults, overlapping generations, division of labour, and cooperative care of young (Wilson, 1971). The eusocial bees comprise three tribes: honey bees (Apini – 8 species), stingless bees (Meliponini – 605 species), and bumble bees (Bombini – 250 species) (Bustamante et al., 2021; M. S.

Engel et al., 2023; Williams et al., 2014). In all tribes, bees live in colonies led by one reproductive female, the queen, with many non-reproductive, female workers. While the queen is the only individual who lays eggs, all workers help to raise offspring by foraging for food, feeding larvae, and regulating the colony environment; males leave the colony to mate and do not provide care to offspring (Michener, 2007).

The associations of social bees with their gut microbes change during development. Larval bees have gut microbiotas that are sporadic both in presence and composition (Hroncova et al., 2015; Martinson et al., 2012; Su et al., 2021; Vojvodic et al., 2013), though the presence of some microbes has been shown to increase survivorship (Corby-Harris et al., 2014). During pupation, when larvae metamorphose into adults, they shed their fore- and hindgut lining, losing most of their gut microbiota (P. Engel & Moran, 2013). Consequently, adult bees emerge from pupation severely microbe-depleted (Martinson et al., 2012). Their gut microbiotas are subsequently re-colonized by specialized microbes that are typically found only in social bee guts and nests (Kwong & Moran, 2016). This recolonization is accomplished primarily by interactions with conspecifics and nest materials (Billiet et al., 2017; Kowallik & Mikheyev, 2021; Su et al., 2021); thus, it is the eusociality of eusocial bees that permits stable, vertical transmission of these gut microbes, and the existence of a core social bee gut microbiota.

The core gut microbiota of adult social bees contains a small number of specialized bacterial taxa that are highly conserved both within and among species (Kwong, Medina, et al., 2017; Kwong & Moran, 2016). These taxa include five main phylotypes (i.e., distinct taxonomic groups): *Bifidobacterium* spp. (Actinobacteria; Bifidobacteriales), *Gilliamella* spp. (Gammaproteobacteria; Orbales), *Snodgrassella* spp. (Betaproteobacteria; Neisseriales), and *Bombilactobacillus* spp. and *Lactobacillus* near *melliventris* (Firmicutes; Lactobacillales; previously referred to as *Lactobacillus* Firm-4 and *Lactobacillus* Firm-5, respectively). Honey bee gut microbiotas tend to contain all five of these phylotypes in addition to *Bartonella apis* and *Frischella perrara,* while many bumble bee species have lost *Bombilactobacillus* and gained symbionts from the genera *Bombiscardovia* and *Candidatus* Schmidhempelia (hereafter *Schmidhempelia*) (Kwong, Medina, et al., 2017); stingless bee species vary widely in the core phylotypes they have maintained (Cerqueira et al., 2021; Kwong, Medina, et al., 2017).

There are three main bee gut compartments: foregut, midgut, and hindgut. Core phylotypes primarily colonize the hindgut. The foregut, or crop, is used for nectar transport and feeding and contains few microbes; those that are present are often obligately or facultatively aerobic environmental taxa. The midgut also contains few taxa as its lining, the peritrophic matrix, is continuously shed, providing an unstable environment for microbial colonization. In contrast, the hindgut is lined by cuticle, which is shed for the final time during metamorphosis and is therefore stable in adult bees. The hindgut environment ranges from microaerophilic to completely anoxic (Zheng et al., 2017), thus most core phylotypes ferment

host dietary sugars for energy, save *Snodgrassella*, which aerobically respires organic acids (i.e., sugar fermentation products) of both dietary and host origin (P. Engel et al., 2012; Kešnerová et al., 2017; Lee et al., 2015; Quinn et al., 2024). Within the hindgut, core phylotypes show a degree of spatial segregation: *Snodgrassella* and *Gilliamella* (as well as *Schmidhempelia* in some bumble bee species) dominate communities in the ileum, while *Lactobacillus*, *Bombilactobacillus*, and *Bifidobacterium* dominate those in the rectum (Kwong & Moran, 2016).

Social bee gut microbiotas support host health and performance in a number of ways. The best-documented benefits are protection from pathogens and parasites (Cariveau et al., 2014; Koch & Schmid-Hempel, 2011; Miller et al., 2021; Motta et al., 2018; Palmer-Young et al., 2019; Steele et al., 2021) and stimulation of the host immune system (Horak et al., 2020; Kwong, Mancenido, et al., 2017; Lang et al., 2022; Steele et al., 2017; Wu, Zheng, Chen, Chen, et al., 2020), but gut microbiotas also promote host weight gain (Zheng et al., 2017) and hormone synthesis (Kešnerová et al., 2017), stimulate the expression of host detoxification genes (Wu, Zheng, Chen, Wang, et al., 2020), and may play additional roles in metal detoxification (Rothman et al., 2019), nutrient processing (Kešnerová et al., 2017; Lee et al., 2018; Zheng et al., 2017, 2019), and host learning, memory, and behaviour (Cabirol et al., 2023; Leger & McFrederick, 2020; Li et al., 2021; Liberti et al., 2022; Z. Zhang et al., 2022). However, social bee gut microbiota community composition and structure can vary with season (Bosmans et al., 2018; Kešnerová et al., 2020) and host species (Cerqueira et al., 2021; Kwong, Medina, et al., 2017), and can be altered by exposure to antibiotics or toxicants (Motta et al., 2018; Raymann et al., 2017; Seidenath et al., 2023). These variations and alterations in the gut microbiota may impact its ability to support host health (Motta et al., 2018).

Still, the vast majority of research on social bee gut microbiotas has been conducted in honey bees, specifically one species: *Apis mellifera*. Bumble bees differ from honey bees with regards to biology, ecology, diversity, and the composition of their microbiotas (Heinrich, 2004; Kwong, Medina, et al., 2017; Michener, 2007), and these differences may limit how well knowledge of social bee gut microbiotas gained from research in honey bees translates to bumble bees and their microbial communities.

In this thesis, I explore alterations and variations in the composition, community structure, and metabolic potential of bumble bee gut microbiotas to expand our knowledge of this important symbiosis.

Chapters 2 and 3 of my thesis are centred around a potential anthropogenic driver of variation in bumble bee gut microbiotas: pesticide exposure. I begin by qualitatively and quantitatively synthesizing literature on the effects of pesticide exposure on bee gut microbiotas (Chapter 2). In this review, I also highlight areas in need of further investigation, such as the effects of herbicide and fungicide exposure on social bee gut microbiotas, and the effects of pesticide exposure in social bee-microbiota systems other

than *Apis mellifera*. In my next chapter, I experimentally test how exposure to the fungicide chlorothalonil, which is commonly used on bumble bee-pollinated tomatoes, alters bumble bee microbiotas (Chapter 3). I also use this experiment as an opportunity to evaluate the use of bumble bee fecal microbiotas as a proxy for gut microbiotas.

In Chapter 4, I investigate how a natural source of disturbance, diapause, can alter bumble bee gut microbiota community structure and function. Before spring colony establishment, bumble bee queens undergo winter diapause; during this period their gut microbiotas are disturbed (Bosmans et al., 2018), but little is known about community dynamics during diapause itself. Bumble bee queens establish their colonies alone and their gut microbiotas seed those of their workers (Su et al., 2021). Thus, it important that bumble bee queen gut microbiotas recover post-diapause to a typical community structure. In this chapter, I use metagenomic sequencing to examine how bumble bee queen gut microbiota community structure and metabolic potential shift during and after diapause. Additionally, I test whether pesticide exposure soon after emergence from diapause can impact post-diapause gut microbiota recovery.

I conduct the research in Chapters 3 and 4 using commercial bumble bee colonies. Bumble bee gut microbiota composition and community structure can vary with colony origin (i.e., whether the colony comes from the wild or a commercial rearing facility) (e.g., Mockler et al., 2018; Motta & Moran, 2023) and bumble bee host species (e.g., Kwong, Medina, et al., 2017; Praet et al., 2018), though it is unclear whether variation in microbiota function follows suit. In Chapter 5, I use metagenomic sequencing to examine how gut microbiota community composition, structure, and metabolic potential vary across bees from two different commercial *Bombus impatiens* suppliers, wild *B. impatiens*, and three other wild bumble bee species sampled in a portion of their native eastern North American range.

In Chapter 6, I discuss and synthesize major results from previous chapters and present directions for future research on bumble bee gut microbiotas.

Though each chapter was written as a stand-alone paper, these four projects together are linked by my goal to provide a better understanding of the bumble bee-gut microbiota symbiosis, and thereby a better understanding of factors that influence the health and performance of a key group of native pollinators.

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Chapter 2

Pesticide-induced disturbances of bee gut microbiotas: A synthesis of studies investigating interactions between pesticides, bee gut microbial communities, and their hosts

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2.1 Abstract

Social bee gut microbiotas play key roles in host health and performance. Worryingly, a growing body of literature shows that pesticide exposure can disturb these microbiotas. Most studies examine changes in taxonomic composition in Western honey bee (*Apis mellifera*) gut microbiotas caused by insecticide exposure. Core bee gut microbiota taxa shift in abundance post-exposure but are rarely eliminated, with declines in Bifidobacteriales and *Lactobacillus* near *melliventris* abundance being the most common shifts. Pesticide concentration, exposure duration, season, and concurrent stressors all influence whether and how bee gut microbiotas are disturbed. Also, the mechanism of disturbance – i.e., whether a pesticide directly affects microbial growth or indirectly affects the microbiota by altering host health – likely affects disturbance consistency. Despite growing interest in this topic, important questions remain unanswered. Specifically, metabolic shifts in bee gut microbiotas remain largely uninvestigated, as do effects of pesticide-disturbed gut microbiotas on bee host performance. Furthermore, few bee species have been studied other than *A. mellifera*, and few herbicides and fungicides have been examined. We call for these knowledge gaps to be addressed so that we may obtain a comprehensive picture of how pesticides alter bee gut microbiotas, and of the functional consequences of these changes.

2.2 Introduction

Bees provide critical pollination services for natural and agricultural plant communities. Despite their ecological and economic importance, populations of some bee species have experienced dramatic declines in recent decades (Goulson et al., 2015; Zattara & Aizen, 2021). These declines are thought to be driven by a combination of factors including land-use change, climate change, increases in pathogen and parasite loads, and pesticide use (Goulson et al., 2015).

Bees are exposed to a variety of pesticides – including herbicides, fungicides, and insecticides – which have a suite of lethal and sub-lethal effects on their health. Bees encounter pesticides while foraging for nectar and pollen (their primary sources of sugar and protein, respectively) and transport these residues back to their nests; residues of multiple pesticides have been found in pollen and wax samples in bee hives and in bees themselves (Botías et al., 2017; Mullin et al., 2010; Traynor et al., 2016). In addition, bee species that nest in the ground, such as some bumble bees, stingless bees, and solitary bees, may be exposed to pesticide residues in soil while constructing and provisioning their nests (Kopit & Pitts-Singer, 2018; Willis Chan et al., 2019). These pesticides are often lethal to bees in sufficiently high doses (Sanchez-Bayo & Goka, 2014), but may also affect bee health at sub-lethal doses by impairing foraging, altering gene expression and caste differentiation, and decreasing weight gain, colony growth, and reproductive output (Bernauer et al., 2015; Boncristiani et al., 2012; Chmiel et al., 2020; Dos Santos et al., 2016; Garrido et al., 2013; Lima et al., 2016; Sandrock et al., 2014).

While a vast amount of research has been dedicated to understanding how pesticides affect bees themselves, we are just beginning to grasp how pesticides affect a key component of bee biology: their gut microbiota. Larval bees have gut microbiotas that are inconsistent in presence and composition both within individuals through time and among individuals within species (Hroncova et al., 2015; Martinson et al., 2012; Vojvodic et al., 2013), though the presence of some microbes has been shown to increase survivorship (Corby-Harris et al., 2014). Larval gut microbiotas are lost when the fore- and hindgut lining is shed during metamorphosis (Engel & Moran, 2013). Adult bees therefore emerge essentially gut microbe-free, and their guts are subsequently re-colonized through contact with environmental microbes and/or social interactions with conspecifics (J. E. Powell et al., 2014; Voulgari-Kokota et al., 2019). However, the associations of adult bees with their gut microbes vary widely among species. At one extreme are solitary bees, representing over 75% of the roughly 20,000 known bee species (Danforth et al., 2019), whose gut microbiotas are both transient and compositionally inconsistent, and are composed primarily of environmental microbes (Cohen et al., 2020; Martinson et al., 2011; McFrederick et al., 2014; Voulgari-Kokota et al., 2019, 2020). These microbes are not thought to be vital to their hosts' health, although much is unknown about their ecology. At the other end of this spectrum are the far less speciose eusocial corbiculate bees (748 known species (Danforth et al., 2019)). This clade of bees

contains three major lineages: honey bees (tribe Apini), bumble bees (tribe Bombini), and stingless bees (tribe Meliponini). The gut microbiotas of these bees are highly intra-specifically conserved, with specialized, host-adapted microbes transmitted among adults through social interaction (Hammer et al., 2021; Kwong & Moran, 2016). These microbiotas are also characterized by their simple composition, consisting of five core anaerobic or microaerophilic bacterial phylotypes: *Gilliamella* spp. (Gammaproteobacteria; Orbales)*, Snodgrassella alvi* (Betaproteobacteria; Neisseriales), *Bifidobacterium* spp. (Actinobacteria; Bifidobacteriales), and *Bombilactobacillus* spp. and *Lactobacillus* near *melliventris* (Firmicutes; Lactobacillales); these last two phylotypes were formerly known as *Lactobacillus* Firm-4 and *Lactobacillus* Firm-5, respectively, and are referred to collectively in this review as the "*Lactobacillus* cluster". Honey bees tend to have all five core phylotypes present along with *Bartonella apis* and *Frischella perrara,* while many bumble bee species seem to have lost the *Bombilactobacillus* phylotype and gained symbionts from the genera *Bombiscardovia* and *Schmidhempelia* (Kwong, Medina, et al., 2017); stingless bee species vary widely in the core phylotypes they have maintained (Kwong, Medina, et al., 2017). Unlike those of solitary bees, social bee gut microbiotas are known to benefit host health. The best-documented benefits are defense against pathogens and parasites (Cariveau et al., 2014; Koch & Schmid-Hempel, 2011; Miller et al., 2021; Palmer-Young et al., 2019) and stimulation of the host immune system (Horak et al., 2020; Kwong, Mancenido, et al., 2017; Steele et al., 2017), but these microbial communities also promote host weight gain (Zheng et al., 2017), stimulate expression of host detoxification genes (Wu, Zheng, Chen, Wang, et al., 2020), and may play additional roles in metal detoxification and nutrient processing (Engel et al., 2012; Kešnerová et al., 2020; Lee et al., 2018; Rothman et al., 2019; Zheng et al., 2017, 2019), although these final two areas require further investigation.

Social bee microbiotas clearly play a key role in host physiology; thus, it is important to understand how they are affected by pesticide exposure. Beginning in the 1970s and intensifying in the past decade, researchers have conducted studies to determine whether and to what extent pesticides can disturb (i.e., alter the community composition of) bee gut microbiotas and if these disturbances have negative effects on host health and performance. In this review, we summarize literature investigating the effects of pesticide exposure on bee gut microbial communities, examining researchers' choices of study taxa, pesticides, and methods, and synthesizing their results. We also highlight gaps in this area of research that remain to be thoroughly investigated and provide recommendations for future studies.

Gut microbial communities of adult social bees are not only much better characterized than those of solitary and larval bees but also appear to have a greater influence on host health and performance. As such, it is unsurprising that studies investigating the effects of pesticide exposure on bee gut microbiotas have focused on the adult stage of social corbiculate bees and, therefore, so shall this review.

2.3 Methods for Investigating Pesticide-induced Disturbance of Bee Gut Microbiotas

2.3.1 General experimental design

Experiments that explore the effects of pesticides on bee gut microbiotas are generally designed in the following manner: 1) they are carried out in-lab with groups of adult bees (isolated from the colony) as the experimental replicates, 2) replicates are grouped into two types of treatments – pesticide-exposed and pesticide-naïve (the exact number of treatment groups depends on the number of pesticides and concentrations tested), 3) bees in the pesticide-naïve group consume sterile pollen and a sterile sugar solution, while bees in the pesticide-exposed group consume sterile pollen and a sterile sugar solution containing the pesticide for a determined duration, 4) at some point during or after pesticide exposure, bees are euthanized, and 5) changes in microbial presence and/or abundance are examined, usually via 16S rRNA gene amplicon sequencing or quantitative polymerase chain reaction (qPCR) (Supplementary Table 2-1). However, some studies deviate from this general framework. For example, some researchers expose bees to pesticides at the hive level (Kakumanu et al., 2016), some conduct their experiments in the field, allowing bees to freely forage on crops or wildflowers (Gilliam & Morton, 1974; Motta et al., 2020), and others expose bee hives to pesticides by placing them next to pesticide-treated or pesticide-free crops (Jones et al., 2017; Wintermantel et al., 2018).

2.3.2 Host taxa choice

To date, the effects of pesticide exposure on bee gut microbiotas have been examined in only five bee species: *Apis mellifera, A. cerana, Bombus terrestris, B. impatiens*, and *Partamona helleri*. Studies on *A. mellifera*, the Western honey bee, vastly outnumber the rest (Figure 2-1A), but only eight of 748 social corbiculate bee species are honey bees (genus *Apis*) (Danforth et al., 2019). Approximately one third of the remaining species are bumble bees (*Bombus* spp.) and most are stingless bees (Meliponini, the tribe that includes *Partamona*) (Figure 2-1B); both of these taxa are major contributors to pollination of both agricultural and natural plant communities (Garibaldi et al., 2013; Greenleaf & Kremen, 2006; Ishimatsu et al., 1989; Pérez-Méndez et al., 2020; Slaa et al., 2006; Winfree et al., 2007). *Apis* bees thus represent less than one percent of known social corbiculate bee species, yet one species from this genus is the sole chosen host for 15 of the 19 modern studies on pesticide–bee gut microbiota interactions (Table 2-1).

Reasons for this large imbalance in host species choice likely include that 1) *A. mellifera* is the most widely managed crop pollinator globally (because of our great dependence on this species for agricultural pollination and honey production, there is great interest in its health); 2) the species is amenable to research (methods and tools for colony maintenance in both field and lab settings are well-developed and colonies are enormous, with workers numbering in the tens of thousands); 3) the gut

microbiota of *A. mellifera* workers is the best-characterized of any bee species (Kwong & Moran, 2016), providing a strong foundation for studies on pesticide-induced disturbance; and 4) *A. mellifera* is widely recognized as a model organism for gut microbiota research in general due to the low diversity of its gut microbial community (Zheng et al., 2018).

While the rationale for choosing *A. mellifera* as the host species for studies on pesticide-induced disturbance of bee gut microbiotas is sound, there are differences among the gut microbiotas and ecologies of social corbiculate bee species that may cause variation in how their microbiotas respond to pesticide exposure. To begin, when comparing core phylotypes across social bee gut microbiotas, overall strain diversity is higher in *A. mellifera* than in other species of honey or bumble bees (Ellegaard et al., 2020; E. Powell et al., 2016). In accordance with the species-area relationship in ecology (Connor & McCoy, 1979), strain diversity in social bee gut microbiotas is thought to be positively associated with colony size (Ellegaard et al., 2020; Kwong, Medina, et al., 2017; E. Powell et al., 2016). As *A. mellifera* colonies are orders of magnitude larger than those of other bee species, even those within the same genus (*A. mellifera*: 50,000+ individuals, *A. cerana*: 5,000+ individuals, *Bombus* spp.: 200+ individuals, Meliponini: 100-8000+ individuals (Wille, 1983)), their gut microbiotas are expected to be more diverse. Additionally, all *Apis* species form new colonies via colony fission in which a queen takes about half of the workers (and all of their resident gut microbes) from her natal colony with her when she leaves to establish a new colony. In contrast, new queens of other social bee species, like bumble bees, establish new colonies alone, creating a genetic bottleneck for their resident microbes. Colony establishment in bumble bees also occurs after hibernation, a period during which body temperature drastically lowers and food consumption ceases, greatly disturbing core gut microbial taxa (Bosmans et al., 2018). The higher gut microbial diversity maintained by *A. mellifera* may be beneficial if different strains of the same phylotype are functionally redundant but vary in their resistance to different environmental stressors. For example, Motta *et al*. (2018) demonstrated that the growth of some *S. alvi* strains—but not others—is inhibited by glyphosate exposure. As stressors change over the lifetime of an individual bee or a whole colony, different strains may become temporarily dominant, yet the overall functioning of the microbial community could be maintained.

Honey bees also differ from other social corbiculate bees in sensitivity to pesticide exposure. The median lethal doses of pesticides for honey bees are generally lower than those for bumble bees and higher than those for stingless bees (perhaps owing to the size differences among these taxa, since doses are often measured in $\mu g\ bee^{-1}$), although stingless bees show an immense range of interspecific variation (Arena & Sgolastra, 2014; Sanchez-Bayo & Goka, 2014). One of the ways that pesticides disturb gut microbiotas is by impairing host health to the point that the host is unable to properly regulate its gut microbiota, leading to dysbiosis (Box 1; Figure 2-2) (Yuan et al., 2019). If social corbiculate bee species

differ in their pesticide sensitivities, the doses of pesticides and durations of exposure that cause gut microbiota dysbiosis via a decline in host health likely vary as well.

Ultimately, *A. mellifera* is but one of hundreds of social corbiculate bee species whose ecologies and physiologies are sufficiently varied that results from studies investigating pesticide-induced gut microbiota disruption in one species are unlikely to apply to all others. Future research on pesticide–bee gut microbiota interactions should endeavour to broaden the choice of host taxa to adequately reflect the diversity of social bees, though studying species that are not managed for agricultural pollination or honey production will present a significant challenge.

2.3.3 Pesticide choice

In total, 29 pesticides, pesticide formulations, and pesticide metabolites have been examined for their effects on bee gut microbiotas. Eighteen of these compounds have been the focus of recent studies (Figure 2-3), and 14 have been shown to affect bee gut microbiota structure *in vivo* (Table 2-1). The most-studied of these compounds, by far, is the herbicide glyphosate: experiments have examined the effects of the pure compound, a major metabolite, and a commercial formulation on honey bee gut microbiotas in lab and field settings (Blot et al., 2019; Motta et al., 2020; Motta & Moran, 2020), as well as the mechanism of disturbance (Motta et al., 2018, 2020), and the disturbance's effect on host performance (Motta et al., 2018). No insecticidal or fungicidal compounds have been investigated in comparable depth, even those that have been examined in multiple studies such as imidacloprid and thiamethoxam. To date, few commercial insecticidal formulations have been tested, some insecticides and fungicides have only been examined in-lab, and the mechanism of disturbance has not been definitively elucidated for any insecticide or fungicide.

While insecticides are currently the most common choice of compound for pesticide-bee gut microbiota studies, increasing the diversity of fungicides and herbicides studied should be a priority. From a conservation standpoint, it is less critical to study the effects of insecticides on bee gut microbiotas as their adverse effects on bee health are already well established (though studies on insecticidal compounds can still provide valuable insights into how bee gut microbiotas are disturbed by pesticide exposure). Herbicides and fungicides, on the other hand, are considered relatively safe for bees, as these compounds tend to have higher lethal doses than bees are likely to encounter while foraging (Mullin et al., 2010; Sánchez-Bayo & Goka, 2016). However, these pesticides still have a wide range of sub-lethal effects on bee health (Chmiel et al., 2020) and may also directly affect bees' resident microbes. Fungicides and herbicides disrupt molecular pathways found in fungi and plants, respectively, but some of these pathways are also found in bacteria, such as the shikimate pathway targeted by glyphosate (Motta et al., 2018). Additionally, fungicides and herbicides may be used as a source of nutrients by bacteria

(Van Eerd et al., 2003). Therefore, though a pesticide may have low toxicity to the bee host, it may still affect host health and performance by disturbing the gut microbiota. Herbicide and fungicide residues are commonly found in bee colony material (Mullin et al., 2010), so research on pesticide-induced disturbance of bee gut microbiotas would benefit from in-depth investigation on a greater variety of compounds from these groups, particularly those that are widely used. Even in the case of herbicides, where the focus on glyphosate reflects its status as the most-used herbicide globally, current motions to limit and ban glyphosate use make it crucial that we test potential replacements for their effects on bee gut microbiotas (Beckie et al., 2020; Cruz et al., 2021; Székács & Darvas, 2018).

Finally, pesticides are often not applied as pure compounds to crops but in formulations (e.g., Roundup® for glyphosate). These formulations contain additional solvents which may themselves affect the bee gut microbiota. For example, exposure to Roundup®, but not glyphosate, decreased the abundance of *Gilliamella* and *Bifidobacterium* bacteria in honey bees (Motta et al., 2020), suggesting that other components of the Roundup® formulation can disturb bee gut microbes. Further applied research should examine the effects of commercial pesticide formulations on bee gut microbiotas so the effects of solvents, on their own or in interaction with the active ingredient, can be better understood.

2.3.4 Measuring responses in the microbiota: Early studies

The first attempts to characterize the effects of pesticides on bee gut microbiotas were a series of studies on Western honey bees (*A. mellifera*) led by Martha Gilliam in the 1970s (Gilliam et al., 1977; Gilliam, Prest, et al., 1974; Gilliam, Wickerham, et al., 1974; Gilliam & Morton, 1974, 1978), followed by a study by Drobníková and Bacílek (1982). All early studies used aerobic culturing techniques to either compare microbes isolated from pesticide-exposed honey bees to those from pesticide-naïve bees (Gilliam et al., 1977; Gilliam, Prest, et al., 1974; Gilliam, Wickerham, et al., 1974; Gilliam & Morton, 1974, 1978), or investigate the effects of pesticides on the *in vitro* growth of bacteria isolated from honey bee guts (Drobníková & Bacílek, 1982). While some core bee gut microbes can grow aerobically (i.e., *Lactobacillus* cluster phylotypes), many cannot, as they are adapted to an anoxic gut environment (Engel et al., 2013). It is therefore likely that the culturing techniques of these early studies failed to capture the true microbial diversity of the guts they examined. However, their results still provide valuable insights into how certain constituents of bee gut microbiotas respond to pesticide exposure (**see Community Profiles of Pesticide-Disturbed Bee Gut Microbiotas: Early Studies**).

2.3.5 Measuring responses in the microbiota: Current methods

Modern studies (i.e., 2016 onwards) primarily use non-culture-based techniques to examine changes in bee gut microbiotas following pesticide exposure (though some studies supplement these

approaches with anaerobic and/or microaerophilic culturing (Blot et al., 2019; Motta et al., 2018). Most studies track changes in taxonomic composition using 16S rRNA gene amplicon or transcript sequencing (Botina et al., 2019; Dai et al., 2018; Kakumanu et al., 2016; Motta & Moran, 2020; Paris et al., 2020), often coupled with qPCR-based methods in order to obtain data on gene or transcript copy counts (Blot et al., 2019; Motta et al., 2018; Raymann et al., 2018; Rouzé et al., 2019). While social bee gut microbiotas have previously been examined using metagenomic methods (Ellegaard et al., 2020; Engel et al., 2012; Zheng et al., 2019), these methods have not yet been used when investigating bee gut microbiota–pesticide interactions.

qPCR approaches may involve using phylotype-specific primers, and primer choice in this case appears to be relatively important: while some taxa show identical shifts in abundance regardless of the primer pair used, shifts in other taxa appear to be primer-dependent. For example, Rouzé *et al*. (2019) found an increase in *Snodgrassella alvi* abundance when using *S. alvi*-specific primers but not Neisseriaceae-specific primers, and Blot *et al*. (2019) found conflicting results after examining *Gilliamella apicola* abundance using two different primer sets. One explanation for why we observe primer-dependent qPCR results is that different primer pairs target different clades within a given phylotype (Blot et al., 2019). For core phylotypes with high strain-level diversity, such as *G. apicola* and *Lactobacillus* cluster spp. (Ellegaard & Engel, 2019), a greater diversity of primers may be required to fully capture the responses of all clades to pesticide exposure.

2.3.6 Measuring changes in functional capabilities

Though taxonomic changes in bee gut microbiota community structure after pesticide exposure have been well-documented using techniques such as 16S rRNA gene amplicon sequencing (DeGrandi-Hoffman et al., 2017; Jones et al., 2017; Kakumanu et al., 2016), qPCR with taxon-specific primers (Blot et al., 2019; Rouzé et al., 2019), active profiling via sequencing of 16S and 18S rRNA transcripts (Motta & Moran, 2020; Paris et al., 2020), and community-level fatty acid profiling (Diaz et al., 2019), functional changes have yet to be studied in depth. Understanding how pesticides affect microbial gene expression and metabolism is as important to our understanding of how pesticide exposure affects bee gut microbiotas as knowledge of taxonomic shifts, if not more so. While resident microbes may confer resistance against pathogen and parasite infection physically through exploitative competition for space and nutrients (McLaren & Callahan, 2020) and by stimulating the host immune system (Horak et al., 2020; Kwong, Mancenido, et al., 2017; Steele et al., 2017), core gut microbe metabolites, such as toxins and short-chain fatty acids, may also protect against infection through direct bacterial antagonism (Steele et al., 2017) and by modifying physiochemical conditions in the host gut (Palmer-Young et al., 2019). Gut microbiota metabolism also maintains low oxygen levels in bee guts, which affects the products of

microbial fermentation (Zheng et al., 2017), and there is evidence of some cross-feeding between core bee gut phylotypes (Kešnerová et al., 2017; Kwong et al., 2014), indicating that microbial metabolism may be important to the proper functioning of a healthy bee gut microbiota. Microbial metabolites may also be absorbed and used by bee hosts, but this remains mostly speculative (Bonilla-Rosso & Engel, 2018; Hammer et al., 2021). Given the many pathways through which microbial metabolism appears to affect the health of the host and the microbial community, it is important to examine whether and how this metabolism changes after pesticide exposure.

It is also important to examine functional changes because changes in the structure of microbial communities do not always reflect changes in community function. On one hand, gut microbiotas often display a high degree of functional redundancy, possibly due to a high frequency of horizontal gene transfer (Moya & Ferrer, 2016; Smillie et al., 2011). This is also true of bee gut microbiotas – four out of five core phylotypes (all except *S. alvi*) are sugar fermenters whose primary metabolic products are organic acids, though they also display some phylotype-specific metabolic pathways (Kešnerová et al., 2017; Zheng et al., 2017), and some toxin-producing genes are found in both *S. alvi* and *G. apicola* (Steele et al., 2017). It is possible that despite shifts in community structure caused by pesticide exposure, the overall function of the bee gut microbiota remains consistent through compensation by functionally similar phylotypes. On the other hand, exposure of human gut microbiotas to various xenobiotics produces significant changes in microbial gene expression even when taxonomic shifts are subtle (Maurice et al., 2013). Core bee gut microbiota phylotypes have a large number of accessory genes (genes that are not found in every strain) (Ellegaard et al., 2015; Engel et al., 2014); therefore, even small shifts in bee gut microbiota community structure after pesticide exposure may alter community gene expression or metabolite production in a biologically significant way.

A study by Kakumanu *et al*. (2016) is the only one so far to examine changes in the functional profiles of bee gut microbiotas before and after pesticide exposure. Using PICRUSt (Langille et al., 2013) to compare the predicted functional composition of pesticide-exposed and pesticide-naïve honey bee gut microbial communities, they found that exposure to the fungicide chlorothalonil increased predicted gene counts in gene families related to oxidative phosphorylation and decreased predicted gene counts in gene families related to sugar metabolism and protease activity (Kakumanu et al., 2016). However, it is important to note that microbial functions inferred from 16S rRNA gene sequences, like those in PICRUSt, are rarely accurate due to the prevalence of horizontal gene transfer in microbial communities. A more appropriate alternative to these inferential methods is to track actual community-level changes in either mRNA transcripts or metabolites in response to pesticide exposure using untargeted or targeted metatranscriptomics or metametabolomics. However, such studies have yet to be conducted, likely because, at the time of writing this review, these methods are typically more expensive than standard gene

amplicon sequencing. A further constraint is that these methods require large amounts of genetic or metabolomic material, and so may only be feasible for bee species with large colony sizes.

Investigating the transcriptome and metabolome of bee gut microbiotas before and after pesticide exposure could also provide insights into whether and how bee gut microbes metabolize pesticides. Certain microbes are able to detoxify xenobiotics, including pesticides (Gressel, 2018; van den Bosch & Welte, 2017), while others can transform xenobiotics into more toxic metabolites (Claus et al., 2016; Collins & Patterson, 2020). These processes may also occur in bee gut microbes, though there is currently little evidence to support this; Raymann *et al*. (2018) examined the production of imidacloprid metabolites by bee gut microbes *in vitro* using targeted metabolomics and found only weak evidence for imidacloprid degradation. Furthermore, because absorption — and therefore potential toxicity — of many pesticides occurs in the midgut (Engel & Moran, 2013), it is likely that 1) many pesticides are absorbed by the bee host before reaching the gut microbiota, which is primarily present in the hindgut, and 2) even if these compounds do reach the hindgut and undergo microbial transformation, many metabolites will not be absorbed by the host. Therefore, with our current knowledge of bee host physiology, it seems premature to conclude that microbial transformation of pesticides affects bee host health.

2.4 Community profiles of pesticide-disturbed bee gut microbiotas

2.4.1 Early studies

Studies on pesticide-induced disturbance of bee gut microbiotas published before DNA sequencing was widely available provided early evidence that a variety of pesticides can affect the presence of microbes in honey bees and the growth of microbes isolated from honey bees *in vitro*, laying the foundation for more in-depth future experiments. Exposure to the herbicide 2,4-D had little effect on the presence of Enterobacteriaceae bacteria or non-yeast fungi in honey bee gut microbiotas (Gilliam, Prest, et al., 1974; Gilliam & Morton, 1974). However, it did decrease the presence and diversity of *Bacillus* bacteria (Gilliam & Morton, 1978). Both 2,4-D and 2,4,5-T, another herbicide, increased the presence and diversity of yeasts (Gilliam et al., 1977; Gilliam, Wickerham, et al., 1974). Drobníková and Bacílek (1982) also demonstrated that yeasts had greater tolerance than bacteria to pesticide exposure. Only one fungicide, folpet, was able to inhibit the growth of yeast isolates; conversely, two fungicides and three insecticides (fenitrothion, pirimiphos-methyl, and pirimicarb) inhibited bacterial growth. Together, these early studies suggest that yeasts in bee guts may be better able than other fungi and bacteria to withstand pesticide exposure.

2.4.2 Recent studies

Recent studies have provided a plethora of information on the taxonomic shifts in bee gut microbiotas after pesticide exposure, mostly based on 16S rRNA gene amplicon sequencing data. We conducted a systematic review of *in vivo* taxonomic shifts recorded in recent studies to: 1) determine whether microbial abundance tends to increase or decrease after pesticide exposure, 2) quantify the magnitudes of these increases and decreases, and 3) identify which microbial taxa are most often disturbed by pesticide exposure.

2.4.2.1 Analysis methods

To synthesize microbial taxonomic shifts recorded in recent studies, we used key word searches on Web of Science and Scopus as well as Google Scholar alerts to find articles in which researchers exposed bees to a pesticide and tracked subsequent changes in their gut microbiotas (Supplementary Table 2-2). We also looked through the references of those articles and relevant reviews for additional papers. From these studies, we extracted the effect sizes of all *in vivo* changes in bacterial abundance between pesticide-exposed and pesticide-naïve bees that were determined by 16S rRNA gene amplicon sequencing, 16S rRNA transcript sequencing, or qPCR with taxon-specific primers (Supplementary Table 2-3). Where possible, we took effect sizes from tables and text; however, most values were extracted from figures using the online software WebPlotDigitizer version 4.3 (Rohatgi, 2020). Effect sizes for taxonomic groups categorized as "Other" were excluded. If multiple figures of the same results at different taxonomic resolutions were provided, effect sizes were extracted from the figure with the highest taxonomic resolution. If data were presented for each replicate in a treatment, effect sizes were extracted for each replicate and subsequently averaged across each treatment (Supplementary Table 2-3). We categorized effect sizes by significance, whether the data were relative abundances or gene/transcript copy counts (Box 2), and whether the microbial taxon was part of the core bee gut microbiota. Taxa were only categorized as "non-core" if there was no possibility of those taxa containing any core phylotypes. We then converted effect sizes to $\log_2$-fold changes in abundance (treatment/control); taxon gains and losses (i.e., where control or treatment abundance of a taxon was zero) were coded as $\log_2$-fold changes of 50 and −50 respectively. We generated two sets of figures using the ggplot package in R (R Core Team, 2023; Wickham, 2016). For the first set (Figure 2-4), we included relative abundance data and gene/transcript copy count data for core taxa in the same figure and compared effect sizes for glyphosate separately from all other pesticides; as most effect sizes are from experiments on glyphosate (71% of all effect sizes, 81% of significant effect sizes), when plotted together overall trends were driven primarily by how taxa responded to glyphosate. For the second set of figures (Figure 2-5), we separated effect sizes from relative abundance data and gene/transcript copy count data but did not separate effect sizes based

on pesticide identity. For the sake of clarity, taxon gains and losses were omitted from both sets of figures. All taxon gains and losses were insignificant.

There are three key components to these results: the direction of the average change in abundance, the magnitude of this change, and the number of pesticides that cause a disturbance. While it would be useful to have a threshold for magnitude above which changes are biologically significant to host performance, we know too little about the functional consequences of shifts in bee gut microbiotas for bee hosts to be able to define such a threshold (see **Performance: The missing piece**).

2.4.2.2 Taxonomic shifts in community composition

Examining relative abundance and gene/transcript copy count data together, pesticides generally cause subtle disturbances in core bee gut microbiotas; core taxa shift in abundance, but they are rarely eliminated. Most shifts in abundance are small (i.e., within a $\log_2$-fold change of 1 or −1) (Figure 2-4) and are not statistically significant (Supplementary Table 2-3).

Bifidobacteriales and Lactobacillales bacteria both decline in abundance by similar magnitudes after exposure to pesticides (Figure 2-4). These taxa are also similarly susceptible to pesticide-induced disturbance: Lactobacillales abundance is significantly disturbed by eight pesticides while Bifidobacteriales abundance is significantly disturbed by seven. Interestingly, the average decline in Lactobacillales abundance and its susceptibility are driven by only one phylotype: *Lactobacillus* near *melliventris*. This phylotype decreases after exposure to most pesticides except for Pristine® (boscalid/pyraclostrobin mixture), which causes increases in abundance, and glyphosate, which can cause changes in both directions. *Bombilactobacillus* spp., on the other hand, increase in abundance by a relatively large magnitude post-pesticide exposure (when considering significant results only) and are significantly disturbed by only three pesticides, with glyphosate-based pesticides in particular having a consistently strong effect.

Neisseriales is similarly resistant to disturbance from pesticides, though glyphosate is a notable exception (Figure 2-4). The large decline in Neisseriales abundance (specifically, *Snodgrassella alvi* abundance) after glyphosate exposure is the best-documented pesticide-induced disturbance in bee gut microbiotas—the exact mechanism of disturbance has even been determined for some *S. alvi* strains (Motta et al., 2018). Besides glyphosate, fipronil is the only other pesticide known to significantly disturb Neisseriales, causing a relatively small increase in abundance (Figure 2-4).

Finally, the average change in abundance of Orbales after exposure to most pesticides is close to zero: pesticides seem equally likely to cause an increase or decrease in abundance (Figure 2-4). Orbales is also relatively susceptible to pesticide-induced disturbance, with six pesticides causing significant shifts in abundance.

In addition to examining the effects of pesticide exposure on core bee gut microbiota taxa, we also examined the average responses of non-core bacteria, not separated by data type (i.e., relative abundances or gene/transcript copy counts) or by pesticide identity (Supplementary Figure 2-1). Most changes in the abundances of non-core taxa are small and insignificant. However, when changes are significant, abundances are more likely to increase than decrease and the changes tend to be large. Whether this signal captured from non-core taxa is noise or is ecologically relevant remains to be determined.

Though taxonomic composition often changes in response to pesticide exposure, alpha diversity is generally consistent between pesticide-exposed and pesticide-naïve bees regardless of the diversity metric used (Kakumanu et al., 2016; Motta et al., 2018; Paris et al., 2020), though some studies report a decrease in alpha diversity in pesticide-exposed groups (DeGrandi-Hoffman et al., 2017; Liu et al., 2019). Effects of pesticides on intra-treatment beta diversity are more varied. When pesticides do cause significant taxonomic shifts in bee gut microbiotas, beta diversity in pesticide-exposed gut microbiotas may increase (Motta et al., 2018, 2020), decrease (Dai et al., 2018; Liu et al., 2019; Motta et al., 2018), or remain the same (Blot et al., 2019; Botina et al., 2019; Motta et al., 2018) when compared to the control group.

2.4.2.3 Relative abundance vs. gene/transcript copy count data

When examining statistically significant shifts in microbial abundance, average responses to pesticide exposure for relative abundance-based and gene/transcript copy count-based data are notably dissimilar (Figure 2-5). Bifidobacteriales, Lactobacillales, and *Lactobacillus* near *melliventris* abundances significantly increased on average when analyzed using relative abundance data but decreased on average with gene/transcript copy count data. This indicates that while these taxa may have increased in abundance relative to other taxa in the community, in some cases they may have decreased in absolute abundance. Similar results were observed in a glyphosate study where the relative abundances of some microbes increased post-pesticide exposure, but their gene copy counts decreased (Motta et al., 2018). Additionally, some pesticides, such as fipronil and thiamethoxam, show significant effects on bee gut microbiotas only when using gene/transcript copy counts and not with relative abundance data (Jones et al., 2017; Paris et al., 2020; Rouzé et al., 2019). In fact, it seems that significant declines in abundance, and indeed significant shifts in general, are more commonly observed using gene/transcript copy count data (Figure 2-5). Based our analysis, and the fact that gene and transcript copy counts more accurately reflect taxonomic shifts in microbial communities (Box 2), researchers may want to prioritize investigating pesticide-induced disturbances in bee gut microbiotas using gene/transcript copy count methods.

2.4.2.4 Intra-phylotype variation

While we have synthesized results at the order and phylotype levels, it is worth noting that recent studies have provided evidence that different strains of the same bacterial phylotype can differ in their tolerance of pesticide exposure. For example, the *in vitro* growth of some *S. alvi* strains is more inhibited by glyphosate exposure than the growth of others (Motta et al., 2018). When examining phylotypes with high strain diversity, using a variety of strains (and, if performing *in vitro* work alongside *in vivo* work, strains isolated directly from the bees used in the study) will help to capture the full variation in a given phylotype's response to pesticide exposure.

2.4.2.5 Hallmarks of bee gut microbiota dysbiosis

In recent years, some hallmarks of bee gut microbiota dysbiosis have been identified, including decreases in *Bifidobacterium* and *Lactobacillus* cluster abundance, increases in the abundance of Gammaproteobacteria, such as Orbales, and proliferation of non-core bacterial phylotypes (Anderson & Ricigliano, 2017; Daisley et al., 2020; Horton et al., 2015; Li et al., 2015). Our synthesis shows that the effects of pesticides on bee gut microbiotas are consistent with only some of these indicators. We found that, indeed, Bifidobacteriales and *Lactobacillus* near *melliventris* abundances tend to decline after pesticide exposure while non-core bacteria proliferate (Figure 2-4, Figure 2-5, Supplementary Figure 2-1). However, we also found that *Bombilactobacillus* abundance does not typically change significantly after pesticide exposure (though if it does, it tends to increase) and that Orbales abundance is equally likely to increase or decrease. The common characteristics of bee gut microbiota dysbiosis were primarily established by studies that investigated disturbances caused by antibiotics (Raymann et al., 2017), sub-optimal diets (Maes et al., 2016), or abnormal initial gut colonizers (Schwarz et al., 2016), not those caused by pesticides whose intended targets are non-bacterial organisms (i.e., insecticides, herbicides, and fungicides). It is possible that disturbances caused by these types of pesticides are not severe enough, particularly compared to antibiotic-induced disturbances, to cause all changes commonly observed in bee gut microbiota dysbiosis. Additionally, the dysbiosis profile is likely highly dependent on the pesticide causing the disturbance. For example, significant changes in abundances of core taxa caused by thiamethoxam exposure follow all hallmarks of bee gut microbiota dysbiosis, but significant changes caused by glyphosate do not (Figure 2-4).

2.4.2.6 Duration of and variability in disturbances

An important but little-studied dimension of pesticide-induced bee gut microbiota disturbance is how long the disturbance persists after pesticide exposure ceases. Diaz *et al*. (2019) found that fatty acid profiles of honey bee gut microbiotas can remain disturbed for at least seven days post exposure to

imidacloprid. Furthermore, Motta *et al.* (2020) demonstrated that honey bee gut microbiotas can remain disturbed for up to four weeks after Roundup® exposure, though it is possible that bees were still being exposed during this time due to pesticide accumulation in hive materials. As adult worker bees generally live around 30–40 days (though longevity varies widely between species and individuals (Free & Spencer-Booth, 1959; Fukuda & Sekiguchi, 1966; Giannini, 1997; Goldblatt & Fell, 1986; Gomes et al., 2015; Smeets et al., 2003)), these disturbance durations can represent a significant portion of an adult bee's lifespan. On the other hand, some evidence suggests that bee gut microbiotas can recover quickly from disturbance. Liu *et al.* (2019) demonstrated that the gut microbiotas of *A. mellifera* workers were disturbed on the seventh day of thiacloprid exposure but had recovered by the thirteenth day of exposure, possibly due to a decrease in pesticide consumption or rapid adaptation of resident microbes. These results suggest that, in some cases, bee gut microbiotas may be able to recover from pesticide-induced disturbance even during exposure.

Another important question is: does exposure to a given pesticide consistently disturb the same bee gut microbial taxa? Across recent studies, we observe that the consistency of disturbances varies among pesticides. Glyphosate, for example, consistently reduces *S. alvi* abundance across multiple concentrations, formulations, seasons, and studies, but its effects on other core taxa members are much more variable (Blot et al., 2019; Motta et al., 2018, 2020; Motta & Moran, 2020). Thiacloprid was also found to consistently disturb *Lactobacillus* near *melliventris* regardless of concentration (Liu et al., 2019). However, in other cases, different microbial taxa are disturbed after exposure to the same pesticide. Rouzé *et al*. (2019) found that exposure to 0.25 μg kg^{-1} of the insecticide fipronil reduced the abundance of *Lactobacillus* cluster spp. and *Bifidobacterium* spp. in honey bee guts but that exposure to 1 μg kg^{-1} increased the relative abundance of *G. apicola* and *S. alvi*, and Paris *et al*. (2020) found that fipronil had no effect on the relative abundance of bee gut microbes on its own. Differences in disturbance profiles were also found for low and high concentrations of the fungicide Pristine® (boscalid/pyraclostrobin mixture) (DeGrandi-Hoffman et al., 2017), and for almost identical concentrations and exposure durations of thiamethoxam (Paris et al., 2020; Rouzé et al., 2019).

One possible explanation for why some pesticides produce consistent disturbances and others do not is the mode of action (Box 1). Glyphosate, for example, directly inhibits *S. alvi* growth by blocking an amino acid synthesis pathway (Motta et al., 2018). As this pathway is consistently present in *S. alvi* strains, the effect of glyphosate on *S. alvi* is consistent as well (though some strains are glyphosate resistant (Motta et al., 2018)). Consistent disturbances may be likely when pesticides directly inhibit microbial growth via pathways that are functionally consistent and invariably present in the affected microbes. On the other hand, many insecticides, such as imidacloprid, thiamethoxam, and fipronil, are thought to disturb bee gut microbial taxa indirectly through a decline in host health (Rouzé et al., 2019).

The exact nature of the decline and how it affects the host's ability to regulate its gut microbiota likely depends on a variety of factors such as the host's health before pesticide exposure, host genetics, and immune system characteristics. Variation in these factors could cause variation in how host health is altered post-pesticide exposure, which in turn could cause variation in the gut microbiota's disturbance profile even within the same host species.

2.5 Additional factors that influence pesticide-induced bee gut microbiota disturbances

Beyond the host species and the identity of the pesticide being examined, numerous factors can affect pesticide-induced bee gut microbiota disturbances. For this review, we focus on four: pesticide concentrations, exposure duration, seasonality, and concurrent stressors.

2.5.1 Pesticide concentration

While it may seem logical that higher concentrations of pesticides would lead to more severe effects, the relationship between concentration and disturbance in bee gut microbiotas appears more complicated. Some studies, such as those of Liu *et al*. (2019) on thiacloprid and Motta and Moran (2020) on glyphosate, have found that disturbances in bee gut microbiotas increase in severity as pesticide concentrations increase. However, other studies have observed disturbances in bee gut microbiotas at low concentrations of a pesticide, but no disturbances at higher concentrations. A pair of studies which exposed honey bees to different concentrations of imidacloprid in sugar syrup for varying lengths of time showed that a concentration of 3.5 μg kg^{-1} could disturb gut microbiotas but a concentration of 500 μg L^{-1} did not (Raymann et al., 2018; Rouzé et al., 2019). All in all, increasing a pesticide's concentration does not consistently increase the magnitude of its effects on bee gut microbial taxa.

It is also important to consider that the concentration of a pesticide delivered in sugar syrup may not reflect the amount that is consumed or absorbed by bee hosts. Most studies track consumption of sugar syrup and are therefore able to estimate the amount of pesticide compound consumed per bee. However, few studies have calculated host body burden, or what concentration of the pesticide accumulates in bee host tissues (though see Liu *et al*. (2019) and Raymann *et al*. (2018)). Calculating body burden is a staple of toxicological studies (Inostroza et al., 2016; Rappaport & Smith, 2010; Van Meter et al., 2014) and so analyzing pesticide residues in bee bodies should be considered a priority for future studies on pesticide-induced disturbance of bee gut microbiotas, especially if the pesticide in question is thought to affect bee gut microbial taxa through its effect on host health. Because gathering such data involves destructive sampling, a larger number of bees would be required for a given experiment; consequently, such studies might be more readily accomplished in bee species with larger colonies.

2.5.2 Exposure duration

The length of pesticide exposure is another factor that plays an important role in pesticide-induced bee gut microbiota disturbances. Most studies on insecticides and fungicides use exposure periods longer than ten days, and, in general, longer periods of exposure appear more likely to disturb bee gut microbial community structure (Table 2-1). For example, three or four days of exposure to imidacloprid did not affect *A. mellifera* or *B. impatiens* gut microbiotas (Raymann et al., 2018; Rothman et al., 2020), but 18 days of imidacloprid exposure disturbed *A. mellifera* gut microbial communities, despite the pesticide's concentration being lower (Rouzé et al., 2019). These results imply that the duration of pesticide exposure may matter more than the concentration when determining whether a pesticide disturbs bee gut microbial taxa. However, a long exposure period for a pesticide does not guarantee gut microbiota disturbance (Wintermantel et al., 2018; Yang et al., 2019), and some studies with short pesticide exposure durations still show perturbations in bee gut microbiotas (Motta et al., 2018, 2020) (Box 2).

Pesticides' modes of action may explain some of the variation in the exposure duration required to induce disturbance (Box 1). Longer periods of exposure may be necessary for pesticides that disturb bee gut microbiotas indirectly through impairment of host health as pesticides need time to accumulate in host tissues and affect host physiology. Imidacloprid, which appears to disturb bee gut microbiotas only after longer exposure durations, is hypothesized to be one such pesticide (Raymann et al., 2017; Rouzé et al., 2019). Conversely, pesticides that directly inhibit microbial growth only need to be taken up by gut microbial cells to cause a disturbance, a process which likely takes significantly less time than affecting the health of an entire host organism. Glyphosate is one pesticide that directly inhibits bee gut microbes, and it can consistently disturb honey bee gut microbiotas after exposure periods of five days or less (Dai et al., 2018; Motta et al., 2018, 2020). Therefore, if a pesticide has strong direct effects on bee gut microbes it may be able to cause disturbances after a shorter exposure duration than pesticides that primarily affect gut microbiotas indirectly through host health.

While longer exposure durations appear more likely to produce observable effects of pesticides on bee gut microbiotas, they may not always be field-realistic. Pesticides that are applied as seed coatings—such as neonicotinoids and some fungicides—may be consistently present in agricultural environments over spring and summer months (Krupke et al., 2012; Lu et al., 2016). However, residues of pesticides applied via spray application may show temporal variation. Generally, there is a period of at least two weeks between pesticide sprays during which time pesticide residues in the environment will degrade and floral turnover may eliminate residue-laden pollen and nectar from the bees' food supply. Therefore, exposure to certain pesticides may be more realistically experienced in pulses and, depending on degradation and floral turnover rates, exposure periods lasting weeks may be unrealistic. Though bee

gut microbiotas appear more likely to be disturbed after chronic pesticide exposure, research that aims to be applicable to pollinator management and policy decisions should ensure that the exposure regimes used in experiments accurately reflect field conditions.

2.5.3 Seasonality

The gut microbiotas of many animals vary seasonally (Carey et al., 2013; Ferguson et al., 2018; Maurice et al., 2015) and those of bees are no exception (Bleau et al., 2020; Bosmans et al., 2018; Kešnerová et al., 2020). In social corbiculate bees, this variation occurs in tandem with changes in host diet, behaviour, and physiology (Fluri et al., 1982; Orčić et al., 2017; Steinmann et al., 2015), including sensitivity to pesticides (Baines et al., 2017; Saleem et al., 2020). The presence of these seasonal differences raises the possibility that interactions between pesticides and bee gut microbiotas may depend on season.

Studies on pesticide–bee gut microbiota interactions that incorporate season into their experimental design demonstrate that which taxa are disturbed by a given pesticide and the extent of the disturbances can vary seasonally. Rouzé *et al*. (2019) compared the abundances of core bee gut microbes after exposure to four insecticides (coumaphos, imidacloprid, thiamethoxam, and fipronil) in honey bees in both summer and winter. Though the direction of changes (i.e., whether taxa increased or decreased in abundance) were consistent between summer and winter, the magnitude of many changes depended on season (Rouzé et al., 2019). Conversely, Blot *et al*. (2019) found that season had a limited effect on glyphosate-induced disturbances in honey bee gut microbiotas. One possible reason for this discrepancy is a difference in the mode of action of the pesticide (Box 1). Glyphosate directly affects microbial growth through interference with amino acid synthesis (Motta et al., 2018), whereas the four insecticides studied by Rouzé *et al*. (2019) were proposed to perturb the gut microbiota through aspecific changes in the host's gut environment. While the mode of action of glyphosate toxicity would not change seasonally, pesticide-induced changes in the host gut environment may differ based on seasonal changes in host physiology, particularly pesticide sensitivity, which in turn may affect which, and to what degree, taxa are disturbed post-pesticide exposure.

It is also important to consider the ecological reality that seasonal studies on pesticide–gut microbiota interactions are meant to reflect. Many bumble bee species hibernate through the winter, consuming no food for months, which eliminates the opportunity for oral exposure to pesticides. Honey bees, on the other hand, may be chronically exposed to pesticides during the winter via stored food, though little is known about actual pesticide concentrations in overwintering hives. Consequently, it is impossible at the moment to determine ecologically realistic pesticide concentrations for studies

examining pesticide–bee gut microbiota interactions in the winter. Until more information on this topic is gathered, it will remain unclear how well results from such studies reflect what is occurring in the field.

2.5.4 Concurrent stressors

In the wild, organisms are rarely exposed to individual stressors. Instead, they often encounter multiple stressors concurrently, and these may act synergistically (Goulson et al., 2015). Paris *et al*. (2020) examined the effects of concurrent exposure to pesticides and the microsporidian parasite *Nosema ceranae* on the gut microbiotas of honey bees. They found that co-exposure to *N. ceranae* and various pesticides had primarily synergistic effects on some constituents of the gut microbiota (i.e., Gammaproteobacteria). In a separate study, *N. ceranae* infection was able to cause similar changes in abundance on its own, though a higher number of spores was used (Rouzé et al., 2019). Still, the study by Paris *et al*. (2020) demonstrates that under certain conditions, although pesticide exposure may not disturb bee gut microbial communities on its own, it may exacerbate disturbances caused by other stressors.

While studies on exposure to single pesticides are important for establishing which pesticides are capable of disturbing bee gut microbiotas, studies that expose bees to multiple concurrent stressors are more realistic. In addition to concurrent pathogen exposure, future studies should consider concurrent exposure to multiple pesticides, as such exposure is the norm for wild and agricultural bee populations (Long & Krupke, 2016; Manning et al., 2017; Mullin et al., 2010; Traynor et al., 2016) and pesticides can sometimes interact synergistically (Pillings & Jepson, 1993; Schmuck et al., 2003; Wade et al., 2019).

2.6 Performance: The missing piece

One of the most important questions concerning pesticide-induced disturbance of bee gut microbiotas is what effect, if any, these disturbances have on host performance. Here we use the term "performance" to encompass the fitness, behaviour, physiology, and development of hosts at both the individual and colony levels. Previous studies investigating the roles of bee gut microbiotas in host performance have largely used comparisons between bees with conventional microbiotas and those that are gut microbiota-free or deficient (Koch & Schmid-Hempel, 2011; Rothman et al., 2019; Wu, Zheng, Chen, Chen, et al., 2020; Zheng et al., 2017). However, it is unclear if the subtler changes caused by pesticide exposure affect host performance in a biologically significant manner.

The most often-tracked performance metric is survival in pesticide-exposed vs. pesticide-naïve bees (Dai et al., 2018; Liu et al., 2019; Motta et al., 2018, 2020; Motta & Moran, 2020; Paris et al., 2020; Raymann et al., 2018; Rothman et al., 2020; Rouzé et al., 2019; Yang et al., 2019; Zhu et al., 2020), but studies have also examined sublethal effects on host behaviour (Botina et al., 2019), individual and

colony development (Dai et al., 2018; Wintermantel et al., 2018), host weight gain (Dai et al., 2018; Liu et al., 2019; Wintermantel et al., 2018), host gene expression (Zhu et al., 2020), and susceptibility to parasite and pathogen infection (Blot et al., 2019; Motta et al., 2018; Paris et al., 2020; Rouzé et al., 2019). The vast majority of these studies examined effects of pesticide-disturbed gut microbiotas on host performance by comparing performance metrics and gut microbiota community structure in pesticide-exposed vs. pesticide-naïve bees (Botina et al., 2019; Wintermantel et al., 2018; Zhu et al., 2020). This experimental design permits researchers to determine that gut microbiota disturbance is not responsible for performance effects if a difference in performance is observed in the absence of changes in gut microbiota structure (Raymann et al., 2018). However, if changes in both gut microbial community structure and performance are observed, it is impossible to determine if performance has been altered due to a pesticide-disturbed gut microbiota or due to direct effects of the pesticide on the host organism; one can conclude that changes in performance are *correlated* with a disturbed gut microbiota, but not that they are *caused* by a disturbed gut microbiota.

There are ways to overcome this hurdle through clever experimental design. Motta *et al*. (2018) conducted an experiment in which groups of honey bees were either positive or negative for: 1) presence of a gut microbiota, 2) glyphosate exposure, and 3) *Serratia marcescens* (an opportunistic bacterial pathogen) infection (Figure 2-6). Through comparisons of survival between these groups, this study showed that the presence of a gut microbiota increases the survival of bees infected with *Serratia* and that exposure to glyphosate eliminates this benefit but does not affect host survival on its own, providing strong evidence that glyphosate-induced disturbance of gut microbiotas increases the lethality of *S. marcescens* in honey bees (Motta et al., 2018). This study provides one framework for establishing that a change in performance is due to pesticide-induced disruption of the gut microbiota and not direct effects of the pesticide on the host.

Another possible methodology that has yet to be tested is inoculating microbe-free bees with either a pesticide-disturbed or pesticide-naïve gut microbiota and comparing performance between the two groups. When adult bees emerge from pupation, they are essentially microbe-free and can therefore be experimentally inoculated with a desired gut microbiota. This approach has been used to compare the impact of host genotype vs. gut microbiota on pathogen infection (Koch & Schmid-Hempel, 2012), determine the metabolic contributions of individual phylotypes in honey bee gut microbiotas (Kešnerová et al., 2017), and confirm effects of pesticides on the growth of individual phylotype strains (Motta et al., 2018), among other experiments. Hypothetically, this approach could be extended to studies on pesticide–gut microbiota–host interactions. Feces or gut homogenates from pesticide-exposed bees could be used to inoculate one group of bees with a pesticide-exposed gut microbiota, and feces or gut homogenates from bees that remained pesticide-naïve used to inoculate another group with a pesticide-naïve gut microbiota.

The performance of the two inoculated groups could then be compared and, as neither group has been directly exposed to the pesticide, any performance differences would be due to differences in gut microbiota structure. One potential drawback is that the method assumes that a pesticide-disturbed gut microbiota will not recover to a "normal" community structure in the absence of pesticide exposure, which may not be the case (see **Duration of and variability in disturbances**). It is also important to note that regardless of experimental design, some metrics of performance remain logistically difficult to measure, such as foraging behaviour and colony-level traits.

An excellent next step in examining causal relationships between pesticide-disturbed gut microbiotas and bee host performance would be to examine a pesticide with a more typical disturbance profile than glyphosate. The most consistent taxonomic changes post-pesticide disturbance are declines in Bifidobacteriales and *Lactobacillus* near *melliventris* abundance. Glyphosate does not consistently cause either of these taxa to decline, instead causing a severe decrease in Neisseriales abundance, the only pesticide to do so. There are vast differences between the Neisseriales core phylotype, *S. alvi*, and the Bifidobacteriales and *Lactobacillus* near *melliventris* phylotypes, including where they are physically located in the gut (Kwong & Moran, 2016), their associations with other core microbes (Bonilla-Rosso & Engel, 2018), and their metabolism (Ellegaard et al., 2015; Ellegaard & Engel, 2019; Kešnerová et al., 2017; Steele et al., 2017). Consequently, knowing that a glyphosate-disturbed gut microbiota affects bee host performance does not provide much information on whether a similar effect will be observed after a decline in *Lactobacillus* near *melliventris* and/or Bifidobacteriales abundance. Future studies should prioritize examining how any of the pesticides that reduce the abundance of these two phylotypes interact with gut microbiotas to influence bee host performance. Not only would this line of investigation provide information for the chosen pesticide, it would also allow inferences about likely impacts of other pesticides that disturb bee gut microbiotas in a similar manner.

In addition to investigating differences in host performance between bees in pesticide-naïve and pesticide-exposed treatments, investigating within-treatment differences in performance can also provide valuable information about the role of bee gut microbes in host performance. A study by Wintermantel *et al*. (2018) found that *G. apicola* abundance was positively correlated with worker weight and male production in bumble bee colonies foraging on clothianidin-exposed fields, but that this correlation was absent from colonies foraging on clothianidin-free fields. This result highlights how, even in the absence of between-treatment differences in gut microbiotas (Wintermantel et al., 2018), bee gut microbes may interact with pesticides to influence host performance.

2.7 Conclusion

Early culture-based studies in the 1970s provided the first evidence that pesticide exposure could disturb bee gut microbiotas, and these findings have since been confirmed by multiple studies conducted within the past decade. Five bee species have been used as hosts for studying pesticide-induced disturbances of bee gut microbiotas, but studies using *A. mellifera* vastly outweigh the rest. The types of pesticides studied are equally unbalanced, with a greater variety of insecticides having been studied than fungicides or herbicides combined—though glyphosate, an herbicide, is the most thoroughly examined individual pesticide by a wide margin. Most modern studies rely on 16S rRNA gene amplicon sequencing to determine differences in taxonomic profiles between pesticide-naïve and pesticide-exposed bee gut microbiotas. Consequently, taxonomic changes in bee gut microbiotas after pesticide exposure are well documented, but data on functional changes are sparse.

Results from modern studies on bee gut microbiota–pesticide interactions reveal that taxonomic shifts post-pesticide exposure are often subtle, with taxa increasing or decreasing in abundance but rarely being eliminated from the community. Members of Lactobacillales (particularly the *Lactobacillus* near *melliventris* phylotype) and Bifidobacteriales are particularly susceptible to pesticide-induced disturbance and generally decline in abundance post-pesticide exposure, while Neisseriales bacteria are far less sensitive (except to glyphosate). Pesticide-induced disturbances may last for days or even weeks after pesticide exposure has ended (Diaz et al., 2019; Motta et al., 2020), potentially encompassing a bee's entire adult lifespan, though recovery may also be possible (Liu et al., 2019). Interestingly, pesticide concentration does not appear to have a large effect on the magnitude or severity of disturbance. Instead, exposure duration appears to be more important, with longer durations of pesticide exposure more likely to cause significant disturbances, though these durations may not be field-realistic for all pesticides. The time required for a pesticide to disturb bee gut microbiotas may depend on the pesticide's mode of action, with pesticides capable of directly affecting gut microbes able to cause disturbances more quickly. The mode of action may also explain seasonal variation in disturbances; the effects of pesticides that are hypothesized to disturb bee gut microbiotas via declines in host health have been shown to vary seasonally (Rouzé et al., 2019), in tandem with seasonal variation in host ecology, physiology, and behaviour, while the effects of pesticides that directly affect gut microbes are more seasonally consistent (Blot et al., 2019). In fact, pesticides that directly affect gut microbes may cause more consistent disturbances in general, across not only seasons but also concentrations (Motta et al., 2020), studies (Blot et al., 2019; Motta et al., 2018), and possibly even species, than pesticides that are hypothesized to indirectly affect gut microbiotas through host health (DeGrandi-Hoffman et al., 2017; Paris et al., 2020; Rouzé et al., 2019). Future work determining the precise mode of action for pesticide-induced disturbances in bee gut microbiotas will clarify these trends and may help to predict the consistency of

disturbances caused by unstudied pesticides with similar mechanisms; i*n vitro* experiments and examining changes in physiochemical gut conditions, host immune gene expression, and host and microbial metabolism after exposure will be useful in this endeavor (Box 1).

Understanding the effects of pesticide-induced bee gut microbiota disturbances on host performance may be the most important question in this field. If pesticide-disturbed gut microbiotas cause bees to perform sub-optimally, this would not only negatively impact the bee hosts and their colonies, but also the pollination services that these bees provide. While many metrics of performance have been measured, most experiments are designed in such a way that it is impossible to tell whether the pesticide-disturbed gut microbiota is causing the difference in performance or if the pesticide is acting on the host itself. To date, the only exception is a study by Motta *et al.* (2018) which provides evidence that a pesticide-disturbed bee gut microbiota, specifically a glyphosate-disturbed gut microbiota, impairs bee host performance. To fully understand the biological consequences of pesticide-disturbed gut microbiotas on bee host performance, future studies should be designed to test causal hypotheses, not simply to establish correlation. These experimental designs will be particularly beneficial in investigating how declines in *Lactobacillus* near *melliventris* and/or Bifidobacteriales abundance affect host performance, as these are the most common changes caused by pesticide exposure in bee gut microbiotas.

Moving beyond conventional methodologies, future studies should also attempt to examine metabolic shifts in the microbial community. Even when taxonomic shifts in gut microbiotas are subtle, such as those observed in bees after exposure to some pesticides, large changes in gene expression can occur (Maurice et al., 2013). This, coupled with the high degree of functional redundancy exhibited by core bee gut microbes (Kešnerová et al., 2017; Steele et al., 2017; Zheng et al., 2017), makes functional changes in bee gut microbiotas worth investigating. Examining these changes may also provide information on the mechanisms through which pesticide-induced disturbances in gut microbiotas affect bee host performance, which may be useful for determining treatments or interventions that can mitigate negative effects.

In addition to more thorough investigations into functional changes, there are other knowledge gaps in this field that future studies can address at the design stage. Specifically, future studies should seek to expand host selection beyond *Apis mellifera* to include social bee species representing different tribes and ecologies, though investigating unmanaged species will be challenging. The strong bias in pesticide choice towards insecticides should also be addressed. Future studies that wish to examine new pesticides should aim to examine the effects of more herbicides and fungicides on bee gut microbiotas, as bees are routinely exposed to pesticides of these types (Mullin et al., 2010), the plant and fungal pathways they disrupt may also be present in resident bee gut microbes (Motta et al., 2018), and knowledge of the effects of more herbicides will be useful when determining safe alternatives for glyphosate (Beckie et al.,

2020; Cruz et al., 2021). More precise measurements are also needed of the amount of pesticides absorbed by bee host tissue (i.e., body burden), especially if the pesticides are thought to disturb bee gut microbiotas by impairing host health. Finally, we recommend that future studies move beyond using only relative abundance data to examine taxonomic shifts and instead incorporate methods that account for changes in total microbial abundance, such as gene or transcript copy counts.

Bees are vital pollinators, both for agricultural crops and natural ecosystems, whose population declines are hypothesized to be, in part, due to pesticide exposure. Pesticides affect not only bees themselves, but also their resident gut microbial communities, to which they are linked in a fundamental symbiosis. Studies which have investigated pesticide-induced disturbances of bee gut microbiotas have provided critical information for understanding the consequences of pesticide exposure for the taxonomic structure of these microbial communities and how this may impact bee performance. However, this interdisciplinary field is still in its infancy, and there are many facets that remain to be investigated in depth. Expanding our knowledge on the interactions between pesticides, bee hosts, and their gut microbiotas will help us generate a more complete picture of not only how pesticides affect bee health and performance, but how toxicants affect host–microbiota symbioses in general.

2.8 Box 1: Mechanisms of pesticide-induced disturbance

There are two principal ways that pesticides can disturb gut microbiotas: 1) by directly affecting the growth of the microbes themselves, and 2) by affecting the host's ability to regulate its gut microbial community, for example by impairing host health or altering the gut environment (Daisley et al., 2020) (Figure 2-2).

Pesticides can both promote and inhibit microbial growth. Some microbes, including those found in the guts of insects routinely exposed to pesticides, are able to use certain pesticides as carbon and energy sources to support growth (Russell et al., 2011). However, pesticides can also be toxic to microbes—for example, by damaging genetic material, causing structural damage, or inhibiting metabolic pathways (De Flora et al., 1984; Muturi et al., 2017; Shahid et al., 2019; Staley et al., 2015). Glyphosate is one such pesticide which negatively affects the growth of the core bee gut microbe *S. alvi* through inhibition of the shikimate pathway and thereby synthesis of aromatic amino acids (Motta et al., 2018). As demonstrated by Motta *et al*. (2018), testing the growth of microbes in the presence of pesticides *in vitro* can help confirm whether pesticides exert direct effects on gut microbes.

In addition to direct effects on gut microbiotas, pesticides may also impair host health in ways that lead to perturbations in the gut microbiota. The immune system has been shown to play important roles in regulating both vertebrate and invertebrate gut microbiotas (Engel & Moran, 2013; Hooper et al., 2012; Nyholm & Graf, 2012). Exposure to a wide array of pesticides alters immune system function in insects (James & Xu, 2012), including bees (Aufauvre et al., 2014; Boncristiani et al., 2012; Brandt et al., 2016, 2017; Di Prisco et al., 2013), which may impair host regulation of the gut microbiota and consequently alter community structure. Pesticide exposure can also alter physical and physiochemical conditions in the midgut of bees (Araujo et al., 2019; Carneiro et al., 2020; Catae et al., 2018); if similar changes occur in the hindgut, the principal location of bee gut microbes, they may further contribute to shifts in gut microbiota structure. This indirect route of gut microbiota disturbance has been hypothesized to be responsible for disturbances caused by some insecticides. Rouzé *et al.* (2019) found that *in vivo* exposure to four different neurotoxic insecticides decreased the abundance of both *Lactobacillus* cluster and *Bifidobacterium* bacteria in honey bee guts. One proposed explanation for these similarities was that the pesticides disturb bee gut microbiotas through aspecific changes in gut homeostasis rather than direct effects on microbial growth.

It is important to note that these two mechanisms are not mutually exclusive: a pesticide may directly affect the growth of some microbial taxa while also impairing host health, which in turn further disturbs the gut microbiota.

2.9 Box 2: Types of data used to evaluate taxonomic shifts

Studies investigating taxonomic shifts in bee gut microbiotas in response to pesticide exposure typically generate two types of abundance data: relative abundances and/or gene/transcript copy counts. Abundance data can be affected at the DNA extraction step (e.g., with preferential extraction of DNA from taxa having cells that are more easily lysed), but also by the sequencing technology. Relative abundances are generated from read count data obtained via high-throughput sequencing. Some sequencing platforms have a set number of reads they can generate, and so the data they create consist of a set of proportions and are known as "compositional data"; converting read counts to relative abundances reflects this compositional nature. Importantly, relative abundances cannot fluctuate independently, making data difficult to properly analyze (Gloor & Reid, 2016; Tsilimigras & Fodor, 2016), and they do not account for changes in the total absolute abundance of the microbial community. Consequently, changes in relative abundances may not fully reflect real taxonomic shifts (Gloor et al., 2017).

Gene and transcript copy counts, on the other hand, are not compositional data and therefore do not have the same constraints. In the set of studies examined in this review, these copy counts are generated in two ways: 1) by using qPCR with taxon-specific primers (Blot et al., 2019; Motta et al., 2020; Rouzé et al., 2019), or 2) by multiplying relative abundance data by total bacterial 16S rRNA gene amplicon counts generated by qPCR (Liu et al., 2019; Motta et al., 2020; Motta & Moran, 2020). Both methods allow the measured abundances of different taxa to fluctuate independently of one another and attempt to account for changes in absolute microbial abundance, which has been shown to fluctuate significantly in bee gut microbiotas post-pesticide exposure, with most studies observing decreases (Liu et al., 2019; Motta et al., 2018; Nogrado et al., 2019). While some studies find no significant changes in total bacterial abundance (Motta et al., 2018), it is unlikely that microbial abundances remain perfectly constant across all replicates and treatments in an experiment, and methodologies should be able to account for these fluctuations.

Absolute microbial abundances generated via qPCR may not always be accurate, primarily due to variation in the number of copies of a gene in a microbial genome, as occurs with the 16S rRNA gene. Nevertheless, gene/transcript copy count data represent an essential complement to compositional data to identify which microbial taxa actually increase or decrease in response to a treatment. The development of digital droplet PCR technology, allowing for absolute quantification of amplicons, and the development of increasingly sophisticated algorithms applied to metagenomic datasets may further improve the ability of researchers to obtain estimates of total microbial abundance.

2.10 Data Availability

All data and scripts are available at github.com/michellehotch/FEMS-2021. Supplementary tables can be found at doi.org/hfjr.

2.11 References

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2.12 Tables

Table 2-1: Summary of recent studies on pesticide-bee gut microbiota interactions.

INSECT = insecticide; FUNG = fungicide; HERB = herbicide. *Lactobacillus* n. *m.* = *Lactobacillus* near *melliventris.*

Pesticide	Pesticide Type	Host Species*	Dose (µg L^{-1})**	Exposure Duration	Methods†	Significantly Affected Core Taxa	Study
Amitraz	INSECT	*Apis mellifera*	1,000	15 and 30 days	16S rRNA gene amp. seq.	None	Yang *et al.* 2019
		Apis cerana	1,000	15 and 30 days	16S rRNA gene amp. seq.	None	Yang *et al.* 2019
Carbaryl powder	INSECT	*Apis mellifera*	250 g of active ingredient per hectare	2 hours	16S rRNA gene amp. seq., qPCR w/ gen. bacteria primers	Present only in exposed: *Snodgrassella alvi*	Nogrado *et al.* 2019
Chlorpyrifos	INSECT	*Apis mellifera*	1,000	15 and 30 days	16S rRNA gene amp. seq.	None	Yang *et al*. 2019
		Apis cerana	1,000	15 and 30 days	16S rRNA gene amp. seq.	None	Yang *et al.* 2019
Clothianidin	INSECT	*Bombus terrestris*	Hives placed along fields with clothianidin-seed-treated crops	21-38 days	qPCR w/ core taxa-specific primers	None	Wintermantel *et al*. 2018
Coumaphos	INSECT	*Apis mellifera*	Two strips in hive	6 weeks	16S rRNA gene amp. seq., PICRUSt	↑ Bifidobacteriales	Kakumanu *et al.* 2016
		Apis mellifera	650 µg kg^{-1} (pesticide/syrup syrup)	18 days	qPCR w/ gen. bacteria and core taxa-specific primers	↓ *Bifidobacterium* spp. ↓ *Lactobacillus* n. *m.* ↓ *Lactobacillus* cluster	Rouzé *et al.* 2019
Fipronil	INSECT	*Apis mellifera*	0.25 µg kg^{-1} 1.0 µg kg^{-1} (pesticide/sugar syrup)	18 days	qPCR w/ gen. bacteria and core taxa-specific primers	↓ *Bifidobacterium* spp. ↓ *Lactobacillus* n. *m.* ↑ *Gilliamella apicola* ↑ *Snodgrassella alvi* ↓ *Lactobacillus* cluster	Rouzé *et al.* 2019
		Apis mellifera	0.5	17 days	16S rRNA gene amp. seq., active profiling w/ 16S rRNA transcript seq.	When co-exposed to *Nosema ceranae*: ↑ Gammaproteobacteria (Orbales)	Paris *et al.* 2020
Imidacloprid	INSECT	*Apis mellifera*	500	3 days	16S rRNA gene amp. seq., qPCR w/ gen. bacteria primers	None	Raymann *et al.* 2018
		In vitro, strains from *Apis mellifera*	1,000,000	72 hours	Single-strain and full gut toxicity assays	None	Raymann *et al.* 2018
		Apis mellifera	0.7	One feeding	Fatty acid profiles	↓ Gram-positive fatty acid biomarkers ↓ Gram-negative fatty acid biomarkers	Diaz *et al.* 2019§

		In vitro, strains from *Apis mellifera*	0.7	72 hours	Single-strain toxicity assays	None		Diaz *et al.* 2019[§]
		Apis mellifera	3.5 µg kg^{-1} (pesticide/sugar syrup)	18 days	qPCR w/ gen. bacteria and core taxa-specific primers	↓ *Bifidobacterium* spp. ↓ *Lactobacillus* cluster	↓ *Lactobacillus* n. *m.*	Rouzé *et al.* 2019
		Bombus impatiens	1	4 days	16S rRNA gene amp. seq.	None		Rothman *et al.* 2020
Nitenpyram	INSECT	*Apis mellifera*	300	14 days	16S rRNA gene amp. seq.	↑ Bacilli ↑ Betaproteobacteria ↑ *Bifidobacterium* spp. ↓ Gammaproteobacteria ↓ *Gilliamella* spp.	↓ *Gilliamella apicola* ↑ *Lactobacillus helsingborgensis* ↓ Orbales ↓ Orbaceae	Zhu *et al.* 2020
Spinosad	INSECT	*Partamona helleri*	810	24 hours	16S rRNA gene amp. seq.	↑ *Gilliamella apicola*		Botina *et al.* 2019
Tau-fluvalinate	INSECT	*Apis mellifera*	Two strips in hive	6 weeks	16S rRNA gene amp. seq., PICRUSt	None		Kakumanu *et al.* 2016
Thiacloprid	INSECT	*Apis mellifera*	200 600 2,000	13 days	16S rRNA gene amp. seq., qPCR w/ gen. bacteria primers	↓ *Lactobacillus* n. *m.*		Liu *et al.* 2019
Thiamethoxam	INSECT	*Apis mellifera*	Hives placed adjacent to crops treated with pesticide	Hives placed in April, sampled after peak flowering	16S rRNA gene amp. seq.	↑ Lactobacillales ↓ Proteobacteria		Jones *et al.* 2017
		Apis mellifera	1.7 µg kg^{-1} (pesticide/sugar syrup)	18 days	qPCR w/ gen. bacteria and core taxa-specific primers	↓ *Bifidobacterium* spp. ↑ *Gilliamella apicola*	↓ *Lactobacillus* cluster ↓ *Lactobacillus* n. *m.*	Rouzé *et al.* 2019
		Apis mellifera	1.5	17 days	16S rRNA gene amp. seq., active profiling w/ 16S rRNA transcript seq.	When co-exposed to *Nosema ceranae*: ↑ Gammaproteobacteria (Orbales)		Paris *et al.* 2020
Boscalid	FUNG	*Apis mellifera*	100	17 days	16S rRNA gene amp. seq., active profiling w/ 16S rRNA transcript seq.	When co-exposed to *Nosema ceranae*: ↑ Gammaproteobacteria (Orbales)		Paris *et al.* 2020
Boscalid/ pyraclostrobin	FUNG	*Apis mellifera*	1,990 11,410	21 days	16S rRNA gene amp. seq.	↓ *Gilliamella* spp. ↑ *Bombilactobacillus*	↑ *Lactobacillus* n. *m.*	DeGrandi-Hoffman *et al.* 2017
Chlorothalonil	FUNG	*Apis mellifera*	10	6 weeks	16S rRNA gene amp. seq., PICRUSt	↓ Lactobacillales		Kakumanu *et al.* 2016
Glyphosate	HERB	*Apis mellifera* (exposed as larvae)	800 4,000 20,000	4 days	16S rRNA gene amp. seq.	↑ Gammaproteobacteria		Dai *et al.* 2018

Apis mellifera	5,000 10,000	5 days	16S rRNA gene amp. seq., qPCR w/ gen. bacteria primers	↓ *Bifidobacterium* spp. ↓ *Bombilactobacillus*	↓ *Lactobacillus* n. *m.* ↓ *Snodgrassella alvi*	Motta *et al.* 2018
Apis mellifera	5,000 10,000	5 days	qPCR w/ gen. bacteria primers and core taxa-specific primers	↑ *Lactobacillus* n. *m.* ↓ *Snodgrassella alvi*		Motta *et al.* 2018
Apis mellifera	169,070 (1 mM)	2 days (with two days in between)	16S rRNA gene amp. seq., qPCR w/ gen. bacteria primers	↑ *Bombilactobacillus* ↓ *Snodgrassella alvi*		Motta *et al*. 2018
Apis mellifera	16,907 (0.1 mM)	5 days	qPCR w/ gen. bacteria primers and core taxa-specific primers, active profiling via 16S rRNA transcript seq.	↓ *Snodgrassella alvi*		Motta *et al.* 2018
Apis mellifera	16,907 (0.1 mM)	5 days	qPCR w/ *S. alvi*- specific primers	↓ growth of: *Snodgrassella alvi* strains		Motta *et al*. 2018
In vitro, strains isolated from *Bombus* and *Apis* spp.	1,690,700 (10 mM)	48 hours	Single-strain toxicity assays	↓ growth of : *Gilliamella apicola* strains *Snodgrassella alvi* strains		Motta *et al.* 2018
Apis mellifera	253,605 (1.5 mM) 1,268,025 (7.5 mM)	15 days	qPCR w/ gen. bacteria and core taxa-specific primers	↓ *Gilliamella apicola* ↑ *Lactobacillus* cluster	↑ *Lactobacillus* n. *m.* ↓ *Snodgrassella alvi*	Blot *et al.* 2019
In vitro, strains isolated from *Apis mellifera*	84,535 (0.5 mM) 253,605 (1.5 mM) 845,350 (5 mM) 2,536,050 (15 mM)	48 hours	Single-strain toxicity assays	↓ growth of: *Bifidobacterium* strains *Gilliamella apicola* strains *Lactobacillus* cluster strains *Snodgrassella alvi* strains		Blot *et al.* 2019
Apis mellifera	169,070 (1 mM) 169,070 (1 mM in Roundup)	5 days	16S rRNA gene amp. seq., qPCR w/ gen. bacteria primers	↓ *Bifidobacterium* spp. ↓ *Gilliamella* spp. ↓ *Snodgrassella* spp.		Motta *et al.* 2020
Apis mellifera	0.1% Roundup	3 days	16S rRNA gene amp. seq., qPCR w/ gen. bacteria primers	↓ *Bifidobacterium* spp. ↓ *Gilliamella* spp.	↓ *Lactobacillus* n. *m.* ↓ *Snodgrassella* spp.	Motta *et al.* 2020
Apis mellifera	0.1% Roundup	Four exposures each one week apart	16S rRNA gene amp. seq., qPCR w/ gen. bacteria primers	↓ *Bifidobacterium* spp. ↓ *Gilliamella* spp.	↓ *Lactobacillus* n. *m.* ↓ *Snodgrassella* spp.	Motta *et al*. 2020
Apis mellifera	0.001% Roundup 0.1% Roundup	Four exposures each one week apart	16S rRNA gene amp. seq., qPCR w/ gen. bacteria primers	↓ *Snodgrassella* spp.		Motta *et al.* 2020
Apis mellifera	0.001% Roundup 0.1% Roundup	One or two exposures	16S rRNA gene amp. seq., qPCR w/ gen. bacteria primers	↑ *Bombilactobacillus* ↓ *Snodgrassella* spp.		Motta *et al*. 2020

		Apis mellifera	Topical exposure: 0.05% Roundup 0.1% Roundup 0.5% Roundup 1.0% Roundup 3.0% Roundup	One spray of ~1.2mL	qPCR w/ *S. alvi*-specific primers	↓ *Snodgrassella* spp. (but only in one of two experiments)	Motta *et al*. 2020
		Apis mellifera	1,690.7 (0.01 mM) 6762.8 (0.04 mM) 11834.9 (0.07 mM) 16,907 (0.1 mM) 169,070 (1 mM)	15 and 20 days	Active profiling w/ 16S rRNA transcript seq., qPCR w/ cDNA	↓ *Snodgrassella alvi*	Motta and Moran 2020
Amino-methylphos-phonic acid (AMPA) (Glyphosate metabolite)	HERB	*Apis mellifera*	166,560 (1.5 mM) 832,800 (7.5 mM)	15 days	qPCR w/ gen. bacteria and core taxa-specific primers	None	Blot *et al*. 2019
		In vitro, strains isolated from *Apis mellifera*	55,520 (0.5 mM) 166,560 (1.5 mM) 555,200 (5 mM) 1,665,600 (15 mM)	48 hours	Single-strain toxicity assays	↓ *Gilliamella apicola*	Blot *et al*. 2019

* Workers were used unless otherwise indicated

** Dose is the concentration of the pesticide in sugar syrup or pollen provided to bees unless otherwise indicated; units are $\mu g\ L^{-1}$ unless otherwise indicated

† Amp. = amplicon, seq. = sequencing, gen. = general

§ Menthol, oxalic acid, and thymol were not included from Diaz *et al.* (2019) as they are primarily marketed as disinfectants, not pesticides

2.13 Figures

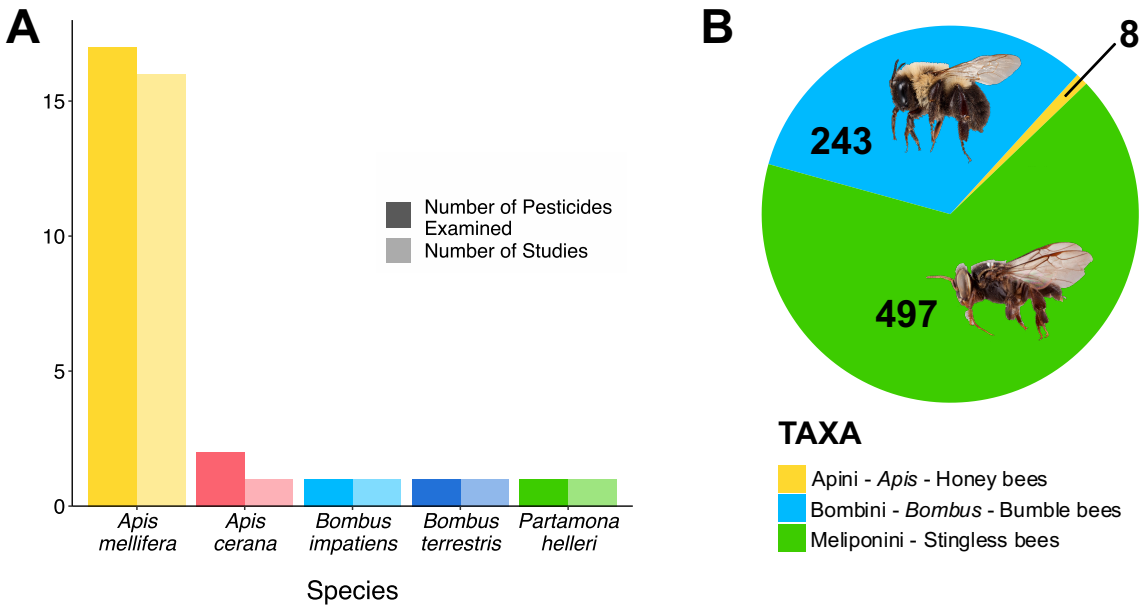


Figure 2-1: Pesticides and bee species examined in studies investigating pesticide-bee gut microbiota interactions.

A) The number of pesticides examined in each host bee species and the number of modern studies (i.e., published 2016 onwards) conducted using each host bee species. B) The proportion of social apid bees belonging to each taxon (tribe). Numbers represent the number of species in each taxon based on data from *Discover Life* (Ascher & Pickering, 2020). Photos of *Bombus impatiens* and *Partamona helleri* from Dr. Laurence Packer (https://www.yorku.ca/bugsrus/resources/resources). Bees are not to scale.

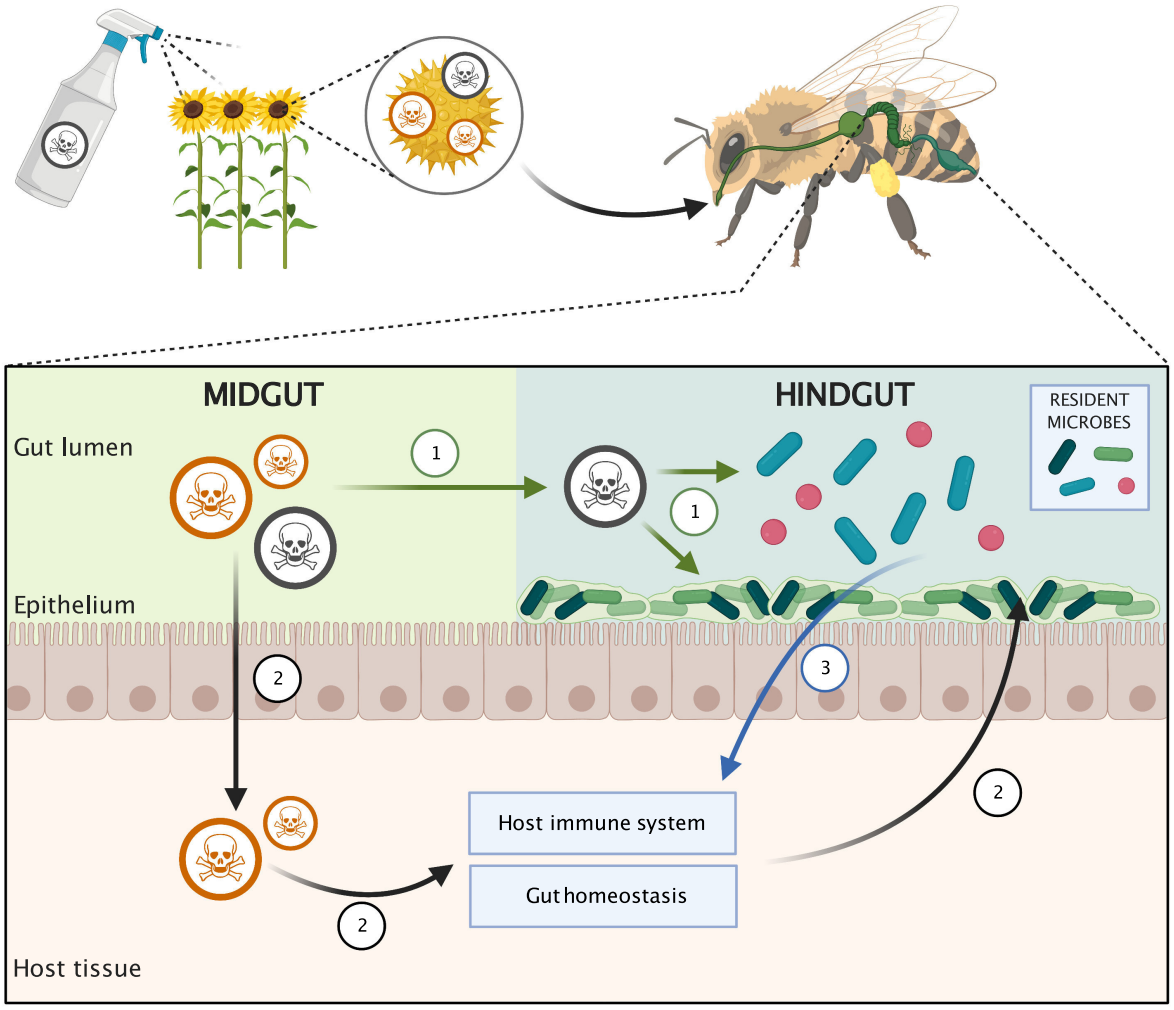


Figure 2-2: Interactions between pesticides, the bee gut microbiota, and the bee host.

Bees consume pollen and nectar which may contain pesticide residues. Once pesticide residues have entered the bee host, two main routes of pesticide-induced disturbance exist: 1) Pesticides directly affect the growth of resident microbes, most of which are found in bee hindguts, and 2) Pesticides are absorbed in the host midgut and affect host health, for example by lowering immune defenses or disturbing gut homeostasis; these changes in turn cause the host to lose its capacity to regulate its gut microbiota and the community becomes disturbed. The disturbed gut microbiota then 3) has further impacts on host health, affecting aspects of host performance such as immune system function, pollen digestion, and the gut environment. Created with BioRender.com.

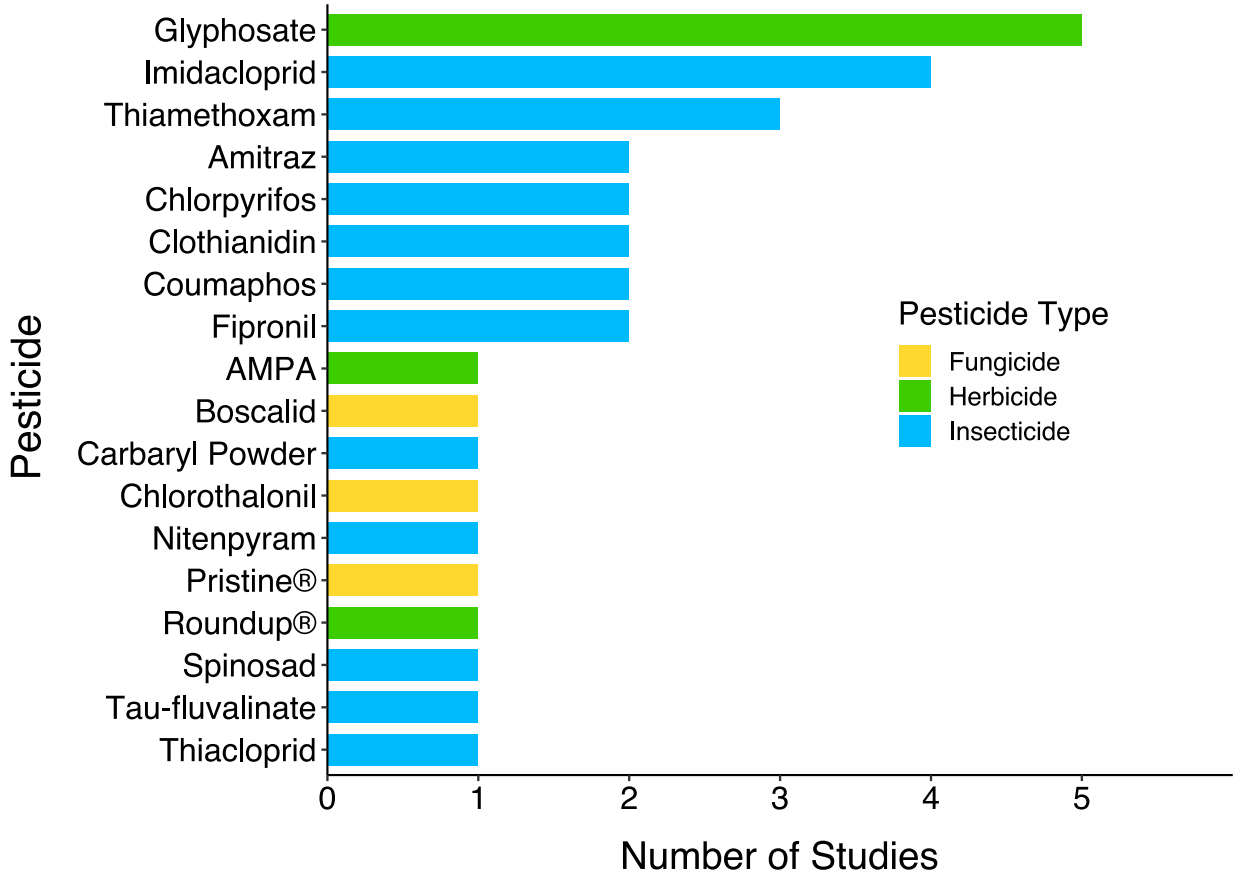


Figure 2-3: Number of studies that have examined each pesticide.

AMPA = amino-methylphosphonic acid.

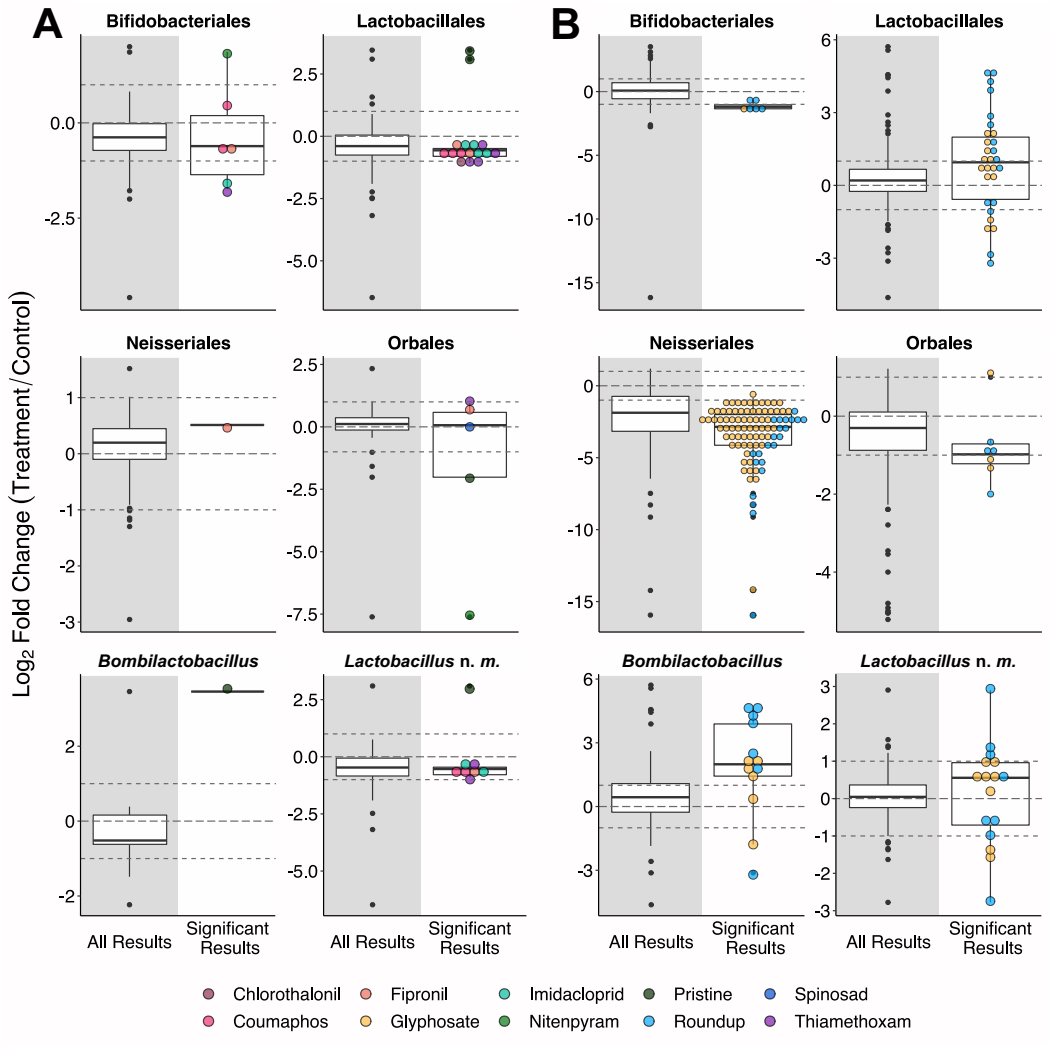


Figure 2-4: Log_2-fold changes in abundance of core bee gut microbiota taxa in response to pesticide exposure.

Log_2-fold changes in abundance of core bee gut microbiota taxa in response to exposure from A) non-glyphosate-based pesticides and B) glyphosate-based pesticides. Log_2-fold changes from both relative abundance and gene/transcript copy count data are displayed. Boxes represent medians and interquartile ranges; the whiskers extend to 1.5 × the interquartile range. Grey panels show box plots for all results regardless of significance (all results: n = 17-272); white panels show box plots for significant results only (significant results: n = 1-92). Dot histograms are shown for significant results with dots coloured by pesticide. Dashed lines represent log_2-fold changes of +1, 0, and −1. *Lactobacillus* n. *m.* = *Lactobacillus* near *melliventris*.

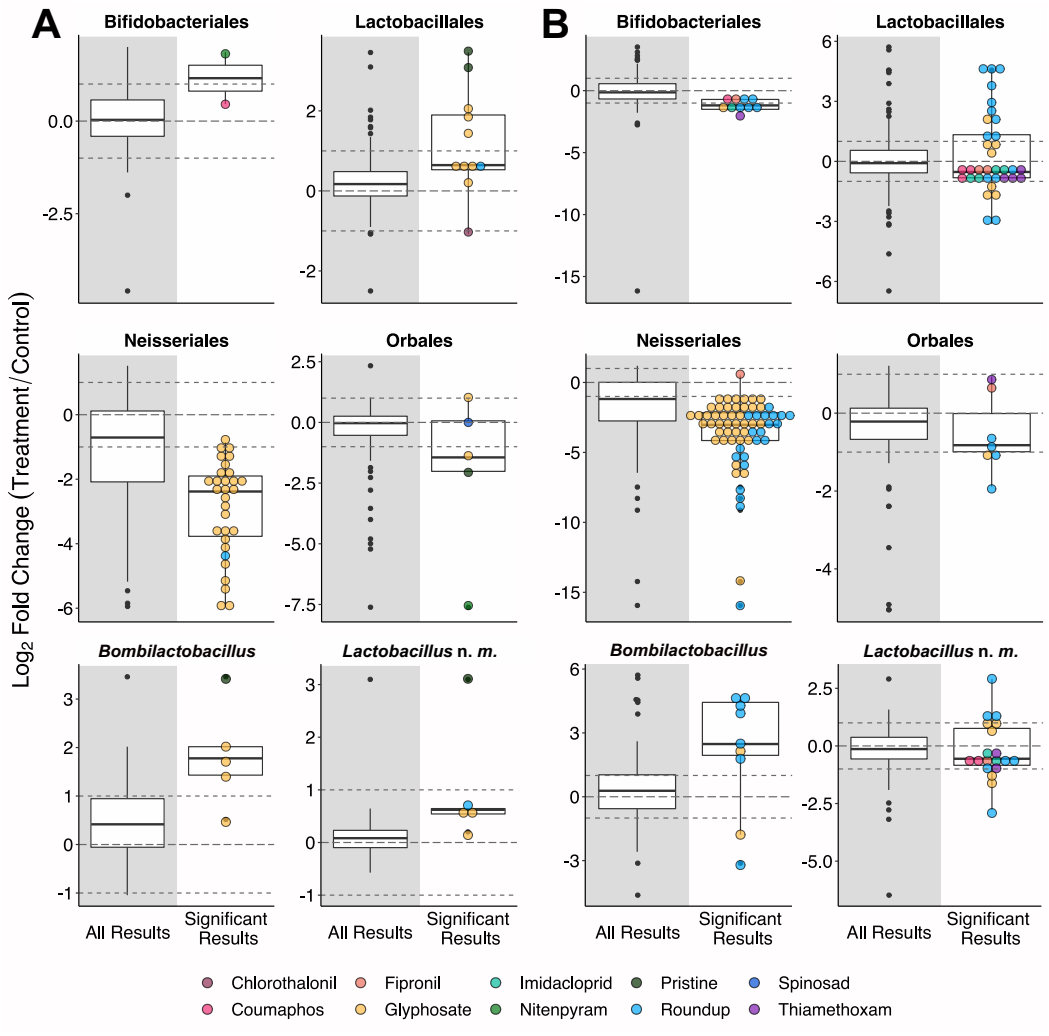


Figure 2-5: Log_2-fold changes in abundance of core bee gut microbiota taxa in response to pesticide exposure separated by data type.

A) shows results from relative abundance data and B) shows results from gene/transcript copy count data. Boxes represent medians and interquartile ranges; the whiskers extend to 1.5 × the interquartile range. Grey panels show box plots for all results regardless of significance (all results: n = 48-238); white panels show box plots for significant results only (significant results: n = 2-63). Dot histograms are shown for significant results with dots coloured by pesticide. Dashed lines represent log_2-fold changes of +1, 0, and −1. *Lactobacillus* n. *m.* = *Lactobacillus* near *melliventris*.

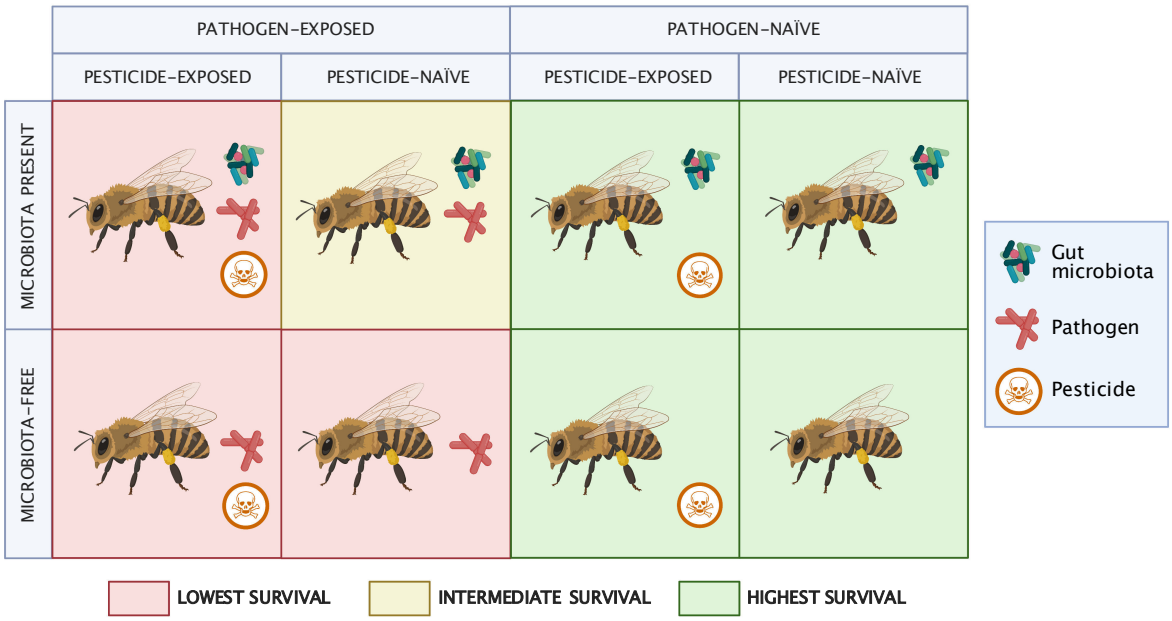
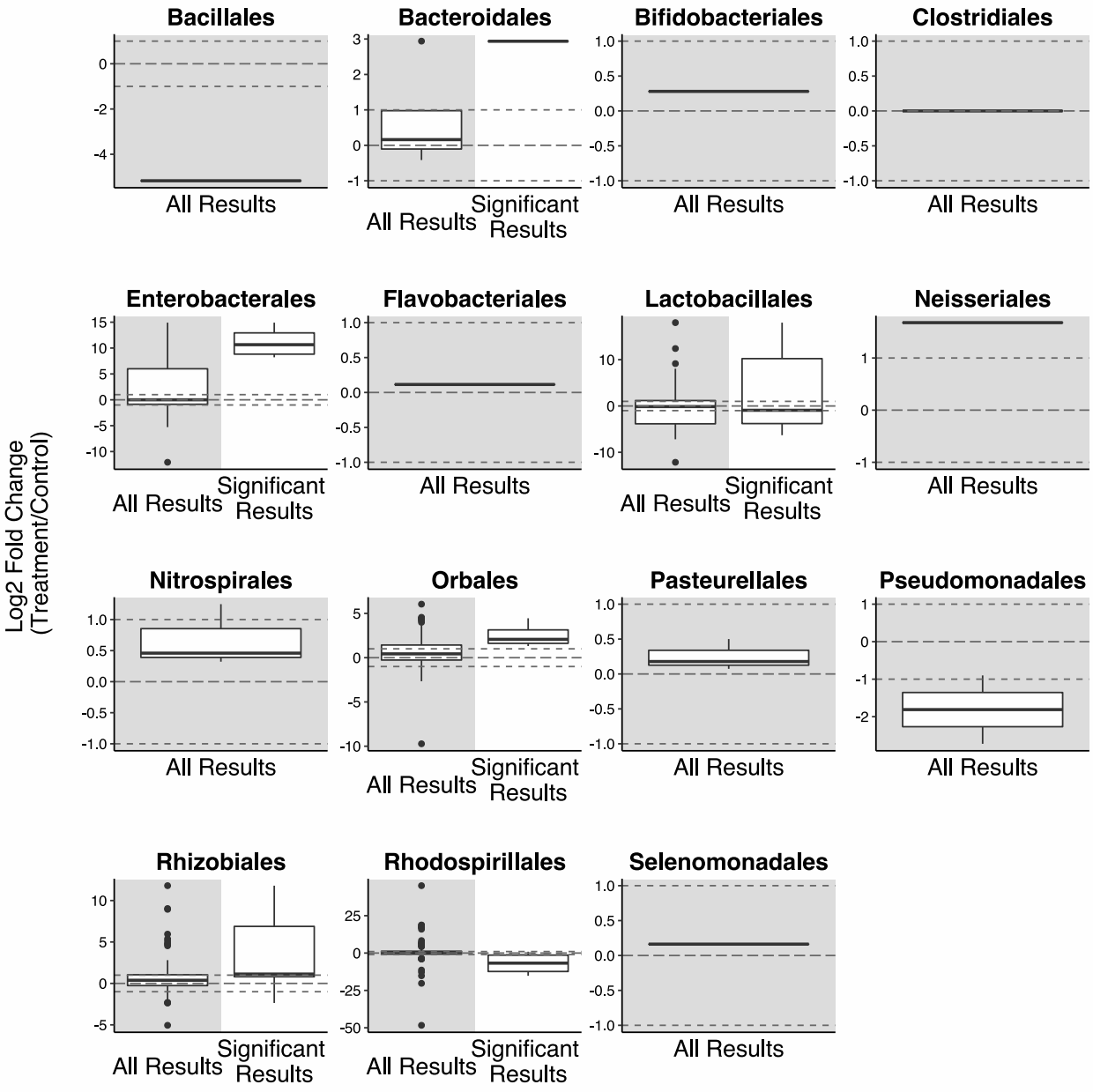


Figure 2-6: Methodology used in Motta *et al.* (2018), with results.

Groups of bees were exposed to combinations of three treatments: inoculation (or not) with a gut microbiota, exposure (or not) to a pesticide, and exposure (or not) to a stressor (i.e., pathogen). Bee survival was compared among groups. Groups with same-coloured backgrounds showed no significant differences in survival. Created with BioRender.com.

2.14 Supplemental Figures



Supplementary Figure 2-1: Log_2-fold changes in abundance of non-core bee gut microbiota taxa in response to pesticide exposure.

Taxa were defined as "non-core" if there was no possibility of that taxon containing any core phylotypes (e.g., the order Nitrospirales, the genus *Frischella* (displayed here as the order Orbales), or the phylotype *Apilactobacillus kunkeei* (displayed here as the order Lactobacillales)). Boxes represent medians and

interquartile ranges; the whiskers extend to 1.5 × the interquartile range. Grey panels show box plots for all results regardless of significance (all results: n = 1-137); white panels show box plots for significant results only (significant results: n = 1-11). Orders without white panels had no significant results. Dashed lines represent $\log_2$-fold changes of +1, 0, and −1.

Chapter 3

Exposure to a fungicide for a field-realistic duration does not alter bumble bee fecal microbiota structure

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3.1 Abstract

Social bees are frequently exposed to pesticides when foraging on nectar and pollen. Recent research has shown that pesticide exposure not only impacts social bee host health but can also alter the community structure of social bee gut microbiotas. However, most research on pesticide–bee gut microbiota interactions has been conducted in honey bees; bumble bees, native North American pollinators, have received less attention and, due to differences in their ecology, may be exposed to certain pesticides for shorter durations than honey bees. Here we examine how exposure to the fungicide chlorothalonil for a short, field-realistic duration alters bumble bee fecal microbiotas (used as a proxy for gut microbiotas) and host performance. We expose small groups of *Bombus impatiens* workers (microcolonies) to field-realistic chlorothalonil concentrations for five days, track changes in fecal microbiotas during the exposure period and a recovery period, and compare microcolony offspring production between treatments at the end of the experiment. We also assess the use of fecal microbiotas as a gut microbiota proxy by comparing community structures of fecal and gut microbiotas. We find that chlorothalonil exposure for a short duration does not alter bumble bee fecal microbiota structure or affect microcolony production at any concentration, but that fecal and gut microbiotas differ significantly in community structure. Our results show that, at least when exposure durations are brief and unaccompanied by other stressors, bumble bee microbiotas are resilient to fungicide exposure. Additionally, our work highlights the importance of sampling gut microbiotas directly, when possible.

3.2 Introduction

Social bees are key pollinators for both natural and agricultural plant communities. Nevertheless, some species are currently in decline due to a variety of factors such as increased parasite loads, lack of floral resources, and pesticide use (Goulson et al., 2015; Janousek et al., 2023; Toledo-Hernández et al., 2022). Pesticides are intensively used in modern agriculture to increase food production. As such, bees present in agricultural environments are often exposed to cocktails of pesticides in the pollen and nectar they consume (Mullin et al., 2010; Tong et al., 2018; Wen et al., 2021), and through contact with soil if they construct underground nests or hibernate underground (Gradish et al., 2019; Rondeau et al., 2022; Sgolastra et al., 2019). Many pesticides, including those that do not explicitly target insects, such as herbicides and fungicides, can harm bee health and fitness by affecting behaviour and physiology (Iwasaki & Hogendoorn, 2021; Lehmann & Camp, 2021; Lu et al., 2020; Tosi et al., 2022). Furthermore, in recent years it has become apparent that the effects of pesticides are not confined bee hosts themselves but may also extend to their gut microbiotas (Favaro et al., 2023; Hotchkiss et al., 2022).

Social bees, including bumble bees, honey bees and stingless bees, have a core gut microbiota that is highly conserved both within and between species (Kwong, Medina, et al., 2017), and is closely linked to host health and performance. This microbial community comprises five bacterial phylotypes present in the bee hindgut: *Bifidobacterium* spp., *Bombilactobacillus* spp*., Gilliamella* spp*.,* and *Lactobacillus* species near *L. melliventris,* which all ferment sugars, as well as *Snodgrassella alvi*, which oxidizes fermentation products (Hammer et al., 2021; Kwong & Moran, 2016). While all five core phylotypes are typically present in honey bees, their presence in bumble bees and stingless bees can be more sporadic, and some bumble bees, such as *Bombus impatiens*, have the additional core phylotype *Candidatus* Schmidhempelia bombi (hereafter *Schmidhempelia bombi*) (Kwong, Medina, et al., 2017; Martinson et al., 2014). The core social bee gut microbiota confers benefits to its hosts primarily via pathogen defense (Cariveau et al., 2014; Koch & Schmid-Hempel, 2011; Miller et al., 2021; Palmer-Young et al., 2019) and immune system stimulation (Horak et al., 2020; Kwong, Mancenido, et al., 2017; Lang et al., 2022; Steele et al., 2017), though core gut microbes also synthesize hormones (Cabirol et al., 2023; Kešnerová et al., 2017), stimulate host detoxification gene expression (Wu et al., 2020), promote host weight gain (Zheng et al., 2017), and appear to play roles in metal detoxification and nutrient processing (Engel et al., 2012; Kešnerová et al., 2017; Lee et al., 2018; Rothman et al., 2019; Zheng et al., 2017, 2019). Recently, it has come to light that various pesticides, including insecticides, herbicides, and fungicides, can alter the social bee core gut microbiota if ingested, decreasing the abundances of core gut microbes and increasing the abundance of non-core community members (Favaro et al., 2023; Hotchkiss

et al., 2022). These alterations are often correlated with reduced host survival (Dai et al., 2018; Liu et al., 2019; Motta et al., 2020; Motta & Moran, 2020; L. Zhu et al., 2020), and, in the case of a glyphosate-disturbed microbiota, increased pathogen-induced mortality (in honey bees) (Motta et al., 2018).

Importantly, alterations in social bee gut microbiota community structure appear more likely to be caused by chronic (i.e., longer than 10 days) than acute (i.e., shorter than one week) pesticide exposure (Hotchkiss et al., 2022; Raymann et al., 2018; Rothman et al., 2020). However, long exposure periods may not be field-realistic for all social bee species. Many pesticides are applied at intervals with labels recommending that applications occur days or weeks apart, though in practice farmers may apply more frequently (Amoako et al., 2012; Mengistie et al., 2017; Viviana Waichman et al., 2007). Due to factors like pesticide degradation, floral turnover, and rainfall, the amount of pesticide residue in floral resources declines between applications, and contaminated nectar and pollen may only be available to foraging bees for a limited time (Choudhary & Sharma, 2008; Gierer et al., 2019; Hicks et al., 2016; Van Scoy & Tjeerdema, 2014). For social bees that store food for long durations, such as honey bees and stingless bees (Roubik, 1993, 2022; Wright et al., 2018), even a short availability window may be sufficient for chronic exposure if stored contaminated pollen and nectar are consumed gradually over weeks or months and pesticides degrade slowly in the nest environment. However, due to differences in nutritional ecology, this is likely not the case for bumble bees.

Bumble bees live “hand-to-mouth”, storing food for only a few days at a time (Heinrich, 2004). Therefore, they will not consume contaminated pollen and nectar long past the date it was collected in the environment, and shorter, acute exposure durations are more field-realistic for these species. These acute durations may not be sufficient to alter bumble bee gut microbial community structure. However, if the microbiota is altered, the subsequent interval between spray dates after which all pesticide-laden food has been consumed but before the pesticide is reapplied may offer an opportunity for the microbiota to recover to its original, unaltered structure. Recovery from pesticide-induced alterations has been previously examined in honey bees with varying results; in some cases, gut microbiota community structure was able to recover after (Motta & Moran, 2023) or even during active pesticide exposure (Liu et al., 2019), yet in others community structure remained altered long after pesticide exposure ceased (Diaz et al., 2019; Motta et al., 2020)—though the latter result may be due to contaminated pollen and nectar remaining in the hive.

In this study, we examined whether pesticide exposure for a short, field-realistic duration could alter the community structure of bumble bee gut microbiotas and, if alterations do occur, whether altered gut microbiotas could recover to a pre-exposure community structure before the next application event. We also assessed whether pesticide exposure over this duration affected bumble bee offspring production.

We used *Bombus impatiens*, which is commercially bred for agricultural pollination in eastern North America, as our study species. For our pesticide, we chose chlorothalonil (CHT), a multi-site, contact activity fungicide (Fungicide Resistance Action Committee, 2018) routinely used on tomato and cucurbit crops which bumble bees are purchased to pollinate (Kurz et al., 2008). Chlorothalonil exposure increases *Nosema bombi* spore loads in *B. impatiens* (Calhoun et al., 2021), increases honey bee larval mortality (W. Zhu et al., 2014), is associated with increased presence of gut pathogens in honey bees and declining bumble bee species (McArt et al., 2017; Pettis et al., 2013), and can result in lower colony biomass, fewer workers, and lighter queens in *B. impatiens* colonies (Bernauer et al., 2015). Furthermore, chlorothalonil can directly inhibit microbial growth through its effects on thiol-dependent enzymes, including glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Long & Siegel, 1975; Tillman et al., 1973), and six weeks of oral exposure alters the relative abundances of core gut microbes in honey bees (Kakumanu et al., 2016). However, the effects of chlorothalonil on bumble bee gut microbiotas are as yet untested.

To serially examine changes in bumble bee gut microbiota community structure during pesticide exposure and recovery without sacrificing bees, we collected, and evaluated the suitability of, fecal microbiotas as a proxy for gut microbiotas. Though not perfect proxies, fecal microbiotas are widely accepted as substitutes for gut microbiotas across the animal kingdom, particularly for species in which sampling the gastrointestinal tract or sacrificing individuals is not feasible, such as larger vertebrates (Ahn et al., 2023; Burnham et al., 2023; Grond et al., 2018; Sommer et al., 2016; Thomas et al., 2015). Fecal proxies are rarely used in insect studies as obtaining gut samples is easy and efficient; all previous work on how pesticide exposure affects social bee gut microbiotas has been conducted using actual gut or whole-bee samples (Al Naggar et al., 2022; Botina et al., 2023; Hotchkiss et al., 2022). However, there are benefits to fecal sampling in lieu of gut sampling even in insects—in our case, for example, sampling fecal microbiotas permits us to serially examine bumble bee microbial communities during our experiment without disturbing social dynamics by euthanizing workers. A previous study using *B. terrestris* workers found that while fecal and gut microbiotas had significantly different community structures, sample type only explained about 5% of variation in structure, and taxonomic composition between samples types was similar (Näpflin & Schmid-Hempel, 2018).

3.3 Materials and Methods

3.3.1 Microcolony establishment

We purchased five *Bombus impatiens* colonies, henceforth referred to as source colonies, from Biobest (Leamington, Ontario, Canada) in three batches: two in early September, two in mid-October, and

one in late November of 2020. We removed all cotton from source colonies upon arrival and kept them in a dark room at 28.5℃ (± 0.09℃) and 23.2% relative humidity (± 0.6%). We provided source colonies with Biobest BioGluc® syrup and sterile honey bee-collected pollen mixed with water *ad libitum*; pollen was sterilized via gamma radiation with an average dose of 17.02 kGy (range: 15.82-17.66 kGy) (Graystock et al., 2016; Klinger et al., 2019) to avoid possibly introducing novel microbes into the source colonies.

From these source colonies we created queenless microcolonies for our experimental replicates. Microcolonies are small groups of worker bees removed from queenright colonies and housed separately. With no queen present, the largest worker establishes dominance and lays unfertilized eggs, which the other workers help to rear to adulthood (Free, 1955). Queenless microcolonies are useful in that they permit multiple replicates from the same genetic background while preserving, to some degree, a realistic colony environment, and they allow researchers to measure the effects of treatments on brood production (Klinger et al., 2019). This technique has been successfully used in a wide variety of studies on bumble bee behaviour, ecology, and gut microbial communities (Billiet et al., 2016; Klinger et al., 2019; McAulay & Forrest, 2019; Meeus et al., 2013), though their use has recently been criticized for nutritional studies (Wynants et al., 2022).

We created twelve microcolonies per source colony in two groups of six: one group seven days after source colony arrival, and another three weeks after arrival (to allow source colonies to produce new workers). For each microcolony, we removed ten workers and housed them in a sterile 470 mL plastic container. We kept microcolonies under the same conditions as source colonies for the duration of the experiment. We permitted workers to acclimate to their microcolony containers for six days (Days -5 to 0). For this entire acclimation period, and the experimental period that followed, we fed microcolonies the same sterile pollen as source colonies *ad libitum*. For the acclimation period we also provided microcolonies with filter-sterilized (pore size 0.2 µm) 30% (w/v) sugar syrup *ad libitum.*

3.3.2 Chlorothalonil solutions

A large range of chlorothalonil (CHT) concentrations have been documented in pollen, ranging from 9 to 98,900 ng/mL with a mean concentration around 90 ng/mL (Bernal et al., 2010; Mullin et al., 2010; Pettis et al., 2013; Rondeau & Raine, 2022; Sanchez-Bayo & Goka, 2014). Consequently, we tested a wide range of concentrations in our study: 1 ng/mL, 10 ng/mL, 100 ng/mL, and 800 ng/mL. We prepared chlorothalonil (PESTANAL, Millipore Sigma, Supelco, Massachusetts, USA) stock solutions in dimethyl sulfoxide (DMSO) at four concentrations, 500 ng/mL, 5000 ng/mL, 50,000 ng/mL and 400,000 ng/mL, to ensure that a consistent volume, 20 µL, was added to sugar solutions.

3.3.3 Treatment establishment

We assigned each microcolony to one of six treatments: control, solvent control, 1 ng/mL CHT, 10 ng/mL CHT, 100 ng/mL CHT, or 800 ng/mL CHT. Microcolonies in CHT treatment groups received filter-sterilized 30% sugar solution spiked with 20 µL of the relevant CHT stock solution, always to a final volume of 10 mL, while the solvent control group received 30% sugar solution spiked with 20 µL of DMSO, and the control group received only sugar solution. We exposed microcolonies to the relevant CHT concentration or solvent for five days beginning the first day after the acclimation period (Day 1), replacing sugar solutions daily. The five-day exposure is designed to mimic a realistic CHT exposure period for *B. impatiens* workers pollinating tomatoes, given the rapid photodegradation of CHT, average floral longevity in tomatoes (3-5 days), and the short food storage duration of bumble bees (Heinrich, 2004; Llop-Tous et al., 2000; Van Scoy & Tjeerdema, 2014). After the five-day exposure period, we provided all microcolonies with filter-sterilized 30% sugar solution (with no CHT or solvent) *ad libitum* for the remainder of a two-week period (Days 6 to14). We recorded the amount of sugar syrup consumed by each microcolony daily to verify that consumption was uniform across treatments.

3.3.4 Fecal sampling

We constructed our microcolony containers with a mesh floor and a sterile collection bowl beneath so that when bees defecated their feces fell through the mesh into the bowl below. On Days 0, 3, 5, 8, and 14 of the experiment, we replaced all bowls for all microcolonies with new bowls sterilized with 10% bleach, 70% ethanol, and 10 minutes of UV light exposure. Following the replacement, every hour for three hours we collected feces from the collection bowls into 2 mL centrifuge tubes using a pipette; we stored fecal samples at -20˚C until DNA extraction, at which point we pooled samples from the same day and microcolony. After collecting fecal samples on Day 14, we euthanized all microcolonies by freezing and stored all adults, larvae, pupae, and eggs at -80˚C until dissections or evaluations of microcolony production.

3.3.5 Dissections for gut-feces comparisons

To evaluate the suitability of fecal microbiotas as proxies for gut microbiotas, we took gut samples from all worker bees in three control microcolonies and three 800 ng/mL CHT treatment microcolonies after the experiment was complete. Within a sterile field, we took all workers from the same microcolony and soaked them for three minutes in a 1% bleach solution to kill cuticular microbes, then subsequently rinsed them three times in sterile water (Engel et al., 2013). We then moved the workers to a sterile dissection dish containing Ringer's solution, dissected the full gut of each worker, and

pooled and froze the guts at -20˚C. We repeated this process for each microcolony, replacing the dissection dish and Ringer's solution between microcolonies, in addition to cleaning the microscope and all dissection tools with 70% ethanol.

3.3.6 Additional gut-feces comparison experiment

In addition to comparing gut vs. fecal microbiota community structure in our original experiment, we also conducted a follow-up experiment to compare fecal and gut microbiotas of pesticide-free microcolonies at additional time points. We purchased three additional *B. impatiens* source colonies in September 2022 and made six queenless microcolonies from each as in the first experiment. We fed these source colonies and microcolonies in an identical manner to those used in the first experiment, save that we never exposed bees to CHT or solvent in this second experiment. The first day after forming the microcolonies, during what was the acclimation period of the first experiment (Day -5), we took fecal samples from two microcolonies from each source colony in an identical manner to the original experiment and euthanized sampled microcolonies by freezing. We took fecal samples from and euthanized two microcolonies from each source colony in the same way again on Days 0 and 14. We dissected and pooled whole guts from the workers of each microcolony as in the first experiment.

3.3.7 DNA extraction and sequencing

We extracted DNA from all fecal and gut samples using the QIAGEN DNeasy PowerLyzer PowerSoil Kit (Hilden, Germany) with the following modifications: 1) we ground pooled gut samples using a pestle before extraction, 2) we incubated samples for 10 minutes at 65˚C, 3) we conducted all centrifugation steps at 13,000 x *g*, and 4) for fecal samples, we eluted DNA in 50 μL of solution C6, as recommended by the manufacturer for samples suspected to have low amounts of DNA, and let the solution sit on the membrane for 5 minutes before centrifugation. Before DNA extractions for fecal samples, we took images of all pooled fecal samples using ImageJ (Schneider et al., 2012) to quantify the volume of each fecal sample. We quantified DNA concentrations using a Qubit 2.0 and an Invitrogen Qubit dsDNA HS Assay Kit. We sent all DNA extracts along with six negative extraction controls to Genome Quebec (Montréal, Québec) for bacterial 16S rRNA gene amplicon sequencing on the Illumina MiSeq platform targeting the V4 region using 515F-Y and 806R primers (Apprill et al., 2015; Parada et al., 2016; Walters et al., 2016).

Though chlorothalonil is a fungicide, we chose to focus on bacteria in this study as all core microbes present in bumble bee gut microbiotas are bacteria, there is little evidence for fungal mutualists in bumble bee gut microbiotas, and fungal presence and community composition in bumble bee guts are

sporadic (Hammer et al., 2021; Rutkowski et al., 2023).

3.3.8 Microbiome analysis

We analyzed 16S rRNA gene amplicon sequences using QIIME 2 v2021.8 (Bolyen et al., 2019). We trimmed primers from all reads using the plugin "cutadept" (Martin, 2011) and denoised reads using Divisive Amplicon Denoising Algorithm (DADA2) (Callahan et al., 2016). We then collapsed reads into amplicon sequencing variants (ASVs) and assigned taxonomy using the BEExact database (Daisley & Reid, 2021). After taxonomic assignment, we removed ASVs with a frequency less than 0.1% of our mean sequencing depth (Comeau et al., 2017), as well as ASVs assigned to mitochondria and chloroplasts. We then used rarefaction analyses to ensure our sequencing depth adequately captured ASV diversity in our samples. Finally, for analyses comparing fecal and gut microbiotas, we used QIIME 2 to generate distance matrices using Bray-Curtis dissimilarities and perform principal coordinates analyses (PCoAs).

3.3.9 qPCR for 16S rRNA gene copy number

We used qPCR to obtain 16S rRNA gene copy numbers from all fecal and gut samples. We generated a four-step standard curve of known 16S rRNA gene copy numbers using DNA extracted from NEB® 5-alpha competent *Escherichia coli* K-12 which we ran in triplicate on every plate. We diluted gut DNA extracts 1:150 and fecal DNA extracts 1:15 in nuclease-free water and ran all samples in triplicate following the protocol in Motta et al. (2018). We used BioRad® SsoFast EvaGreen Supermix with a BioRad® CFX96 real-time system and C1000 thermocycler, and analyzed run data with Bio-Rad® CFX Maestro software (v.2.3). Six fecal samples had insufficient volume to conduct qPCR after sending DNA for sequencing and as such they were excluded from qPCR analysis.

After calculating the total 16S rRNA gene copy number for each sample, we multiplied that number by the relative abundance of each taxon present in that sample, correcting for the average number of 16S rRNA gene copies per genome in the given bacterial genus or family according to the *rrn*DB version 5.8 (Stoddard et al., 2015). For fecal samples not being compared to gut samples, we further corrected copy numbers by dividing by fecal volume to obtain 16S rRNA gene copy number per microlitre of feces; we then used these values to generate a distance matrix of fecal microbiotas using the vegan package in R (Oksanen et al., 2022).

To avoid confusion when discussing abundance data, we henceforth refer to relative abundance data transformed using total 16S rRNA gene copy counts as gene copy count data and use the term "relative abundance" only when discussing relative ASV abundances obtained directly from Qiime2.

3.3.10 Microcolony fitness measurements

We assessed microcolony fitness to determine whether it was affected by CHT treatment. We thawed frozen microcolonies on ice. We counted and weighed all eggs, larvae, pupae, and workers who survived to Day 14. We did not weigh dead larvae nor include them in our counts; we identified these by their brown or black colouration.

3.3.11 Statistical analyses

We conducted all data visualization and statistical analyses in R version 4.3.1 (R Core Team, 2023). We fit all linear and generalized linear models described in this section using the lme4, lmerTest, and glmmTMB packages (Bates et al., 2015; Brooks et al., 2017; Kuznetsova et al., 2017), examined their model assumptions using the performance package (Lüdecke et al., 2021), and conducted post-hoc tests using the package emmeans (Lenth, 2023). For mixed effects models, we treated source colony as random when it had five levels and fixed when it had fewer.

We analyzed sugar syrup consumption using a linear mixed model with volume consumed in past 24 hours as the dependent variable and treatment, day of experiment, age of source colony, and all two- and three-way interactions, as categorical, fixed, independent variables. We included source colony ID and microcolony ID as nested random effects. We simplified the initial model by dropping interactions and then main effects if we found them to be insignificant ($P > 0.05$).

We analyzed microcolony production using a series of mixed effects models. We used linear mixed effects models to examine adult weight, egg weight, and larvae weight, and generalized linear mixed effects models with either a Poisson or negative binomial distribution to examine adult count, egg count, larval count, and pupal count. The first model tested for each production metric contained treatment, age of source colony, and their interaction as fixed, independent variables and source colony ID as a random effect; we again dropped insignificant terms.

While we collected data on pupae weights, only approximately one quarter of microcolonies produced pupae during our experiment. As we only had a small amount of data for this production measure and sample sizes were uneven across treatments and ages, we chose to exclude this measure from analysis.

We analyzed alpha diversity (Shannon index) of fecal and gut microbiotas using linear mixed effects models. For the analysis of fecal microbiotas alone, the model contained treatment, day, age, and their interactions, as well as source colony ID and microcolony ID as nested random effects. For the analysis of fecal and gut microbiotas together, the initial model contained sample type, day, and their interaction, as well as source colony ID as fixed predictors; microcolony ID was included as a random

effect. We dropped insignificant interactions. We used the same method to compare total 16S rRNA gene copy counts across fecal microbiotas and between fecal and gut microbiotas, and to compare the absolute 16S rRNA gene copy counts of all core taxa plus the non-core taxon *Enterobacteriaceae* across fecal microbiotas. Models for fecal microbiota included treatment, day, age, and their interactions, as well as source colony ID and microcolony ID as nested random effects. To compare fecal and gut microbiotas, we ran models including the fixed predictors treatment (first experiment) or day (second experiment), sample type, and their interaction, as well as microcolony ID (random) and source colony ID (fixed).

We used the adonis2 function in the vegan package to conduct permutational multivariate analyses of variance (PERMANOVAs) to analyze microbial community structure (Oksanen et al., 2022). We ran all models with 9,999 permutations, dropping insignificant terms and rerunning the simplified models until all terms were significant. Our response variables were Bray-Curtis dissimilarity matrices generated using vegan or QIIME 2. For fecal microbiotas, predictor variables were day, treatment, age, and their interactions, with data stratified by source colony. For comparing fecal and gut microbiotas from our first experiment we included treatment, sample type, and their interaction, source colony ID, and microcolony ID as predictors, and for the second experiment we included day, sample type, and their interaction, source colony ID, and microcolony ID. Microcolony ID was included as a fixed effect in these models as adonis2 does not permit the inclusion of random effects. When our final models were selected, we evaluated homogeneity of group dispersions using the betadisper function in the vegan package with type set to “median” (Oksanen et al., 2022). We conducted post-hoc comparisons using the pairwiseAdonis package for PERMANOVA models (Martinez Arbizu, 2020) and the TukeyHSD function for group dispersions.

To compare relative abundances of microbial taxa in fecal and gut microbiotas, we conducted an analysis of community structures of microbiomes with bias correction (ANCOMBC). For this, we used the ancombc2 function in the ANCOMBC package (Lin & Peddada, 2020) with Bonferroni correction and an alpha of 0.05. Based on results from the PERMANOVAs run previously for fecal vs. gut comparisons, we included sample type and source colony ID as fixed effects for the first experiment, and sample type, day, and source colony ID as fixed effects for the second experiment. We also tested if 16S gene copy counts for taxa in fecal samples were correlated with those in gut samples using Pearson’s product-moment correlations. Finally, we compared the average Bray-Curtis dissimilarity between fecal and gut microbiotas originating from the same microcolony and those originating from different microcolonies using a permutation test in the lmPerm package (Wheeler, 2010).

3.4 Results

3.4.1 Syrup consumption

We established and maintained 60 queenless microcolonies for durations of three weeks over the course of four months. Sugar solution consumption was not affected by pesticide treatment at any point during the experiment ($F_{5,44} = 1.5$, $P = 0.21$; Supplementary Figure 3-1). Sugar solution consumption did vary based on day and age of source colony (day:age interaction: $F_{19,1101} = 3.5$, $P = 5.8\times0^{-07}$; Supplementary Figure 3-1); however, post-hoc tests revealed no significant differences between age groups during the pesticide exposure period (Days 1 to 5) (Supplementary Figure 3-2).

3.4.2 Microcolony performance and production

We examined microcolony performance and production on the final day of the experiment (Day 14). There was no significant main effect of chlorothalonil treatment on any production metric (all $\chi^2 <$ 3.20, df = 5, all $P > 0.05$; Figure 3-1), though the model for average larva weight contained a significant interaction between treatment and age of source colonies at the time of microcolony creation ($\chi^2 = 11.3$, df = 5, $P = 0.046$). Post-hoc tests revealed that the average larva weight of microcolonies made with three-week-old source colonies and exposed to 100 ng/mL CHT was significantly higher than that of all other three-week-old treatments except for 1 ng/mL CHT, and that the average larva weight of DMSO microcolonies made with one-week-old source colonies was significantly higher than those made with three-week-old source colonies (Figure 3-1F). The number of workers and the number and average weight of eggs were also consistent across source colony ages ($\chi^2 = 0.0001$–1.5, df = 1, $P = 0.21$–0.99) (Figure 3-1). Average worker weight as well as larva and pupa counts did vary significantly with age of source colonies (df = 1; worker weight: $\chi2 = 4.10$, $P = 0.043$; larva count: $\chi2 = 25.60$, $P = 4.19\times10^{-07}$; pupa count: $\chi2 = 6.37$, $P = 0.012$); in all cases, microcolonies formed with workers from younger source colonies performed better (i.e., heavier workers, higher average larva and pupa counts at the end of the experimental period) (Figure 3-1).

3.4.3 Fecal microbiota diversity and community structure

We obtained 74 ASVs across 299 fecal microbiota samples with a median read frequency of 25,082; one microcolony on one sampling day and all negative controls had low read counts (<1000 reads and <100 reads, respectively) and were removed from downstream analysis. Rarefaction analyses revealed that we sufficiently captured ASV diversity in all remaining samples.

Of the 74 observed ASVs, approximately 94% of reads from fecal samples assigned to one of eight ASVs: *Acinetobacter, Bifidobacterium, Bombilacotobacillus, Bombiscardovia, Enterobacteriaceae*, *Lactobacillus*, *Schmidhempelia*, or *Snodgrassella* (Figure 3-2). We did not assign any sequences in this or subsequent experiments to *Gilliamella*; this absence of *Gilliamella* has been previously noted in other *B. impatiens* colonies from Biobest (Motta & Moran, 2023). Alpha diversity varied significantly with day ($F_{4,229} = 4.1$, $P = 0.0034$), with alpha diversity on Day 14 higher than on Days 0, 3 or 5 ($P = 0.041$; $P = 0.004$; $P = 0.010$; Supplementary Figure 3-3A).

Total microbial 16S rRNA gene copy counts varied with a day:age interaction ($F_{4,2161832} = 3.3$, $P = 0.011$), but not treatment ($F_{5,43} = 0.90$, $P = 0.49$; Figure 3-2C, Supplementary Figure 3-4A). Similarly, 16S rRNA gene copy counts of all individual core taxa and *Enterobacteriaceae* did not vary by treatment (all $F < 1.91$, all $P > 0.11$), and most did not vary by day or age with the exception of *Bombilactobacillus* and *Enterobacteriaceae* (Supplementary Figure 3-4). Specifically, *Bombilactobacillus* gene copy count varied with day (Supplementary Figure 3-4D) and *Enterobacteriaceae* gene copy count varied with a day:age interaction (Supplementary Figure 3-4B).

Fecal microbial community structure did not vary by treatment (PERMANOVA, stratified by source colony; $F = 0.96$, df = 5, $P = 0.25$) but did vary by day sampled ($F = 2.9$, df = 4, $P = 0.0001$) and age of source colony upon microcolony formation ($F = 4.05$, df = 1, $P = 0.0002$, Figure 3-3). Tests for homogeneity of group dispersions revealed that levels of both age ($F = 9.35$, df = 1, $P = 0.003$) and day ($F = 2.5$, df = 4, $P = 0.045$) had significantly different dispersions, though all post-hoc comparisons for day were insignificant after correcting for multiple comparisons. Fecal samples from microcolonies made with three-week old workers were less dispersed than those made with one-week old workers ($P = 0.002$) (Figure 3-3B), and this difference in dispersion likely contributed to the significant differences observed in the PERMANOVA. However, despite their statistical significance, day and age explained little variation in the fecal microbiota community structure data, with R^2 values of 0.039 and 0.013, respectively.

Instead, when removed as a stratifying variable and included in the PERMANOVA, source colony ID explained approximately 18% of variation in fecal community structure ($R^2 = 0.18$; $F = 16.4$, df = 4, $P = 0.0001$; Figure 3-3B). Source colonies did not have significantly different group dispersions ($F = 1.8$, df = 4, $P = 0.17$; Figure 3-3B).

3.4.4 Fecal vs. gut microbiota comparisons

In the paired samples taken from our pesticide exposure experiment, fecal microbiotas consistently had significantly lower total 16S rRNA gene copy counts (i.e., total microbial abundance)

than gut microbiotas (median fecal gene count = 1.9×10^8, median gut gene count = 5.5×10^9; $F = 29.7$, df = 1, $P < 0.0001$; Figure 3-4B). We observed 31 ASVs across six fecal microbiota and six gut microbiota samples with a median read frequency of 24,691. Rarefaction analyses revealed that we sufficiently captured ASV diversity in all samples. Over 94% of sequences from the six fecal samples and over 99% of sequences from the six gut samples were assigned to one of eight phylotypes: *Acinetobacter, Bifidobacterium, Bombilacotobacillus, Bombiscardovia, Enterobacteriaceae*, *Lactobacillus*, *Schmidhempelia*, or *Snodgrassella* (Figure 3-4A). When we compared fecal and gut microbial community structure at Day 14, we found that structure did not vary by treatment (PERMANOVA; $F = 2.0$, df = 1, $P = 0.13$) but did vary by sample type ($F = 10.7$, df = 1, $P = 0.0001$) and source colony ID ($F = 4.5$, df = 2, $P = 0.008$; Figure 3-4C-E); we also found that microcolony ID explained no additional variation in microbiota community structure ($F = 0.32$, df = 2, $P = 0.95$). Neither sample type ($F = 1.3$, df = 1, $P = 0.27$) nor source colony ($F = 1.5$, df = 2, $P = 0.28$) had significantly different dispersions. Sample type explained approximately 39% of variation ($R^2 = 0.39$) and source colony ID explained approximately 33% ($R^2 = 0.33$). *Lactobacillus* and *Bombilactobacillus* both had significantly higher relative abundances in gut microbiotas with log-fold changes of 1.13 (ANCOM; adjusted $P = 0.03$) and 1.75 (ANCOM; adjusted $P = 0.004$), respectively (Figure 3-4A). Meanwhile, *Enterobacteriaceae* was significantly more abundant in fecal samples with a log-fold change of 2.67 (ANCOM; adjusted $P = 0.008$; Figure 3-4A).

The significant difference we found between fecal and gut microbiota community structures in the PERMANOVA prompted us to conduct the second experiment comparing the fecal and gut microbiota community structures of 18 microcolonies not exposed to pesticide. In that experiment, we observed 23 ASVs across all microbiota samples with a median read frequency of 79,491, and rarefaction analyses revealed that we sufficiently captured ASV diversity in samples; negative controls had <100 reads each and were removed from further analysis.

As in the first (pesticide-exposure) experiment, fecal microbiotas had consistently lower 16S rRNA gene copy counts than gut samples (median fecal gene count = 2.1×10^8, median gut gene count = 1.1×10^9; $F = 21.95$, df = 1, $P < 0.0001$; Figure 3-5B). Over 98% of sequences were assigned to one of six phylotypes: *Bifidobacterium, Bombilacotobacillus, Bombiscardovia, Lactobacillus*, *Schmidhempelia*, or *Snodgrassella* (Figure 3-5A). Alpha diversity (Shannon index) did not vary based on sample type ($F_{1,17} = 2.0$, $P = 0.18$) or source colony ID ($F_{2,13} = 0.32$, $P = 0.73$), but did vary by day ($F_{2,13} = 4.6$, df = 2, $P = 0.030$), with alpha diversity being significantly lower on Day -5 than on Day 14 ($P = 0.047$; Supplementary Figure 3-3B). Microbial community structure varied with sample type ($F = 28.7$, df = 1, $P = 0.0001$), day ($F = 18.8$, df = 2, $P = 0.0001$), source colony ID ($F = 11.2$, df = 2, $P = 0.0001$), and microcolony ID ($F = 7.25$, df = 13, $P = 0.0001$), with sample type explaining 14% of variation ($R^2 =$

0.14), day explaining 19% ($R^2 = 0.19$), source colony explaining 11% ($R^2 = 0.11$), and microcolony ID explaining an additional 47% (Figure 3-5D-F). Tests for homogeneity of dispersions showed that samples taken on different days had significantly different dispersions ($F = 9.8$, df = 2, $P = 0.0005$), but different sample types ($F = 1.0$, df = 1, $P = 0.32$) and source colonies ($F = 0.13$, df = 2, $P = 0.88$) did not. As in our original experiment, *Bombilactobacillus* relative abundance was significantly higher in gut than fecal microbiotas (ANCOM; adjusted $P = 0.001$; Figure 3-5A), though with a smaller log-fold change of 0.92; *Lactobacillus* was no longer differentially abundant. Furthermore, $\log_{10}$ 16S rRNA gene copy counts in fecal and gut samples were not correlated for any core taxa (t = 0.45–1.54, df = 16, $P = 0.14$–0.65; Figure 3-6). Finally, fecal and gut microbiota pairs originating from the same microcolony were more similar than unmatched pairs (permutation test with 10,000 permutations, $P < 0.0001$; Figure 3-5C).

3.5 Discussion

3.5.1 Chlorothalonil exposure does not affect fecal microbiota community structure

We found that exposure to chlorothalonil for a short, field-realistic duration does not alter bumble bee fecal microbiota community structure, regardless of concentration (Figure 3-2, Figure 3-3). This contrasts with previous research which found that exposure to 10 ng/mL of chlorothalonil (one of our tested concentrations) increased the relative abundance of Rhizobiales and Pasteurellales bacteria and decreased the relative abundance of Lactobacillales in honey bee gut microbiotas (*Apis mellifera*) (Kakumanu et al., 2016). However, Kakumanu et al. (2016) used a chronic exposure period of six weeks for their experiment. A six-week exposure period is field-relevant for honey bees, as even though chlorothalonil is often applied at intervals honey bees store food for long durations (Wright et al., 2018) and would therefore have access to chlorothalonil-contaminated food even after chlorothalonil residues had degraded in floral pollen and nectar between pesticide applications. Bumble bees, on the other hand, store food for short durations and only have access to pesticide-contaminated food while pesticide residues are present in nectar and pollen (Heinrich, 2004). We therefore chose a shorter exposure duration of five days for our experiment to reflect these differences in field-realistic exposure periods driven by host ecology. We believe that this difference in exposure period played a key part in the observed difference in results, alongside other potential factors such as differences in pesticide toxicity to the host (Mundy-Heisz et al., 2022; Sanchez-Bayo & Goka, 2014). The lack of change in bumble bee fecal microbiota structure that we observed here aligns with a previously noted pattern that long pesticide exposure durations tend to more readily alter social bee gut microbiota community structure than short exposure durations (Hotchkiss et al., 2022), though we did not test this hypothesis directly in our study,

and comparing results across studies involving different species should be done cautiously. Future studies investigating interactions between pesticides and social bee gut microbiotas should directly compare acute vs. chronic exposure periods to further investigate this mechanism.

While our chosen exposure regime and chlorothalonil concentrations are field-realistic for our study species, there are other aspects of our experiment that are less so. In our study, we performed one "application event" (i.e., one exposure period) of pure chlorothalonil. In a real agricultural setting chlorothalonil would be reapplied throughout the growing season as a commercial formulation, and likely alongside other pesticides. It is possible that repeated acute exposure periods, combined with exposure to additional chemicals in the formulation as well as other pesticides, could alter bumble bee gut microbiota community structure. Furthermore, we found that *Gilliamella* spp. were not present in our bumble bee microbiotas. *Gilliamella* spp. comprise a core social bee gut microbiota phylotype found in honey bees, stingless bees, and commercial and wild *Bombus* spp., including *B. impatiens* (Cariveau et al., 2014; Hammer et al., 2021; Koch & Schmid-Hempel, 2011; Kwong, Medina, et al., 2017; Kwong & Moran, 2016; Li et al., 2015). In social bees, *Gilliamella* spp. aid in sugar detoxification (Zheng et al., 2016), are associated with declines in pathogen infection intensity (Cariveau et al., 2014; Wintermantel et al., 2018), and cross-feed and compete with other core microbes (Kešnerová et al., 2017; Kwong et al., 2014). It is therefore possible that the bumble bee microbiotas in our study did not respond to chlorothalonil exposure as they would have if *Gilliamella* spp. were present. However, a lack of *Gilliamella* spp. does not seem uncommon in commercial Biobest *B. impatiens* colonies: all our source colonies were missing this phylotype despite being purchased at different times, and *Gilliamella* spp. were also missing from Biobest colonies purchased by another lab in the United States (Motta & Moran, 2023). Biobest is one of the largest suppliers of commercial *B. impatiens* colonies in North America, and if their colonies often lack *Gilliamella* spp. then the results from this study would accurately reflect how the microbiotas of many commercial *B. impatiens* workers respond to chlorothalonil exposure.

While chlorothalonil exposure had no effect on fecal microbiota community structure, we did see an effect of source colony age, day of sample collection (age of microcolony), and source colony identity (Figure 3-2, 3-3). However, despite statistical significance, neither age of source colony nor day explained much variation in microbiota structure—only about one and five percent, respectively. Conversely, source colony ID explained approximately 18% of all variation in fecal microbiota community structure (Figure 3-3B). We purchased our five source colonies at different times during fall 2020, though even colonies purchased at the same time (1 and 2, 3 and 4) had significantly different fecal microbiota community structures. Genetic differences or differences in the gut microbiotas of the founding queens for each colony may have contributed to differences in observed structure. Regardless of the cause, researchers

should be aware that the microbiotas of commercial bumble bee colonies can vary substantially, even if purchased from the same supplier at the same time, and these differences should be accounted for in statistical analyses.

3.5.2 Chlorothalonil exposure does not affect microcolony production

In our experiment, chlorothalonil exposure for a field-realistic period had no effect on the number or average weight of adults, eggs, and pupae produced by bumble bee microcolonies at any of our tested concentrations (Figure 3-1). There was also no effect of chlorothalonil exposure on the number of larvae (Figure 3-E), and though average larva weight was affected, it did not change consistently with chlorothalonil concentration (Figure 3-1F). Our results contrast with those from a previous study by Bernauer et al. (2015) which found that chlorothalonil exposure lowered *B. impatiens* colony biomass, worker number, and queen weight. However, they sprayed flowers with a high concentration of chlorothalonil and allowed bees to forage on those flowers for four weeks, reapplying the pesticide after two weeks (Bernauer et al., 2015). The difference in the number of exposure events and chlorothalonil concentration, or the use of colonies vs. microcolonies (Wynants et al., 2022), could explain our differing results (Bernauer et al., 2015); exposure to higher concentrations of chlorothalonil than we used also increases larva mortality in honey bees (W. Zhu et al., 2014). Notably, a more recent study by Calhoun et al., which used a chlorothalonil concentration comparable to our study (100 ng/mL), found that 12 days of exposure did not affect adult body size or eclosion time in *B. impatiens* (Calhoun et al., 2021). Our results coupled with those of Calhoun et al. (2021) suggest that exposure to chlorothalonil at concentrations closer to the mean concentrations observed in pollen (~90 ng/mL) may be insufficient to impair host health and colony performance, at least on relatively short time scales.

Conversely, age of source colony at microcolony creation affected multiple performance and production metrics. Average worker weight, larva count and pupa count were all significantly higher in microcolonies made from younger source colonies (Figure 3-1). This may be related to the reduced sugar syrup consumption we observed in microcolonies made with three-week-old source colonies compared to those made with one-week-old source colonies, though it is unclear if microcolonies were less productive because they consumed less sugar syrup or if they consumed less sugar syrup because they were less productive. As bumble bee workers can live for months in captivity (Heinrich, 2004), older source colonies will tend to have older workers on average, and so individual senescence likely also contributed to the reduction in microcolony production.

3.5.3 Bumble bee fecal and gut microbiotas have different community structures

We found that *B. impatiens* fecal and gut microbiotas are structurally distinct. In the first round of our fecal vs. gut microbiota experiments, microbiota community structure varied significantly among source colonies, likely for the reasons discussed previously for fecal microbiotas, and with sample type (gut vs. fecal; Figure 3-4A, D-E). We also found three differentially abundant taxa: relative abundances of *Lactobacillus* and *Bombilactobacillus* were higher in gut microbiotas, while *Enterobacteriaceae* relative abundance was higher in fecal samples. While our knowledge of core gut microbe localization is limited in bumble bees, in honey bees *Lactobacillus* and *Bombilactobacillus* are most abundant in the rectal lumen (Martinson et al., 2012; Powell et al., 2014). If these taxa are similarly located in bumble bee guts, then perhaps they have adaptations that make them less likely to be excreted in feces so that they may remain in their optimal environment; it is worth noting, however, that in other animal systems the relative abundance of Firmicutes bacteria is often higher in fecal than rectal and cecal microbiotas (Ahn et al., 2023; Ingala et al., 2018; Mukhopadhya et al., 2022; Stanley et al., 2015). On the other hand, *Enterobacteriaceae* bacteria are non-core, environmentally-derived members of social bee gut microbiotas, and certain members can be pathogenic to hosts (Hammer et al., 2021; Kwong & Moran, 2016). Bacteria in *Enterobacteriaceae* may therefore lack adaptations to avoid being excreted in feces or may be more abundant in feces as dead, excreted cells, killed by the host immune system.

Our second experiment, which aimed specifically to compare fecal and gut microbiotas, confirmed many of the observations we made during the first. Fecal and gut microbiotas in the second experiment were both dominated by the same core gut microbial taxa as in the first experiment, though they lacked *Enterobacteriaceae* and *Acinetobacter* (Figure 3-A). Their absence is likely due to differences in the starting microbiotas of source colonies, as even in our first experiment high *Acinetobacter* and *Enterobacteriaceae* abundances were linked to specific source colonies (Figure 3-A, B). Once again, we observed that fecal and gut microbiota community structures were significantly different (Figure 3-5E), though they were more structurally similar in this second experiment than in our first (compare Figure 3-4D and Figure 3-5E). Furthermore, the day fecal and gut microbiotas were sampled had no impact on this relationship, though fecal and gut microbiotas collectively were more structurally similar from Day 0 onwards (Figure 3-5D). This shift is possibly due to workers and their microbiotas acclimating to more similar living conditions and a simpler diet (i.e., sugar syrup vs. BioGluc®), although, curiously, we did not see this pattern in the first pesticide exposure experiment. As in our first comparison, *Bombilactobacillus* relative abundance was higher in gut than fecal microbiotas, likely for the reasons previously discussed (Figure 3-5A), but *Lactobacillus* relative abundance was consistent between sample types and *Enterobacteriaceae* was not detected.

Our results are comparable to those of previous studies comparing animal fecal and gut microbiotas. A study in *B. terrestris* found, as ours did, that bumble bee fecal and gut microbiotas are structurally distinct but similarly dominated by core social bee gut microbes (Näpflin & Schmid-Hempel, 2018), though in our study microbiota type explained almost four times as much variation in microbial community structure as in the latter study, perhaps due to differences in host species or sample size. Several studies in other insects, mammals, and birds have also found that fecal microbiotas contain similar microbial taxa to gut microbiotas but that certain taxa are differentially abundant between microbiota types (Bodawatta et al., 2020; Kakumanu et al., 2018; Näpflin & Schmid-Hempel, 2018; Yan et al., 2019; Yasuda et al., 2015); however, some studies in birds and bivalves show different patterns (Griffin et al., 2021; Ingala et al., 2018).

We found that fecal and gut 16S rRNA gene copy counts were not correlated for any core taxa. Other studies validating the use of fecal microbiotas as a proxy for gut microbiotas in humans and other animals have found high, significant correlations between measures of microbial abundance in fecal and gut samples (Kakumanu et al., 2018; van den Bogert et al., 2011; Yasuda et al., 2015), though lower correlations have been observed in others (Griffin et al., 2021; Yan et al., 2019). In our study, 16S rRNA gene copy counts in fecal microbiotas spanned four orders of magnitude while in gut microbiotas they only spanned two (Figure 3-5B). This large variation in the number of microbes excreted in feces likely contributes to the poor correlation between 16S rRNA gene copy counts (and, by proxy, absolute microbial abundance) in bumble bee gut and fecal microbiotas.

Overall, bumble bee fecal and gut microbiotas are dominated by the same taxa, and examining bumble bee fecal microbiotas as a proxy for gut microbiotas is an acceptable method, particularly if sacrificing bees to obtain actual gut samples is undesirable or not feasible. For example, for our experiment it allowed us to examine changes in bumble bee microbiotas over time without sacrificing workers, which would have altered social dynamics within our microcolonies and possibly affected production, especially if we had sacrificed the dominant, egg-laying worker acting as "queen" (Free, 1955). However, fecal and gut microbial 16S rRNA gene copy counts are poorly correlated across core taxa and, as with all fecal microbiota proxies, there are differences in community structure between the two microbiota types. Therefore, interpreting changes in bumble bee fecal microbiota community structure, especially changes in the abundances of specific taxa, as changes in bumble bee gut microbiotas should be done carefully and with proper within-study comparisons.

With regards to our first experiment, we are confident in concluding that the absence of an effect of chlorothalonil exposure on bumble bee fecal microbiota community structure is a strong indicator that chlorothalonil also had no effect on gut microbiota community structure. Despite low correlations

between 16S rRNA gene copy counts (i.e., microbial abundances) in fecal and gut microbiotas, both microbiotas are dominated by the same core microbes, albeit at different relative and absolute abundances. Therefore, if chlorothalonil had affected any core microbial taxa in the bumble bee gut microbiota, we very likely would have observed some change in fecal microbiota community structure, though we would not have been able to determine if those changes accurately reflected the alterations occurring in the gut microbial community. However, we observed no effect of chlorothalonil exposure on bumble bee fecal microbiota community structure, and therefore conclude that chlorothalonil treatment also had no, or minimal, impact on bumble bee gut microbiotas.

3.6 Conclusion

We found that exposure to the fungicide chlorothalonil for a field-realistic period does not disturb *B. impatiens* fecal microbiota community structure, nor does it affect microcolony production. Though our experiment was limited in scope (i.e., we simulated one application of a single pesticide), our study provides insight into how the health and fitness of bumble bees—key native, commercial pollinators—may be resilient against short-term exposure to a fungicide often used on crops that they pollinate. As global pesticide use is expected to continue to increase in the coming decades (Deutsch et al., 2018; Sharma et al., 2019; Tudi et al., 2021; Zhang, 2018), additional well-designed, field-realistic, ecologically relevant studies on how pesticides affect the health and performance of animals, including pollinators, will be crucial for minimizing the negative environmental impacts of pesticides.

Furthermore, our research shows that bumble bee fecal microbiota community structure does not perfectly reflect gut microbiota community structure, a pattern echoed across a large body of research examining fecal and gut microbiotas across the animal kingdom. As insect gut samples are relatively easy to obtain when compared to those of other animal taxa, such as vertebrates, we recommend that future studies examining social bee gut microbiotas use whole or partial gut samples whenever possible to obtain accurate results. However, if gut samples cannot be obtained, then studies should include proper within-study fecal and gut microbiota comparisons so that results may be properly interpreted.

3.7 Data availability

All data and R scripts are available at github.com/michellehotch/AEM-2023. Sequences are available through the NCBI Sequence Read Archive (PRJNA1022150).

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3.9 Figures

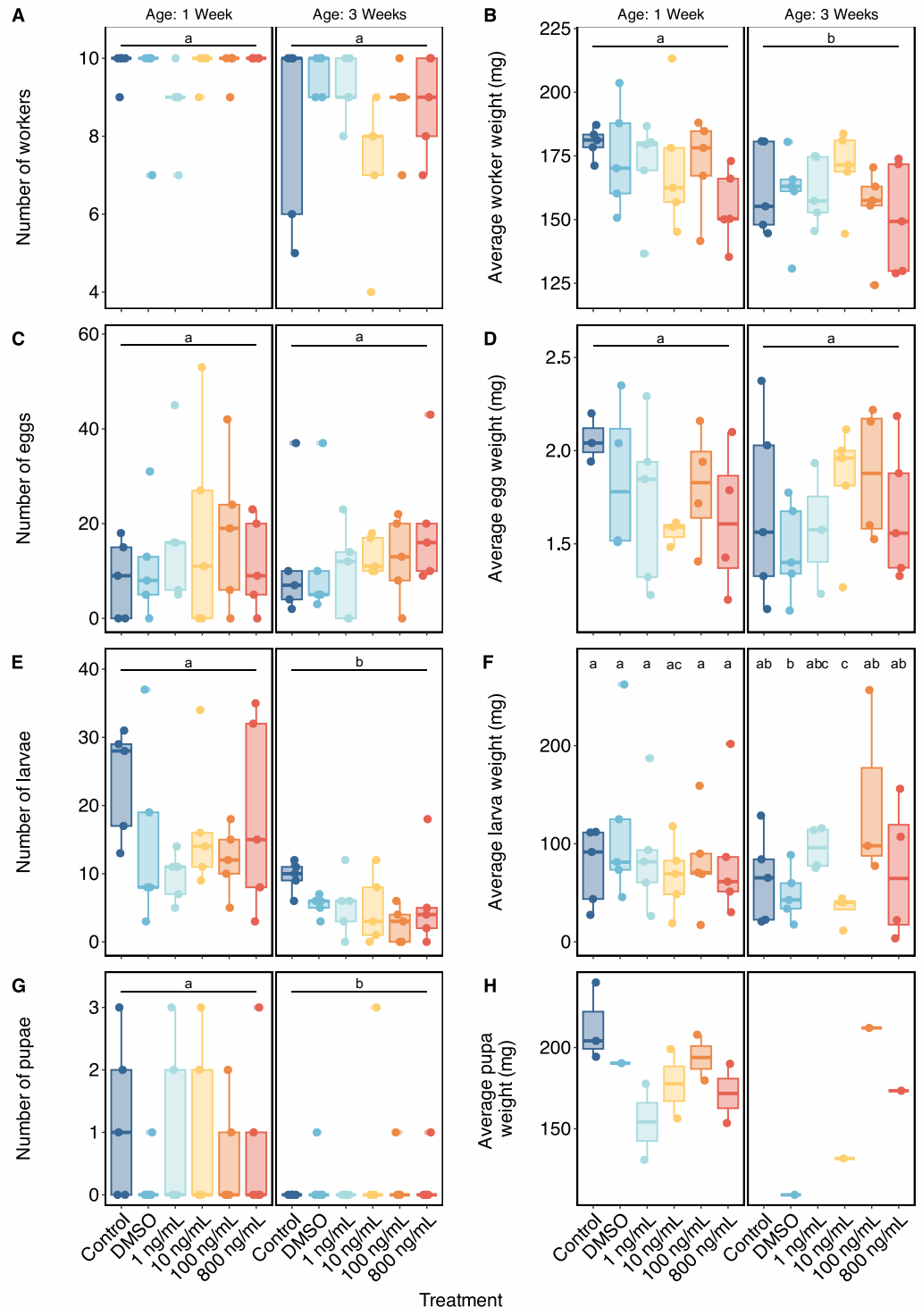

A
Age: 1 Week
Age: 3 Weeks
Number of workers
B
Average worker weight (mg)
C
Number of eggs
D
Average egg weight (mg)
E
Number of larvae
F
Average larva weight (mg)
G
Number of pupae
H
Average pupa weight (mg)
Control
DMSO
1 ng/mL
10 ng/mL
100 ng/mL
800 ng/mL
Treatment

Figure 3-1: Number and average weight of *B. impatiens* workers, eggs, larvae, and pupae in queenless microcolonies on Day 14.

Number and average weight of *B. impatiens* workers (A, B), eggs (C, D), larvae (E, F) and pupae (G, H) in queenless microcolonies on Day 14. All panels are faceted by age of source colonies when the microcolonies were created (one week or three weeks). For panels presenting count data (A, C, E and G) n = 60; for panels presenting averages (B, D, F, and H) n = 16–60. Boxes represent medians and interquartile ranges; the whiskers extend to 1.5 × the interquartile range. Treatments sharing a letter do not differ significantly. For panel F, pairwise comparisons were only conducted when the age and/or treatment of the pair matched (i.e., DMSO Age 1 and DMSO Age 3, or Control Age 1 and DMSO Age 1, but not Control Age 1 and DMSO Age 3). For panel H, there were insufficient data to conduct a formal analysis.

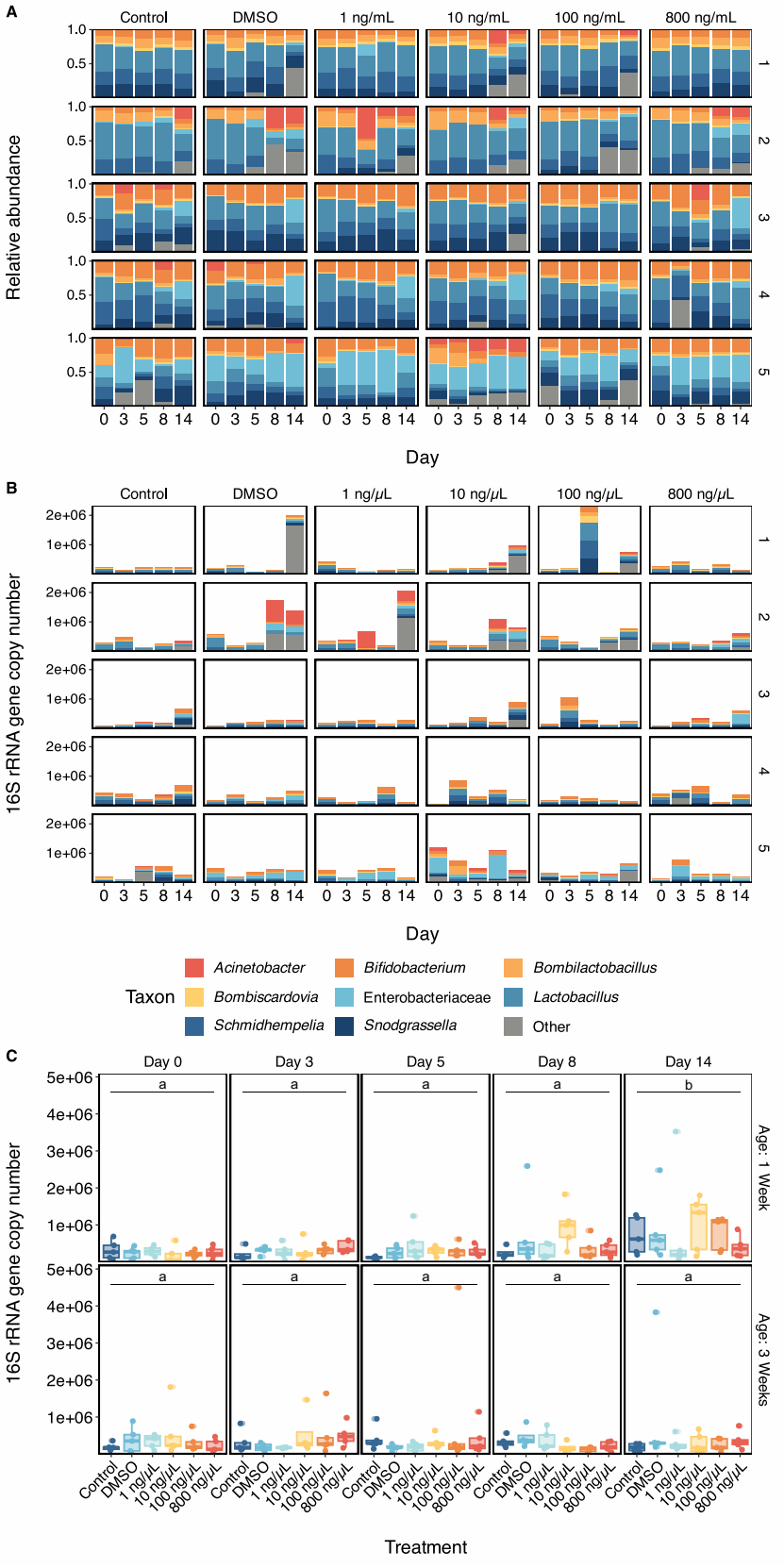

A
Control
DMSO
1 ng/mL
10 ng/mL
100 ng/mL
800 ng/mL
Relative abundance
1.0
0.5
0 3 5 8 14
Day
B
Control
DMSO
1 ng/μL
10 ng/μL
100 ng/μL
800 ng/μL
16S rRNA gene copy number
2e+06
1e+06
Day
Taxon
Acinetobacter
Bifidobacterium
Bombilactobacillus
Bombiscardovia
Enterobacteriaceae
Lactobacillus
Schmidhempelia
Snodgrassella
Other
C
Day 0
Day 3
Day 5
Day 8
Day 14
16S rRNA gene copy number
5e+06
4e+06
3e+06
2e+06
1e+06
a
b
Age: 1 Week
Age: 3 Weeks
Control
DMSO
1 ng/μL
10 ng/μL
100 ng/μL
800 ng/μL
Treatment

Figure 3-2: Microbial community structure and abundance in bumble bee microcolony fecal microbiotas.

A) Stacked bar plot of relative abundances of microbial phylotypes in bumble bee (*B. impatiens*) microcolony fecal microbiotas (n = 299; n = 9-10 per time point per treatment) faceted into columns by treatment and rows by source colony ID (1 through 5). B) Stacked bar plot of 16S rRNA gene copy counts of microbial phylotypes in *B. impatiens* microcolony fecal microbiotas (n = 293; n = 9-10 per time point per treatment) faceted into columns by treatment and rows by source colony ID. For panels A and B, taxa with <1% mean abundance across all samples are grouped into "Other". C) Box plot of total 16S rRNA gene copy counts in *B. impatiens* microcolony fecal microbiotas over time faceted by treatment (n = 293; n = 9–10 per time point per treatment). Boxes represent medians and interquartile ranges; the whiskers extend to 1.5 × the interquartile range. Pairwise comparisons were only conducted for pairs in which day and/or age matched (i.e., Day 0 Age 1 and Day 0 Age 3, or Day 0 Age 1 and Day 8 Age 1, but not Day 0 Age 1 and Day 8 Age 3).

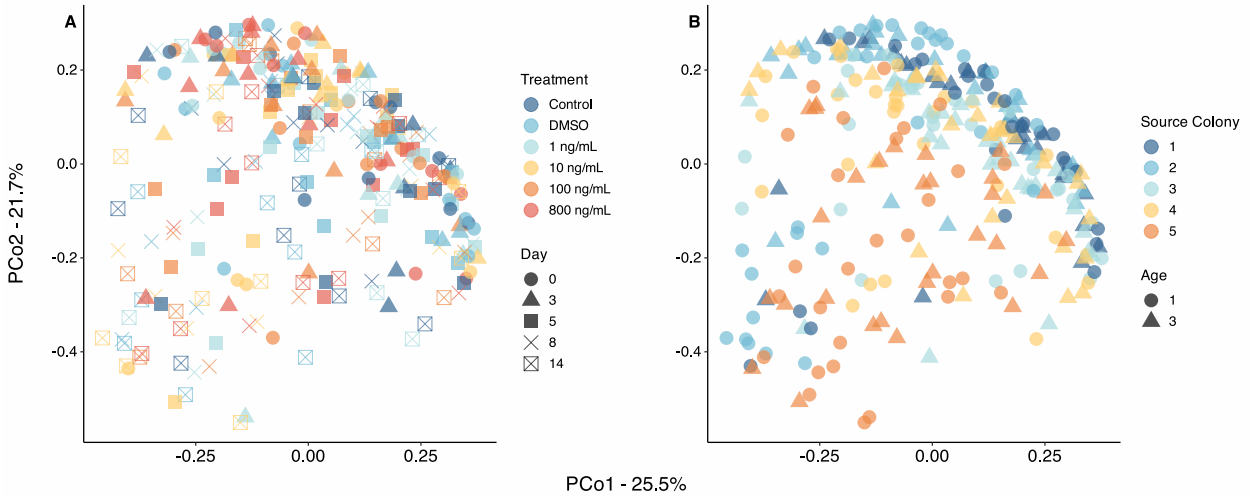


Figure 3-3: Principal coordinates analyses of bumble bee (*B. impatiens*) microcolony fecal microbiotas.

Principal coordinates analyses of bumble bee (*B. impatiens*) microcolony fecal microbiotas (n = 293 total; n = 9–10 per time point per treatment) using Bray-Curtis dissimilarities coloured by A) pesticide treatment and day, and B) source colony ID and age (in weeks). Variables are displayed in groups of two for ease of visualization. Structure does not vary by treatment (PERMANOVA: $F = 0.96$, df = 5, $P = 0.25$), but does vary by age (PERMANOVA: $F = 4.05$, df = 1, $P = 0.0002$) and day (PERMANOVA: $F = 2.92$, df = 4, $P = 0.0001$). Day 0 communities were significantly different from Days 5, 8, and 14 communities, Day 3 communities were significantly different from Day 8 and Day 14 communities, and Day 5 communities were significantly different from Day 14 communities (all $F > 2.13$, df = 1, all $P = < 0.02$). When removed as a stratifying variable, all source colonies had different community structures from one another (PERMANOVA: $F = 16.40$, df = 4, $P = 0.0001$; post-hoc tests: all $F > 2.35$, df = 1, all $P < 0.036$).

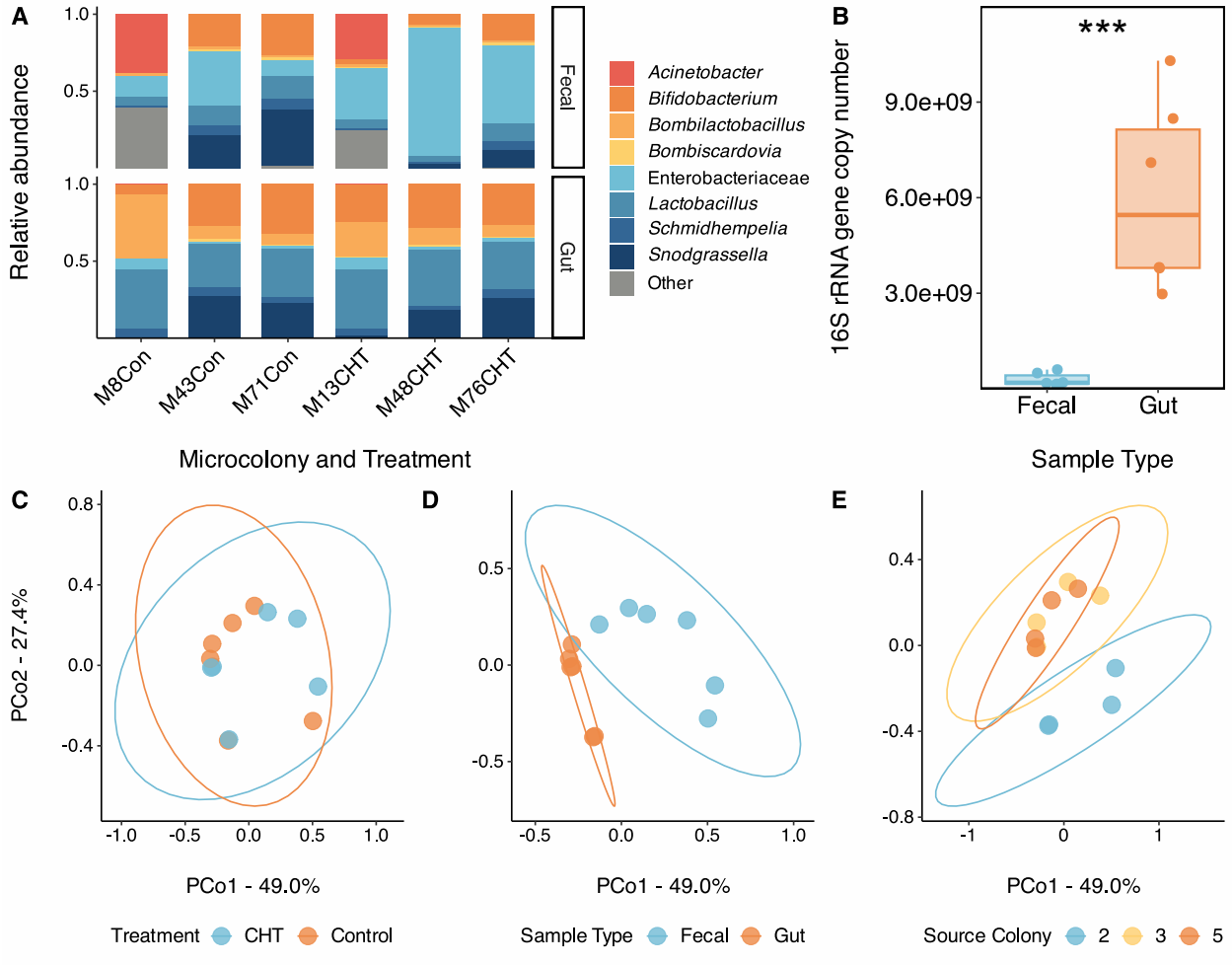


Figure 3-4: Comparisons of microbial community structure and abundance of bumble bee microcolony fecal and gut microbiotas from the CHT exposure experiment.

A) Stacked bar plot of relative abundances of microbial phylotypes in bumble bee (*B. impatiens*) microcolony fecal and gut microbiotas (n = 12; n = 6 per sample type). Samples are labelled "M", then the microcolony ID, then treatment (Con = Control, CHT = 800 ng/mL CHT). Taxa with <1% mean abundance are grouped into "Other". B) Box plot of total 16S rRNA gene copy counts in *B. impatiens* microcolony fecal and gut microbiotas (n = 12, n = 6 per sample type). Boxes represent medians and interquartile ranges; the whiskers extend to 1.5 × the interquartile range. Median fecal gene count = 1.9×10^{8}, median gut gene count = 5.5×10^{9}. C-E) Principal coordinates analyses of bumble bee microcolony gut and fecal microbiotas using Bray-Curtis dissimilarities coloured by C) pesticide treatment (n = 12; n = 6 per treatment), D) sample type (n = 12; n = 6 per sample type), and E) source colony ID (n = 12; n = 4 per source colony). Community structure did not vary by treatment

(PERMANOVA; $F = 2.02$, df = 1, $P = 0.13$) but did vary by sample type (PERMANOVA: $F = 10.70$, df = 1, $P = 0.0001$) and source colony ID (PERMANOVA: $F = 4.46$, df = 2, $P = 0.008$). Specifically, structures of microbiotas from microcolonies made from Source Colony 2 were significantly different from those made from Source Colonies 3 or 5 (2 v. 3: $F = 5.44$, df = 1, $P < 0.01$; 2 v. 5: $F = 7.11$, df = 1, $P < 0.01$).

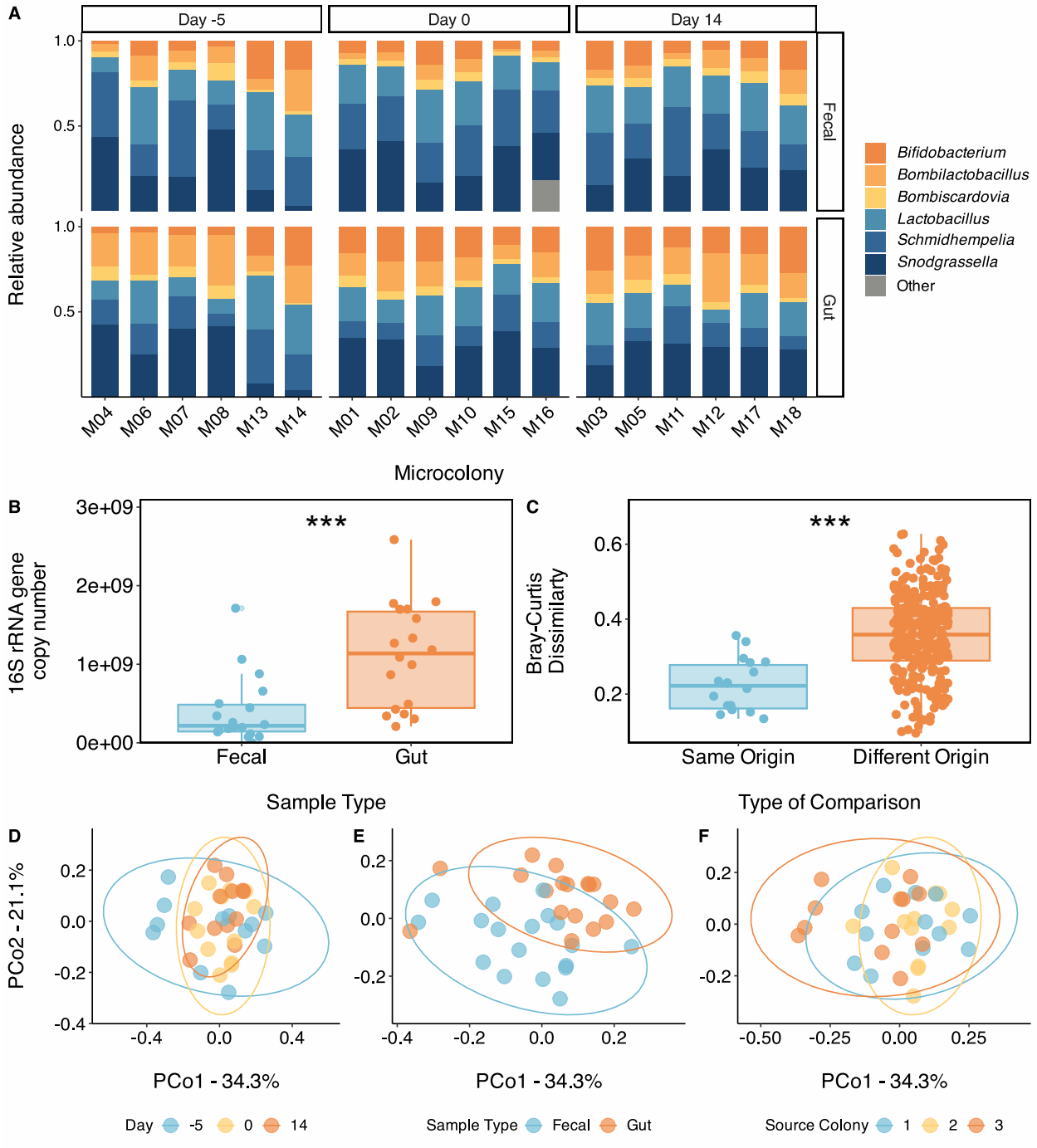


Figure 3-5: Comparisons of microbial community structure and abundance of bumble bee microcolony fecal and gut microbiotas from the fecal vs. gut microbiota follow-up experiment.

A) Stacked bar plot of relative abundances of microbial phylotypes in bumble bee (*B. impatiens*) microcolony fecal and gut microbiotas from the second fecal vs. gut microbiota comparison experiment

taken at three different time points (n = 36; n = 6 per time point per sample type). Taxa with <1% mean abundance are grouped into "Other". B) Box plot of total 16S rRNA gene copy counts in *B. impatiens* microcolony fecal and gut microbiotas from the second fecal vs. gut microbiota comparison experiment (n = 36; n = 6 per time point per sample type). Median fecal gene count = 2.1×10^8, median gut gene count = 1.1×10^9. C) Box plot of Bray-Curtis dissimilarities of fecal and gut microbiota pairs from the same microcolonies ("same origin"; n = 18) and fecal and gut microbiota pairs from different microcolonies ("different origin"; n = 307). Boxes represent medians and interquartile ranges; the whiskers extend to 1.5 × the interquartile range. D-F) Principal coordinates analyses of bumble bee (*B. impatiens*) microcolony fecal and gut microbiotas from the second fecal vs. gut microbiota comparison experiment using Bray-Curtis dissimilarities coloured by B) day (n = 36; n = 12 per day), C) sample type (n = 36; n = 18 per sample type), and D) source colony ID (n = 36; n = 12 per source colony). Community structure varied with sample type (PERMANOVA: $F = 28.70$, df = 1, $P = 0.0001$), day (PERMANOVA: $F = 18.81$, df = 2, $P = 0.0001$), and source colony ID (PERMANOVA: $F = 11.21$, df = 2, $P = 0.0001$). Day -5 communities were significantly different from Day 0 and 14 communities (all $F > 6.09$, df = 1, all $P < 0.01$). Source Colony 3 communities were significant different from Source Colony 1 and 2 communities (all $F > 3.18$, df = 1, all $P < 0.03$).

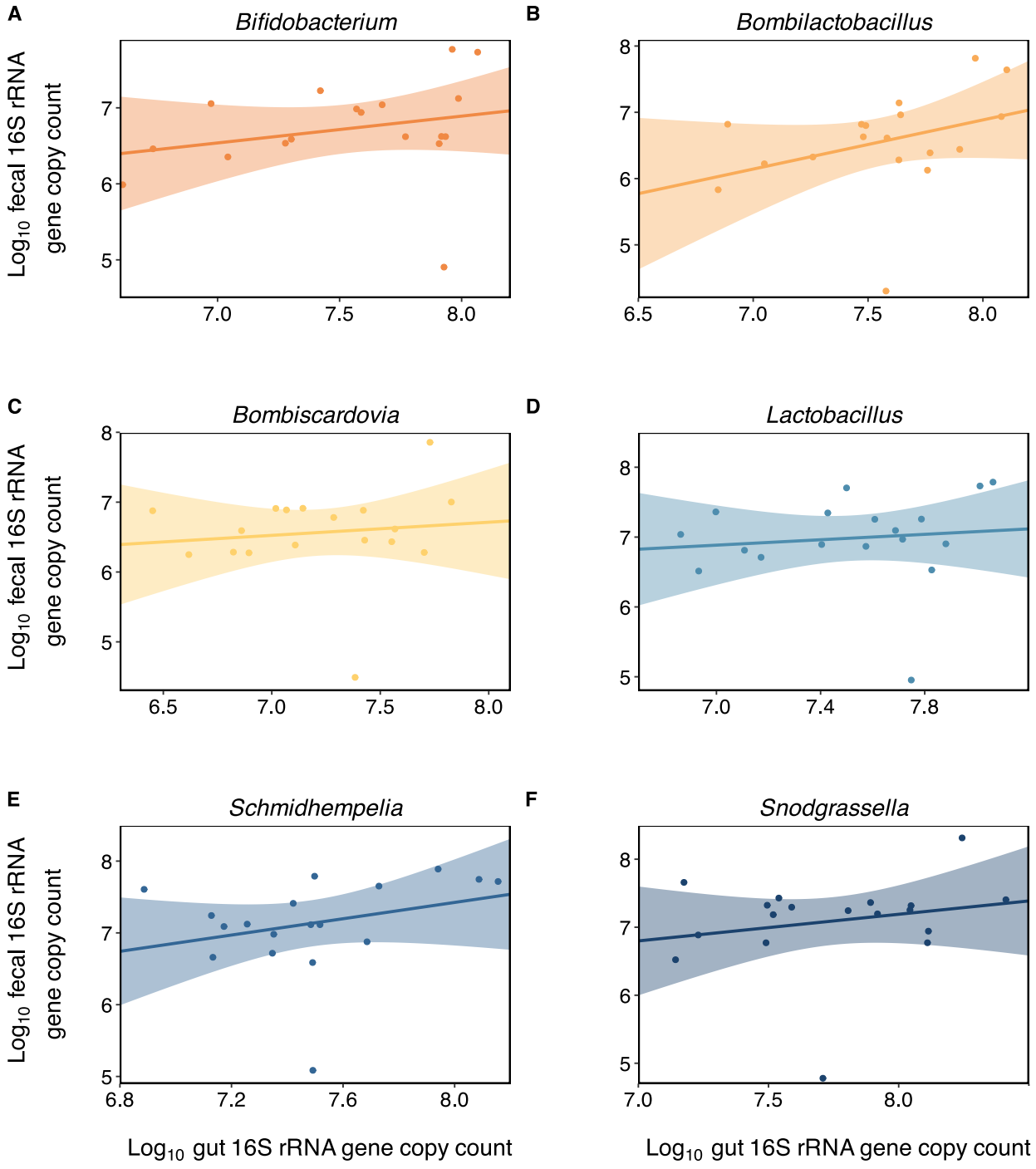
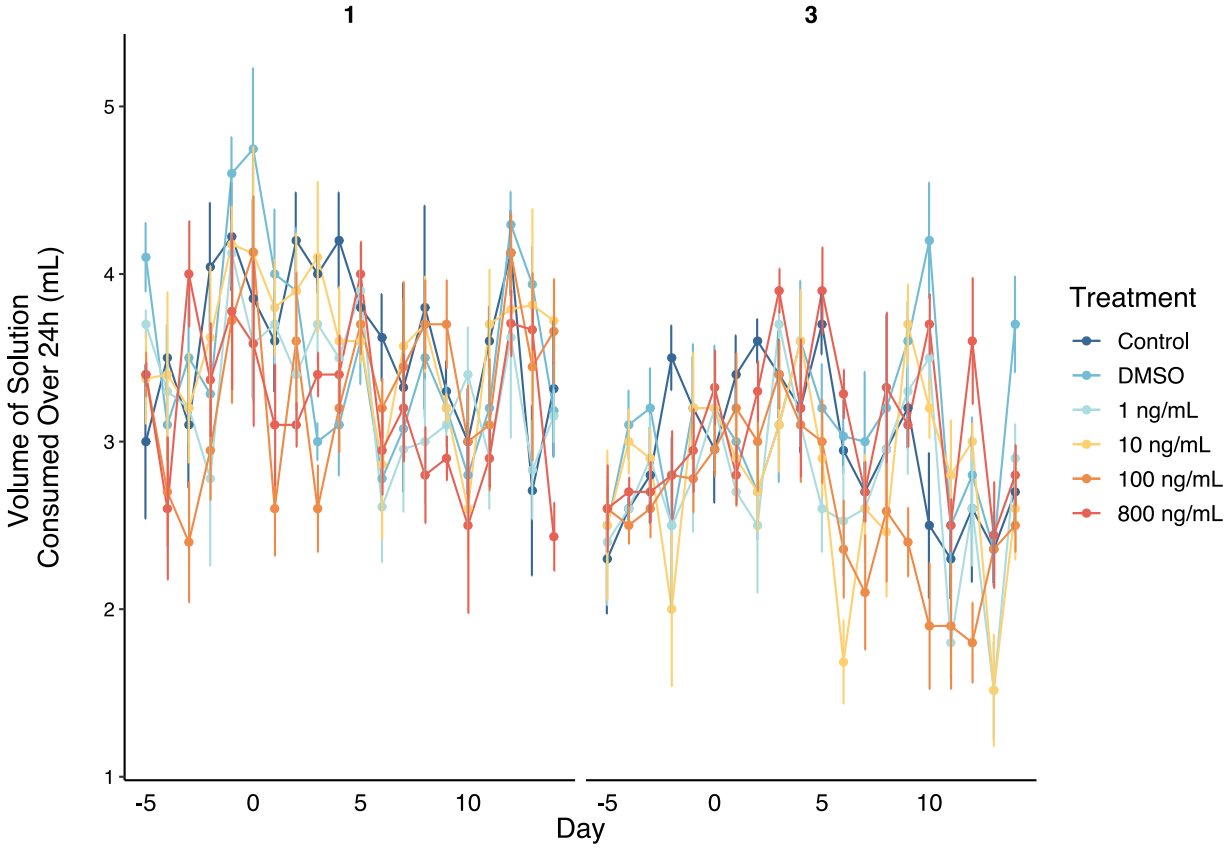


Figure 3-6: Correlations between $\log_{10}$ 16S rRNA gene copy counts in gut microbiotas and fecal microbiotas for various core taxa in *B. impatiens* gut microbiotas.

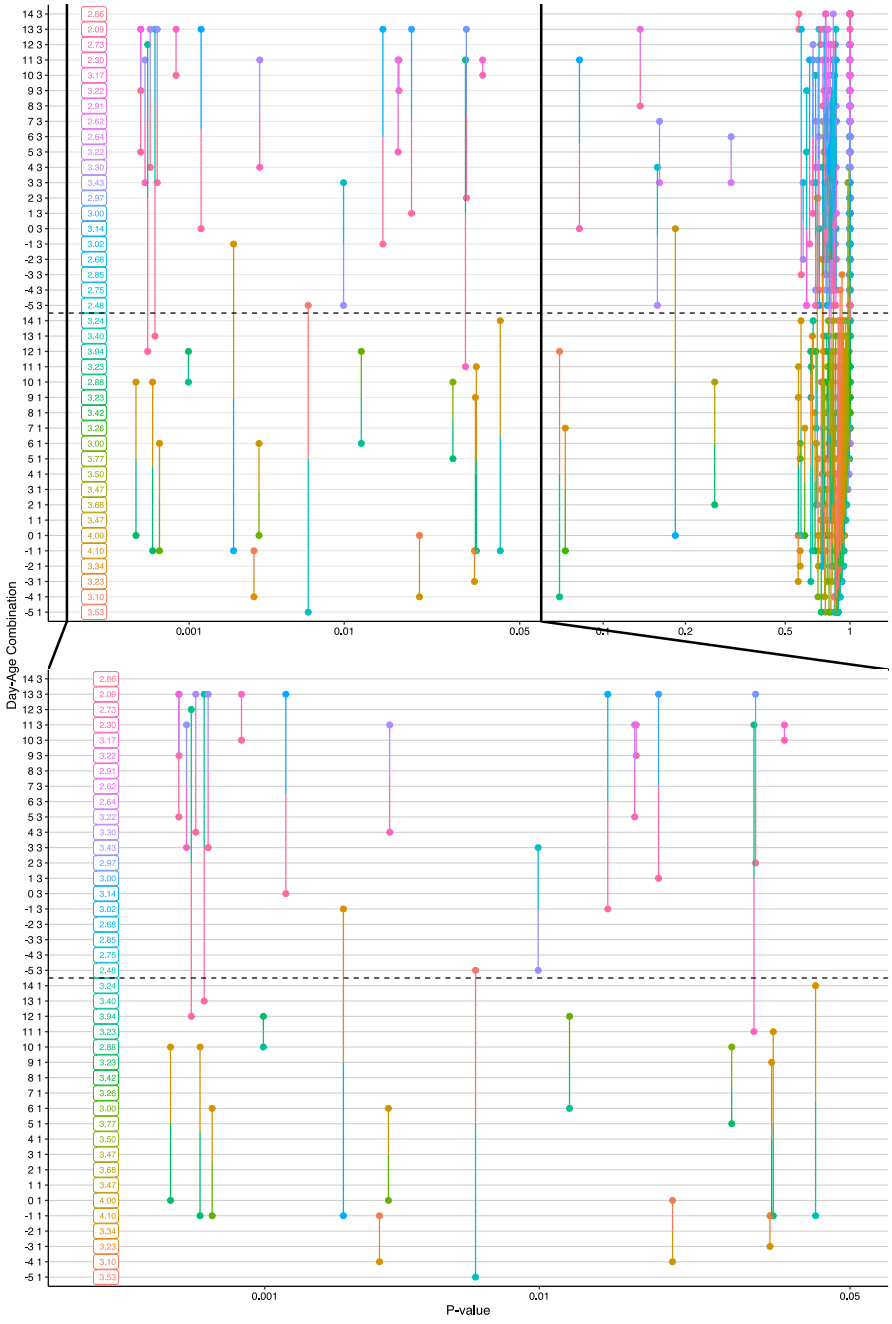
Trendlines are drawn using the lm ("linear model") smoothing method in ggplot2. n = 18 per taxa.

3.10 Supplementary Figures



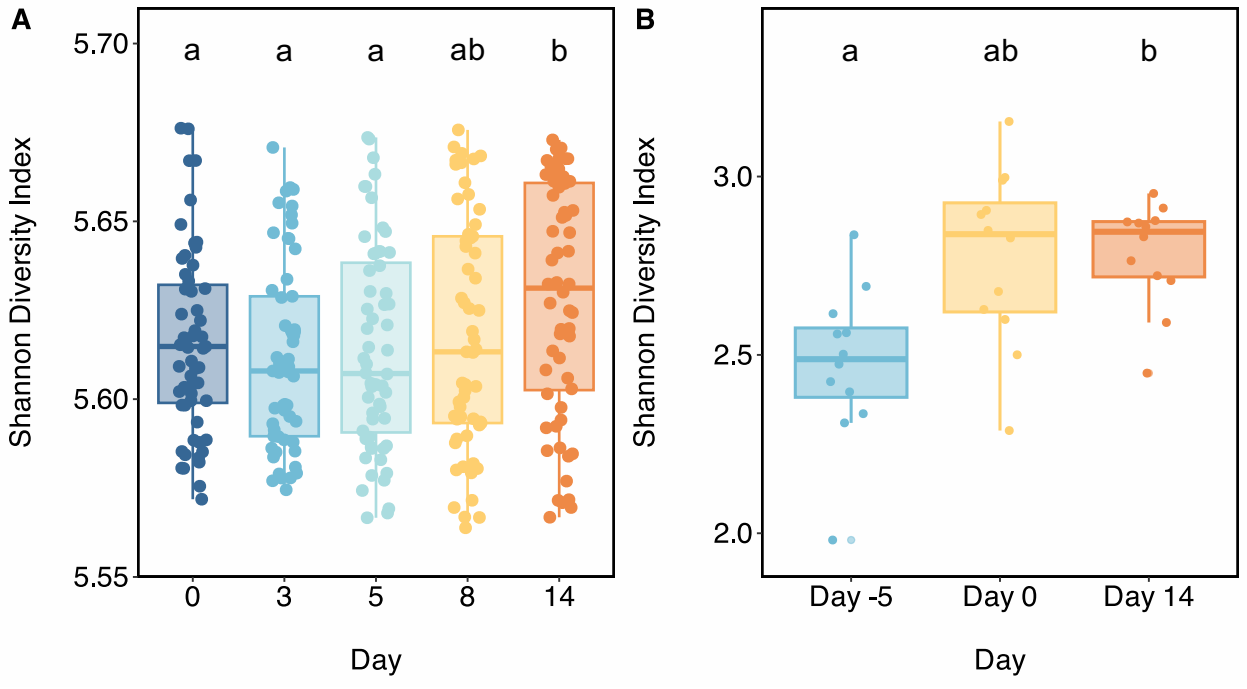
Supplementary Figure 3-1: Volume of sugar solution consumed by *Bombus impatiens* microcolonies in the preceding 24 hours over the course of the experiment.

Dots represent means and bars extend to mean±SE. Figure is faceted by age of the source colonies when the microcolonies were formed (one or three weeks old). Days -5-0 = before pesticide exposure, Days 1-5 = during pesticide exposure, Days 6-14 = after pesticide exposure.



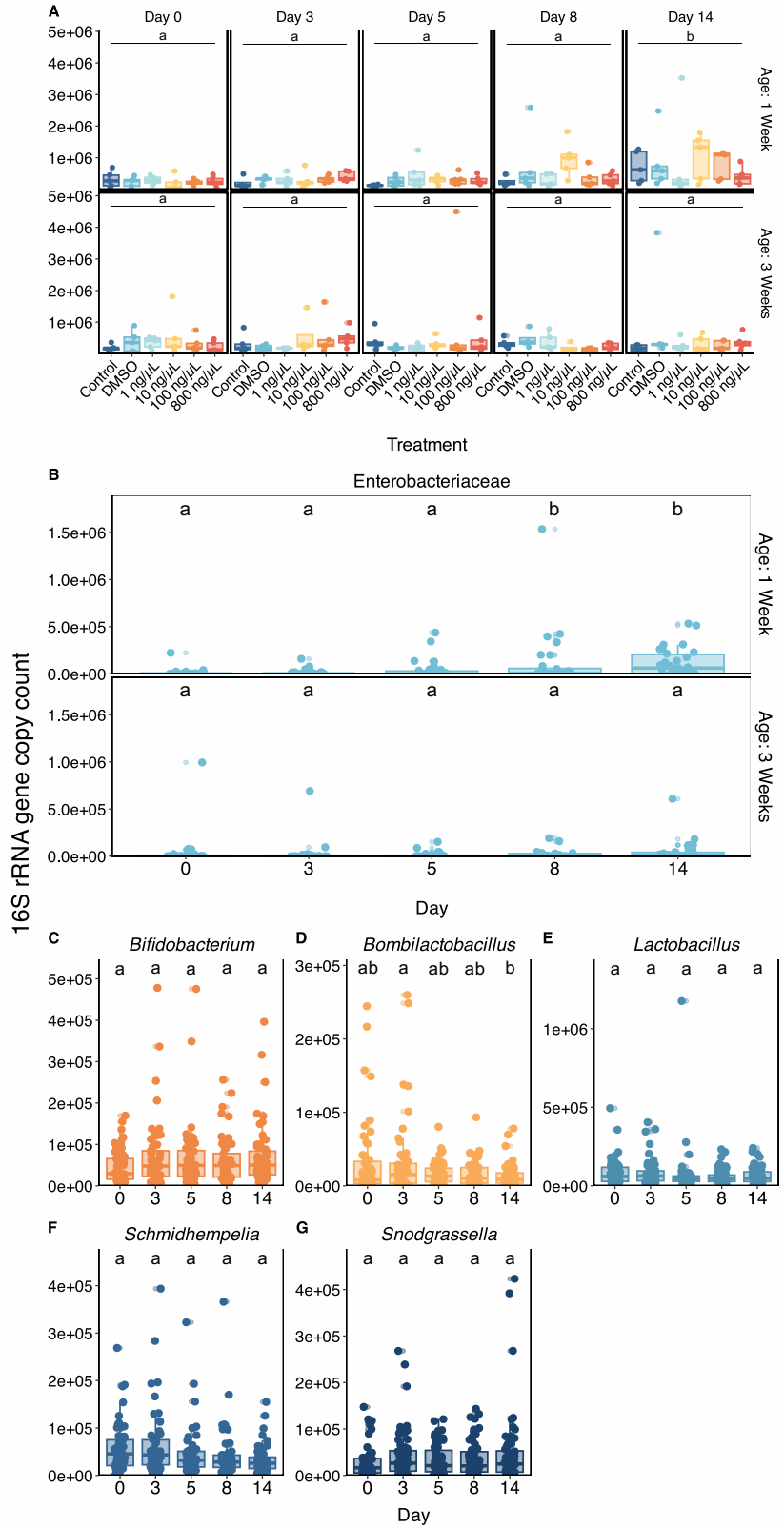
Supplementary Figure 3-2: Pairwise p-value plot of comparisons of sugar syrup consumed in the previous 24 hours between different day:age combinations of B. impatiens microcolonies.

Values on the left are marginal means estimated using the emmeans R package.



Supplementary Figure 3-3: Alpha diversity (Shannon diversity index) for microbiotas in Chapter 2.

A) *B. impatiens* microcolony fecal microbiotas (n = 293; n = 57-60 per day) by day in the first (pesticide exposure) experiment. B) *B. impatiens* microcolony fecal and gut microbiotas from the second (fecal-gut comparison) experiment (n = 36; n = 12 per day). Boxes represent medians and interquartile ranges; the whiskers extend to 1.5 × the interquartile range.


A
Day 0
Day 3
Day 5
Day 8
Day 14
Age: 1 Week
Age: 3 Weeks
Control
DMSO
1 ng/µL
10 ng/µL
100 ng/µL
800 ng/µL
Treatment
B
Enterobacteriaceae
16S rRNA gene copy count
Day
C
Bifidobacterium
D
Bombilactobacillus
E
Lactobacillus
F
Schmidhempelia
G
Snodgrassella

Supplementary Figure 3-4: Boxplots of 16S rRNA gene copy counts in *B. impatiens* fecal microbiotas.

Boxplots of 16S rRNA gene copy counts in B. impatiens fecal microbiotas for A) all bacteria and B-G) specific core taxa (n = 293; n = 57–60 per day). Boxes represent medians and interquartile ranges; the whiskers extend to 1.5 × the interquartile range. For panels A and B, pairwise comparisons were only conducted for pairs in which day and/or age matched (i.e., Day 0 Age 1 and Day 0 Age 3, or Day 0 Age 1 and Day 8 Age 1, but not Day 0 Age 1 and Day 8 Age 3).

Chapter 4

Changes in bumble bee queen gut microbiotas during and after diapause

This chapter has been submitted to *Insect Molecular Biology*.

4.1 Abstract

Bumble bees are key pollinators with gut microbiotas that support host health. After bumble bee queens undergo winter diapause, which occurs before spring colony establishment, their gut microbiotas are disturbed, but little is known about community dynamics during diapause itself. Queen gut microbiotas also help seed worker microbiotas, so it is important that they recover post-diapause to a typical community structure, a process that may be impeded by pesticide exposure. We examined how bumble bee queen gut microbiota community structure and metabolic potential shift during and after diapause, and whether post-diapause recovery is affected by pesticide exposure. We euthanized commercial *Bombus impatiens* queens before and during diapause. Additionally, we allowed some queens to recover from diapause for one week before euthanasia, exposing half to the common herbicide glyphosate. Using whole-community, shotgun metagenomic sequencing, we found that core bee gut phylotypes dominated communities before, during, and after diapause, but that two phylotypes, *Schmidhempelia* and *Snodgrassella*, ceased to be detected in queen microbiotas during late diapause and recovery. Despite fluctuations in taxonomic community structure, metabolic potential remained constant through diapause and recovery. Also, glyphosate exposure did not affect post-diapause microbiota recovery. However, metagenomic assembly quality and our ability to detect microbial taxa and metabolic pathways varied with microbial abundance, which was substantially reduced during diapause. Our study offers new insights into how bumble bee queen gut microbiotas change taxonomically and functionally during a key life stage, and provides guidance for future microbiota studies in diapausing bumble bees.

4.2 Introduction

Bumble bees are key pollinators in both wild and agricultural ecosystems. Their health, like that of other eusocial, corbiculate bees, is supported through a symbiosis with a core community of microbes living in their hindgut (Hammer, Le, Martin, et al., 2021; Kwong & Moran, 2016; Motta & Moran, 2023b). The social bee gut microbiota contains five core phylotypes: *Bifidobacterium* spp., *Bombilactobacillus* spp., *Gilliamella* spp. (along with *Candidatus* Schmidhempelia spp., hereafter *Schmidhempelia*, in many bumble bees), *Lactobacillus* spp. near *melliventris*, and *Snodgrassella* spp., with the first four phylotypes primarily fermenting sugars and the last metabolizing sugar fermentation products (Kwong & Moran, 2016). These phylotypes are well conserved both within and across social bee species including honey bees, bumble bees, and stingless bees, though certain lineages have gained additional core phylotypes or lost others (Cerqueira et al., 2021; Kwong, Medina, et al., 2017). The social bee gut microbiota benefits its hosts primarily by defending against pathogen infection (Cariveau et al., 2014; Koch & Schmid-Hempel, 2011; Miller et al., 2021; Palmer-Young et al., 2019) and stimulating the host immune system (Horak et al., 2020; Kwong, Mancenido, et al., 2017; Lang et al., 2022; Steele et al., 2017), though it is also capable of synthesizing hormones (Kešnerová et al., 2017), stimulating host detoxification genes (Wu et al., 2020), and increasing host weight gain (Zheng et al. 2017); it may also play roles in metal detoxification and nutrient processing (Engel et al., 2012; Kešnerová et al., 2017; Lee et al., 2018; Rothman et al., 2019; Zheng et al., 2017, 2019). Given these benefits, it is important for social bees to maintain their gut microbiotas. However, bumble bee ecology presents an inherent obstacle to this maintenance in the form of winter diapause.

The three tribes of eusocial corbiculate bees (i.e., honey bees, stingless bees, and bumble bees) differ in how they withstand periods of adverse environmental conditions, and, consequently, how their gut microbiotas shift during these periods. Both honey bees and stingless bees form perennial colonies that remain active year-round. During winter, regardless of latitude, honey bees maintain colony temperatures above 20˚C by forming thermoregulating clusters (Heinrich 1981) and workers regularly consume honey, feeding both themselves and their gut microbes. Stingless bee colonies, primarily found in tropical environments, do not experience low winter temperatures to the same degree as temperate honey bee colonies, but they can still experience seasonal food scarcity as abundances of floral resources fluctuate. In response, stingless bee colonies may enter reproductive diapause and shift from collecting pollen and nectar to primarily nectar, but again workers are regularly consuming food (Dos Santos et al., 2014). While seasonal changes in stingless bee gut microbiotas have yet to be examined, winter honey bee gut microbiotas generally contain the same core phylotypes as summer microbiotas, though at

different relative and absolute abundances, and total microbial abundance is the same as or higher than in summer microbiotas (Castelli et al., 2022; Kešnerová et al., 2020; C. Li et al., 2022).

In contrast to honey bees and stingless bees, bumble bee species in temperate environments have annual colony life cycles. In the late summer and early fall, bumble bee colonies produce a new generation of queens. Newly eclosed queens spend a few days in their natal colonies before leaving, providing them sufficient time to acquire their gut microbiota from interactions with their nest mates and the nest environment (Billiet et al., 2017; Hammer, Le, Martin, et al., 2021). After this brief period they leave to mate and forage on pollen and nectar, accumulating large lipid and glycogen stores in their fat bodies that allow them to survive winter diapause, a prolonged period of decreased body temperature, lowered metabolism, and fasting (Carnell et al., 2020; Röseler & Röseler, 1986; Woodard et al., 2019). Queens then dig hibernacula and remain there for four to ten months, depending on the species (Heinrich, 2004), during which time their body temperature lowers to a few degrees above zero (Alford, 1969; Heinrich, 2004; Keaveny et al., 2022; Yoon et al., 2010, 2013) and they consume no food. Bumble bee core gut microbes spend non-diapause months living in a saccharide-rich environment at warmer temperatures (in general, both bumble bee abdominal temperatures during flight and bumble nest temperatures are at least a few degrees higher than ambient air temperature, sometimes much warmer (Heinrich, 1977)), and so diapause conditions present a challenge for resident gut microbes that is unique among social bee tribes. Thus, unsurprisingly, bumble bee gut microbiotas also experience seasonal shifts in community structure (Bosmans, Pozo, Verreth, Crauwels, Wäckers, et al., 2018).

While we know that diapause alters bumble bee gut microbiotas, overall, little is known about microbial community fluctuations during this period. One study profiled *B. terrestris* guts after diapause and found high relative abundances of core gut microbes, but lacked pre-diapause controls (Su et al., 2021). Another study examined ileal (i.e., upper hindgut) microbiotas of *Bombus impatiens* queens before and immediately after a four-month diapause and found that diversity and relative abundance of non-core microbes increased after diapause while total microbial abundance declined by approximately 50% (Bosmans, Pozo, Verreth, Crauwels, Wäckers, et al., 2018). Restructuring of gut microbiotas is also observed in other animals that overwinter in diapause-like states, such as hibernating mammals, including ground squirrels, lemurs, bats, and bears (Carey et al., 2012, 2013; Carey & Assadi-Porter, 2017; Greene et al., 2022; Sommer et al., 2016; Xiao et al., 2019), fasting reptiles (Costello et al. 2010), and overwintering insects such as crickets and beetles (Ferguson et al., 2018; Hou et al., 2021; J. Wang et al., 2017). However, only ileal microbial communities of bumble bees have been examined with proper pre-diapause controls, and only after diapause, not during. Three of the core social bee gut microbiota phylotypes (*Bifidobacterium* spp., *Bombilactobacillus* spp., and *Lactobacillus* spp. near *melliventris*)

reside primarily in the rectum, the lower part of the bee hindgut. Therefore, to date we have an incomplete picture of how bumble bee gut microbiota community structure changes during and after diapause, and it is unknown whether taxonomic changes are accompanied by shifts in gut microbiota metabolism.

Furthermore, little is known about bumble bee gut microbiota community dynamics after diapause. When bumble bee queens emerge from diapause in the spring, each will found a new colony, raising the first generation of workers on her own. As bumble bee gut microbiotas are primarily seeded through interactions with conspecifics and hive materials (Billiet et al., 2017; Hammer, Le, Martin, et al., 2021; Su et al., 2021), bumble bee queens are a key source of core gut microbes for the workers they raise in early spring. Therefore, recovery of the queen gut microbiota to a "standard" gut microbiota community structure is important to support the health of the colony as a whole. One study which sampled wild *B. terrestris* queen ileal microbiotas in the early spring (i.e., soon after the end of diapause) found high relative abundances of the core bee gut microbes *Snodgrassella* and *Gilliamella* (Bosmans, Pozo, Verreth, Crauwels, Wilberts, et al., 2018). Other studies examining the gut microbiotas of lab-reared queens one to three months post diapause found that gut microbiotas at this time are dominated by core gut microbes (L. Wang et al., 2019), but that some phylotypes, such as *Gilliamella* spp., may be lost during diapause (Koch et al., 2013). These studies imply that post-diapause recovery of queen gut microbiotas to a core-microbe-dominated state is possible. However, none of these studies profiled gut microbiotas during diapause, nor did they investigate gut microbiota community dynamics early in diapause recovery. Moreover, as wild queens must actively forage in the early spring to feed both themselves and their young, they may be exposed to stressors that could impede post-diapause gut microbiota recovery, such as pesticides, which are known to alter social bee gut microbiotas (Hotchkiss et al., 2022). Bumble bee queen gut microbiotas may even be especially susceptible to disturbances during this period as communities are in flux, transitioning from a diapause to active community structure as queens resume activity and food consumption.

In this study we use metagenomics to compare bumble bee (*B. impatiens*) queen gut microbiota taxonomic community structure and metabolic potential before, during, and after diapause to better understand bumble bee gut microbiota community dynamics during this unique time in the bumble bee colony life cycle. Additionally, we compare post-diapause bumble bee queen gut microbiota recovery in the presence and absence of an external stressor, glyphosate exposure. Glyphosate is the most used herbicide, globally (Duke and Powles 2008; Benbrook 2016), and exposure has a consistent effect on social bee gut microbiotas: both in its pure form and as part of commercial formulations, glyphosate decreases the abundance of the core social bee gut microbe *Snodgrassella alvi*, though some *S. alvi* strains are resistant and abundance declines are more consistently reported in honey bees than bumble bees (Blot

et al., 2019; Cullen et al., 2023; Helander et al., 2023; Motta et al., 2018, 2020; Motta & Moran, 2020, 2023a; Straw et al., 2023). *Snodgrassella alvi* resides primarily in the ileum where it forms a biofilm, and likely cross feeds, with *Gilliamella* spp. (Hammer, Le, Martin, et al., 2021) and helps maintain an anoxic environment that supports the growth of other core, anaerobic microbes (Engel et al., 2013; Kwong et al., 2014; Zheng et al., 2017). Due to these roles played by *S. alvi*, we expected that glyphosate exposure could interfere with post-diapause bumble bee gut microbiota community dynamics.

4.3 Materials and Methods

4.3.1 Queen rearing

We purchased three *Bombus impatiens* colonies, henceforth referred to as natal colonies, from Biobest (Leamington, Ontario, Canada) in the fall of 2020 and winter of 2021. We removed all cotton from natal colonies upon arrival and kept them in a dark room at 28.5 ℃ (± 0.09 ℃) and 23.2% relative humidity (± 0.6%). We fed natal colonies Biobest BioGluc® syrup and balls of honey bee pollen (Hawkins Honey, Ontario, Canada) mixed with water *ad libitum*. Pollen balls were sterilized with gamma-irradiation (average dose = 16.43 kGy; range = 12.82-17.7 kGy) before being fed to bees.

We checked natal colonies daily for newly emerged queens and males. When queens emerged, they were weighed (Day 0) and tagged with unique IDs consisting of a letter corresponding to their natal colony (W, R, or Y) and a number. After tagging, we released queens back into their natal colonies for five days; this period is similar to the duration wild queens spend in their natal colonies (Woodard *et al.*, 2019) and is sufficient for gut microbiota acquisition via conspecific and nest contact (Billiet et al., 2017; Hammer et al., 2023; Meeus et al., 2013). After five days (Day 5), we removed queens from their natal colonies and housed them in sterile plastic containers with sibling queens. Whenever males emerged, we removed them from their natal colonies immediately to avoid intra-colony matings and housed them in sterile plastic containers with sibling males. We kept queens and males in these containers on a 12:12 h light:dark cycle and fed them filter-sterilized (pore size 0.2 µm) 30% (w/v) sugar solution and sterilized pollen *ad libitum.*

Two days after each queen's removal from her natal colony (Day 7), we attempted to mate her with a male from a different colony. We released two to four queens and at least twice the number of males into a flight cage (40 cm x 40 cm x 60 cm) under bright light and observed bees for mating events. However, few of our queens mated successfully (<10%), and so we ultimately used exclusively unmated queens for our experiment.

4.3.2 Diapause sampling

We kept queens in sterile plastic containers until ten days post-tagging (Day 10). On Day 10, we once again weighed queens and randomly assigned them to one of five time-points/treatments (hereafter "treatments"): pre-diapause, two-month diapause, four-month diapause, diapause-recovery control, or diapause-recovery with glyphosate. We assigned more queens to treatments with a diapause stage to account for mortality over diapause.

We euthanized queens in the pre-diapause group (11 total) directly after weighing by freezing them at -80˚C. We then placed the remaining queens individually in sterile 50 mL Falcon tubes with holes poked in the lids for ventilation. We placed these vials in an incubator at 14˚C for two days to serve as an acclimation period and to allow queens to defecate before diapause. After this two-day period, we moved all queens in their Falcon tubes to containers in an incubator set at 4˚C for diapause (Yoon et al., 2010, 2013); we maintained humidity in containers at >80% for the duration of diapause.

We sampled two-month queens after two months of diapause, and four-month, recovery control, and recovery + glyphosate queens after four months of diapause. At sampling time-points, we removed queens from the incubator and allowed them to warm up to room temperature to verify they were alive. we weighed live queens and then immediately euthanized them by freezing at -80˚C, and for recovery treatments we weighed live queens and moved them on to the recovery experiment. We excluded all queens that died during diapause from further analysis. Across all treatments that underwent diapause, 12 queens were alive in the two-month treatment (five dead), 12 in the four-month treatment (12 dead), 11 in the recovery control treatment (9 dead), and 13 in the recovery + glyphosate treatment (10 dead).

4.3.3 Recovery experiment

We used queens in the recovery control and recovery + glyphosate groups to examine how pesticide exposure affects the recovery of bumble bee queen gut microbiotas post-diapause. After diapause, we placed queens from these treatments in individual sterile plastic containers in a dark room at 28.5℃ (± 0.09℃) and 23.2% relative humidity (± 0.6%) for a one-week recovery period. During this time, we provided control queens with sterile 30% (w/v) sugar solution *ad libitum*, and glyphosate-treatment queens with a sterile glyphosate–30% sugar solution *ad libitum*. We prepared the glyphosate-sugar solution fresh daily by spiking 10 mL of sugar solution with a 1 mg/mL stock solution of glyphosate (PESTANAL®, analytical standard) to a final concentration of 0.1 mM glyphosate. Regardless of treatment, we replaced all sugar solutions daily and recorded the volume of sugar solution consumed each day by each queen. Additionally, on the first day of the recovery period, we provided all queens with 1 g balls of sterile honey bee pollen mixed with water. We replaced these pollen balls daily until we

observed signs of egg laying, in which case we left the original ball and added a 0.5 g pollen ball which we replaced daily instead. After one week, we euthanized all queens by freezing at -80˚C.

4.3.4 Dissections

We dissected all queens in a sterile field using a dissecting microscope. We first soaked queens for three minutes in a 1% bleach solution to kill any cuticular microbes then rinsed them three times in sterile water (Engel et al., 2013). We then moved queens to a dissection dish containing sterile Ringer's solution (2.16 g NaCl, 0.051 g CaCl2, 0.111 g KCl in 300 mL of water) and removed each queen's entire gut. We froze guts at -20˚C until DNA extractions. For each new dissection, we used fresh Ringer's solution and a new sterile dissection dish; we also cleaned the microscope with 70% ethanol and flame-sterilized all dissection tools between dissections.

4.3.5 DNA extractions and sequencing

We extracted DNA from a subset of five, randomly-selected queen guts per treatment using the QIAGEN DNeasy PowerLyzer PowerSoil Kit (Hilden, Germany), following protocol with the following modifications: 1) gut samples were masticated using a pestle before extraction, 2) after 60 µL of solution C1 was added and samples were vortexed, samples were incubated in a water bath for 10 minutes at 65˚C, 3) all centrifugation steps were conducted at 13,000 x *g*, and 4) solution C6 sat on the membrane for 5 minutes before the samples were centrifuged for elution; we did not enrich for prokaryotic cells before extraction as we were interested in observing changes in fungal communities in addition to bacterial and archaeal communities during diapause. We quantified DNA using a Qubit 2.0 with the Invitrogen Qubit dsDNA HS Assay Kit following protocol with the modification that the DNA was incubated in the dye/buffer mixture for five minutes. We sent extracted DNA and a negative control to Génome Québec for PCR-free metagenomic shotgun sequencing using the NovaSeq 6000 system; the negative control did not contain sufficient DNA for sequencing.

4.3.6 Metagenomic bioinformatics

We examined the quality of raw reads using FastQC v0.11.9 (Andrews, 2019), and trimmed and filtered out low-quality reads using fastp v0.23.2 with default settings (Chen et al., 2018). After this filtering step, we had a median of 90 million paired-end reads per sample (range: 73.6 million – 117.8 million). We then used the bwa-mem command in bwa v0.7.17 to map trimmed reads against a *B. impatiens* genome (NCBI PRJNA61101) to remove host reads (H. Li, 2013; Sadd et al., 2015), and used Kraken2 v2.1.2 to map host-filtered reads against the Kraken2 standard plant reference library to remove

pollen-associated sequences (Wood et al., 2019). After filtering out reads that mapped to the host genome and Kraken2 plant reference library, we had a median of 773,000 paired-end reads per sample (range: 508,723 – 3,601,528); >99% of paired reads were lost at this step (Supplementary Table 4-1).

We assembled fully filtered reads using metaSPAdes v3.15.4 with paired-end library type and default settings, including k: 21, 33, 55 (Nurk et al., 2017). We performed individual assemblies by queen and coassemblies by treatment with the goal of comparing individual assemblies to coassemblies to 1) see if we observe additional taxa in coassemblies and 2) verify patterns in taxonomic and metabolic diversity observed in individual assemblies. We assessed assembly quality using quast v5.0.2 and multiqc v1.9 (Ewels et al., 2016; Gurevich et al., 2013). We then used the anvi-script-reformat-fasta program from Anvi'o v7.1-dev to reformat the assemblies for further analysis and remove contigs less than 300 bp (Eren et al., 2020; Shell & Rehan, 2022). For each assembly, we generated contig databases in Anvi'o using anvi-gen-contigs-database program (Eren et al., 2020). We calculated contig coverages using bwa-mem to map filtered reads against contigs, samtools v1.17 to convert mapping output to the bam file type, and Anvi'o's anvi-init-bam, anvi-profile, and anvi-export-splits-and-coverages to calculate and extract the coverage for each contig for each assembly (Danecek et al., 2021; Eren et al., 2020; H. Li, 2013).

After calculating the coverage for each contig in each assembly, we wanted to obtain a high-level taxonomic overview of our individual assemblies to determine whether any contigs were being assigned to eukaryotes (i.e., whether host and pollen contamination was still present). To accomplish this, we used blast+ v2.14.1 to assign taxonomy to all contigs in individual assemblies based on alignment against the blast+ nt database (Camacho et al., 2009). We selected the top taxonomic hit for each contig in each assembly, filtered for taxonomic hits with e-values $< 1\times10^{-50}$ and percent identities >90%, and then calculated the mean coverage for each of six high-level taxa of interest – bacteria, fungi, plants, invertebrates, and vertebrates. We found that most contigs were assigned to invertebrates, indicating that substantial host contamination remained in our assemblies (Supplementary Figure 4-1A), and that fungal contigs were inconsistently present in individual assemblies (Supplementary Figure 4-1B). Although our initial plan was to examine taxonomic and metabolic changes in bacterial and fungal communities in bumble bee queen gut microbiotas, due to the inconsistent presence of fungal contigs, the complexity of eukaryotic genes and genomes, and low number of reads post-host and plant filtering, we decided to limit our analysis to bacterial communities.

To eliminate eukaryotic contigs from our assemblies, we used the "dplyr" package (v1.1.2) in R (v4.3.1) (R Core Team, 2023; Wickham et al., 2023) to generate a list of all contigs assigned to the kingdom "bacteria" by blast+ for each individual assembly; we did not filter assignments by e-value or percent identity at this step. We then used those lists to subset only bacteria-assigned contigs from each of

the individual assemblies. We also repeated the blast+ assignment and bacteria filtering step for the coassemblies.

At this point in our analysis, we had four types of assembly: 1) individual queen assemblies that contained all contigs 300 bp or longer (full individual assemblies), 2) individual queen assemblies that contained all contigs 300 bp or longer assigned to bacteria in blast+ (bacterial individual assemblies), 3) coassemblies by treatment that included all contigs 300 bp or longer (full coassemblies), and 4) coassemblies by treatment that included all contigs 300 bp or longer assigned to bacteria in blast+ (bacterial coassemblies). To ensure that we did not lose any bacterial sequences when filtering out eukaryotic contigs, we analyzed taxonomic community structure of all assembly types in Anvi'o (Eren et al., 2020) using the anvi-estimate-scg-taxonomy program and compared the results of full vs. bacterial assemblies. The anvi-estimate-scg-taxonomy program estimates bacterial and archaeal taxonomic composition based on single-copy core genes in the Genome Taxonomy Database (GTDB) (Parks et al., 2020). First, the program determines how many copies of each single-copy gene in the GTDB are present in each assembly. It then uses the most common and abundant single-copy gene across all assemblies to assign taxonomy and calculate coverage for each taxon. In our study, the most common single-copy gene for all assembly types was ribosomal protein S7, so we assigned taxonomy with this gene. To evaluate the sensitivity of taxonomic profiles to single-copy gene choice, we also examined taxonomic profiles generated using the second most common single-copy gene, ribosomal protein S2.

We used the adonis2 function in the vegan package in R (Oksanen et al., 2022) to conduct permutational multivariate analyses of variance (PERMANOVA) to compare the community structure of assemblies which contained only bacterial contigs to those using all contigs. Our response variables were Bray-Curtis dissimilarity matrices generated using vegan, and our predictor variable was contig type. We ran models with 9,999 permutations and found no significant differences in taxonomic community structure between assemblies containing all or only bacterial contigs (individual assemblies: $F_{1,61} = 0.03$, $p = 0.99$; coassemblies: $F_{1,18} = 0.003$, $p = 0.99$; Supplementary Figure 4-2); in fact, for ribosomal protein S7, community structure was identical between contig types (Supplementary Figure 4-2C,D). Consequently, for our results and discussion we focus on taxonomic community structure analyses conducted using assemblies containing bacterial contigs only. Moreover, we likewise used assemblies containing only bacterial contigs to analyze queen gut microbiota metabolism.

We analyzed metabolic potential at the contigs level using Anvi'o's anvi-estimate-metabolism program (Veseli et al., 2023) which uses the KO database from the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2012; Kanehisa & Goto, 2000). We included all pathways with a pathwise module completeness of >0.5 in our analysis, removed modules assigned to the "Signature

modules" class and "Carbon fixation" subcategory, and calculated the relative coverage for each remaining module using average coverage values provided in the anvi-estimate-metabolism output. We then used the program anvi-compute-metabolic-enrichment with our individual assemblies to identify KEGG modules (i.e., metabolic pathways) that were consistently present (i.e., enriched) in some treatments and primarily absent in others (Shaiber et al., 2020). For this function, we set the module completion threshold to 0.5.

4.3.7 qPCR for 16S rRNA gene copy number

We used qPCR to obtain 16S rRNA gene copy numbers from all gut samples. We generated a four-step standard curve of known 16S rRNA gene copy numbers using DNA extracted from NEB® 5-alpha competent *Escherichia coli* K-12 which we ran in triplicate on every plate. We diluted gut DNA extracts 1:150 in nuclease-free water and ran all samples in triplicate following the protocol in Motta et al. (2018). We used BioRad® SsoFast EvaGreen Supermix with a BioRad® CFX96 real-time system and C1000 thermocycler, and analyzed run data with Bio-Rad® CFX Maestro software (v.2.3).

After calculating the total 16S rRNA gene copy number for each sample, we multiplied that number by the relative coverage of each taxon present in that sample, correcting for the average number of 16S rRNA gene copies per genome in the given bacterial genus or family according to the *rrn*DB version 5.8 (Stoddard et al., 2015). We then used these values to generate distance matrices of queen gut microbiotas using the vegan package in R and Bray-Curtis dissimilarity (Oksanen et al., 2022).

To avoid confusion when discussing abundance data, we henceforth refer to relative coverage data transformed using total 16S rRNA gene copy counts as "gene copy count data" and use the terms "relative coverage" or "relative abundance" only when discussing untransformed relative coverage data.

4.3.8 Statistical analyses

We conducted all statistical analyses in R v4.3.1 (R Core Team, 2023). We fit all linear models described in this section using the "lm" function in the base R stats package and examined all model assumptions using the performance package (Lüdecke et al., 2021). We performed any post-hoc tests of linear models using the "TukeyHSD" function in the stats package. When we included natal colony in models, we coded it as a fixed factor as it did not have enough levels to be coded as random. We visualized all data using the ggplot2 package (Wickham, 2016).

We used a series of linear models to compare queen weight and weight change across treatments during different experimental periods. First, we compared weight before diapause and weight change from eclosion to diapause entry between all treatments to ensure that treatments contained queens of

similar physical condition. Second, we compared weight at the end of diapause and weight change during diapause between the four-month diapause, recovery control, and recovery + glyphosate treatments to verify that queens in these treatments finished diapause in similar condition. Lastly, we compared weight change after diapause (i.e., during the recovery period) and weight at the end of recovery between the two recovery treatments to determine whether these measurements were affected by glyphosate exposure. Each model contained weight or weight change during the relevant time period as the dependent variable and treatment and natal colony ID as independent variables. We analyzed sugar solution consumption during the recovery experiment using a linear model with volume consumed in the past 24 hours as the dependent variable and natal colony and an interaction between day (coded as categorical) and treatment as independent variables; the interaction was insignificant and was dropped from the final model.

We analyzed how 16S rRNA gene copy counts and the number of paired reads post-filtering varied across treatments using linear models with $\log_{10}$ 16S rRNA gene copy count and $\log_{10}$ number of paired reads as the dependent variables, respectively, and treatment and natal colony ID as independent variables. For the 16S rRNA gene copy model, we also ran a model without an outlier in the recovery control treatment. We also tested whether 16S gene copy counts were correlated with the number of reads post-filtering using Pearson's product-moment correlation.

We calculated alpha diversity, specifically the Shannon index, for bumble bee queen gut microbiotas using the "diversity" function in the vegan package (Oksanen et al., 2022). We then used a linear model to determine whether alpha diversity varied with treatment, with Shannon index as the dependent variable, and treatment and natal colony ID as independent variables. As data for this model appeared heteroscedastic, we also ran this model with a ranked dependent variable.

We used the "adonis2" function in the vegan package to conduct PERMANOVAs to analyze how queen gut microbial community structure varied with treatment (Oksanen et al., 2022). We ran models with 9,999 permutations. Our response variables were Bray-Curtis dissimilarity matrices generated using vegan, and our independent variables were treatment and natal colony ID. We evaluated homogeneity of group dispersions using the "betadisper" function in the vegan package with type set to "median" (Oksanen et al., 2022). We conducted post-hoc comparisons using the pairwiseAdonis package for PERMANOVA models (Martinez Arbizu, 2020) and the "TukeyHSD" function for group dispersions. To identify which microbial taxa were differentially abundant across treatments, we conducted an analysis of community structures of microbiomes with bias correction (ANCOMBC). For this, we used the "ancombc2" function in the ANCOMBC package (Lin & Peddada, 2020) with Bonferroni correction and an alpha of 0.05. Before running the analysis, we added a small constant (+1) to all zero values in the

queen ID x taxon matrix to obtain p-values and test statistics for taxa which contained structural zeros. We included treatment and natal colony ID as fixed effects for the model.

We investigated how treatment affected KEGG module category composition (i.e., high-level metabolic composition) in queen gut microbiotas using the "adonis2" function in the vegan package (Oksanen et al., 2022). We ran the PERMANOVA with 9,999 permutations. Our response variable was a Bray-Curtis dissimilarity matrix of module category coverages generated using vegan, and our independent variables were treatment and natal colony ID. We evaluated homogeneity of group dispersions using the "betadisper" function in the vegan package with type set to "median" (Oksanen et al., 2022). To examine which specific KEGG modules (i.e., metabolic pathways) were differentially enriched across treatments, we used the Anvi'o program anvi-compute-metabolic-enrichment with the module completion threshold set to 0.5 (Eren et al., 2020; Shaiber et al., 2020). To be conservative, we used unadjusted p-values to determine significance as adjusted q-values were approximately an order of magnitude less than unadjusted p-values.

4.4 Results

4.4.1 Queen weight and sugar solution consumption

We found no significant differences in pre-diapause weight or relative weight change between treatments (weight: $F_{4,18} = 0.32$, $P = 0.85$; weight change: $F_{4,18} = 0.57$, $P = 0.69$; Supplementary Figure 4-3A,D). We likewise found no difference in weight after diapause or weight loss during diapause between the four-month and recovery treatments (weight: $F_{2,10} < 0.01$, $P = 0.99$; $F_{2,10} = 0.86$, $P = 0.45$; Supplementary Figure 4-3B,E), nor in weight change during recovery or weight after recovery between the two recovery treatments (weight: $F_{1,6} = 0.01$, $P = 0.88$; weight change: $F_{1,6} = 0.02$, $P = 0.88$; Supplementary Figure 4-3C,F). Queens from natal colony 1 weighed more than queens from other natal colonies before diapause (natal colony ID: $F_{2,18} = 5.13$, $P = 0.02$; both pairwise P-values = 0.03), but there were no differences between queens from different natal colonies for any other metrics (all $F < 1.7$, all $P > 0.2$). Daily sugar solution consumption by queens during the recovery experiment did not differ by treatment ($F_{1,60} = 0.84$, $P = 0.36$), but did differ by day ($F_{6,60} = 3.07$, $P = 0.011$; Supplementary Figure 4-4A) and natal colony ID ($F_{2,60} = 18.72$, $p < 0.001$; Supplementary Figure 4-4B). Specifically, sugar solution consumption was significantly higher on Day 6 than Day 2 (Tukey HSD, $P = 0.004$) and was significantly lower in queens from natal colony 2 overall (all $P < 0.001$).

4.4.2 Microbial abundance through diapause

We found that 16S rRNA gene copy count varied significantly with treatment ($F_{4,18} = 10.8$, $P < 0.001$) but not among natal colonies ($F_{2,18} = 1.74$, $P = 0.20$). Specifically, 16S rRNA gene copy counts in pre-diapause queen microbiotas were significantly higher than those in the two-month diapause, four-month diapause, and recovery + glyphosate treatments (means: pre-diapause = 6.5×10^8, two-month diapause = 4.8×10^7, four-month diapause = 1.1×10^7, recovery + glyphosate = 5.0×10^7; all $P < 0.05$; Figure 4-1A). Gene counts of queen microbiotas from the recovery control treatment were also significantly higher than those of queen microbiotas in the four-month diapause treatment (means: four-month diapause = 1.1×10^7, recovery control = 1.7×10^8; $P = 0.03$; Figure 4-1A). However, the recovery control treatment contained an outlier with a 16S rRNA gene copy count almost 3.5× that of the treatment mean. With this outlier removed, treatment still affected 16S rRNA gene copy count ($F_{4,17} = 13.5$, $P < 0.001$), but pre-diapause 16S rRNA gene copy counts were significantly higher than those in all other treatments (all $P < 0.01$) and all other pairwise comparisons were insignificant. The number of paired reads after filtering for host and plant sequences also differed with treatment ($F_{4,18} = 6.56$, $P = 0.002$) but not natal colony ID ($F_{2,18} = 2.85$, $P = 0.08$), with the number of paired reads post-filtering higher in pre-diapause queens than in any other treatment (all $P < 0.05$; Figure 4-1B). 16S rRNA gene copy counts and the number of paired reads post-filtering were significantly correlated ($r = 0.67$, $P < 0.001$; Figure 4-1C).

4.4.3 Sequencing and Metagenomic Assemblies

Metagenomic assemblies performed at the level of individual bumble bee queen gut microbiotas had a mean length of 3.2 million base pairs, contained an average of ~9,200 contigs, and had a mean N50 and L50 of 15,500 base pairs and 180 contigs, respectively. However, average quality metrics for metagenomic assemblies varied based on treatment both for assemblies performed at the level of individual bumble bee queen gut microbiotas (Supplementary Table 4-2) and coassemblies by treatment (Supplementary Table 4-3), with pre-diapause assemblies having the best quality statistics on average and four-month diapause assemblies having the worst.

After selecting bacterial contigs only, the number of contigs in individual assemblies decreased by 88% on average (range: 46.7 – 99.9%), but the size of the longest contig in each assembly only decreased by an average of 27% (range: 0 – 81.4%), and N50 values for all assemblies increased (Supplementary Table 4-4). For coassemblies, the number of contigs decreased by 87% on average (range: 73.8 – 97.5%) after selecting for bacterial contigs, but the size of the longest contig in each assembly was constant and all N50 values increased (Supplementary Table 4-5).

4.4.4 Taxonomic profiles through diapause

We assigned taxonomy to metagenomic assemblies of bumble bee queen gut microbiotas using the single-copy gene ribosomal protein S7. Of our 25 individual assemblies with bacterial contigs, we did not find ribosomal protein S7 sequences in nine. All assemblies without S7 sequences belonged to gut microbiotas sampled during or after diapause with $< 3.0\times10^8$ 16S rRNA gene copies, and all but one of these assemblies contained fewer than 100 bacterial contigs. Across the 16 queen gut microbiotas where taxonomy could be assigned, we identified seven taxa: six were core bumble bee gut microbial phylotypes – *Bifidobacterium* (along with *Bifidobacteriaceae*), *Bombilactobacillus*, *Lactobacillus*, *Schmidhempelia* and *Snodgrassella* – and the last was the non-core phylotype *Enterobacteriaceae* (Figure 4-2A,B). In all but one gut microbiota, the only taxa identified were core phylotypes. The same taxa were also observed in the coassemblies by treatment (Figure 4-2C), though one phylotype, *Lactobacillus*, that was absent in all individual four-month assemblies was identified in the coassembly of four-month queen gut microbiotas. The *Gilliamella* phylotype, another core bacteria in bumble bee gut microbiotas, was not identified in any of our queen gut microbiotas, though its absence has been noted in commercial *B. impatiens* colonies in other studies (Hotchkiss et al., 2024; Motta & Moran, 2023a). Alpha diversity did not vary with treatment (Supplementary Figure 4-5), whether the dependent variable was ranked to account for heteroscedasticity in the data ($F_{4,9} = 2.3$, $P = 0.14$) or unranked ($F_{4,9} = 2.2$, $P = 0.16$), nor did it vary with natal colony ID (unranked: $F_{2,9} = 2.5$, $P = 0.13$, ranked: $F_{2,9} = 2.4$, $P = 0.14$). Taxonomic profiles assigned with ribosomal protein S2 were similar to those assigned with S7, though relative coverages differed slightly and some individual assemblies lost or gained a microbial phylotype (Figure 4-2A, Supplementary Figure 4-6A). Coassembly profiles were also similar across both single copy genes, though *Lactobacillus* was identified at four months using protein S7 but not S2 (Figure 4-2C, Supplementary Figure 4-6B).

Microbial community structure varied with treatment (PERMANOVA: $F_{4,9} = 4.4$, $P = 0.0003$; homogeneity of multivariate dispersions: $F_{4,11} = 2.37$, $P = 0.12$) but not among natal colonies (PERMANOVA: $F_{2,9} = 1.8$, $P = 0.09$; Figure 4-2D); the same result was observed with the taxonomic profiles assigned using ribosomal protein S2 (treatment: $F_{4,8} = 3.5$, $P = 0.0001$; natal colony ID: $F_{2,8} = 1.9$, $P = 0.067$; homogeneity of multivariate dispersions: $F_{4,10} = 0.94$, $P = 0.47$; Supplementary Figure 4-6C). Specifically, the community structure of bumble bee queen gut microbiotas before diapause was significantly different than the structure of any other treatment (all $F_{1,4} > 3.5$, all $P < 0.05$). The two recovery treatments did not differ in community structure ($F_{1,4} = 1.12$, $P = 0.4$). Four phylotypes, *Bombilactobacillus, Lactobacillus, Schmidhempelia,* and *Snodgrassella*, were differentially abundant between treatments in the ANCOMBC global test (*Bombilactobacillus*: W = 15.8, $q = 0.006$;

Lactobacillus: W = 20.6, $q = 0.002$; *Schmidhempelia*: W = 111.5, $q < 0.0001$; *Snodgrassella*: W = 50.6, $q < 0.0001$). After conducting pairwise comparisons between all treatments and correcting for multiple comparisons, we found that *Schmidhempelia* 16S rRNA gene copy counts were significantly higher in pre-diapause microbiotas than in four-month (W = -7.14, $q < 0.001$), recovery control (W = -6.68, $q = 0.001$), or recovery + glyphosate (W = -6.21, $q = 0.002$) microbiotas, and that *Snodgrassella* 16S rRNA gene copy counts were significantly higher in pre-diapause microbiotas than in four-month (W = -5.78, $q = 0.005$) or recovery + glyphosate (W = -4.96, $q = 0.012$) microbiotas. No pairwise comparisons for *Bombilactobacillus* or *Lactobacillus* were significant.

4.4.5 Metabolic profiles through diapause

Of the 25 queen gut microbiotas we examined, metabolic modules with ≥50% pathwise completeness could not be found in eight, and one microbiota only contained one pathway; these nine microbiotas were the same nine which contained no copies of the ribosomal protein S7 or S2 genes in our taxonomic analyses. In the 16 remaining microbiotas, we identified 143 modules with ≥50% pathwise completeness from eight KEGG module categories (Figure 4-3A). The number of modules identified per queen gut microbiota ranged from 1–120, and the microbiotas with the five highest module counts all belonged to the pre-diapause treatment (Figure 4-3A). At the level of KEGG module category, microbiotas did not differ in metabolic structure by treatment (PERMANOVA: $F_{4,10} = 1.8$, $P = 0.14$; homogeneity of multivariate dispersions: $F_{4,12} = 1.06$, $P = 0.43$) or natal colony ID (PERMANOVA: $F_{2,10} = 0.26$, $P = 0.92$). Metabolic profiles were similar for coassemblies by treatment (Supplementary Figure 4-7).

We found 63 KEGG modules (i.e., metabolic pathways) from 19 module subcategories that were differentially enriched across treatments (Figure 4-3B, Supplementary Figure 4-8). All differentially enriched modules were enriched in the pre-diapause treatment; of those, 11 were also enriched in the recovery treatment, 10 in the recovery + glyphosate treatments, four in the two-month diapause treatment and one in the four-month diapause treatment (Supplementary Figure 4-8).

4.5 Discussion

4.5.1 Microbial abundance decreases through diapause

We found that average microbial abundance in bumble bee queen gut microbiotas decreases during diapause by an order of magnitude, falling from 6.5×10^8 16S rRNA gene copies before diapause to approximately 1.7×10^7 after four months (Figure 4-1A). Additionally, we found little difference in

average gene copy counts between two and four months into diapause, implying that microbial abundance declines within the first two months of diapause and then plateaus. Studies on gut microbiotas in analogous animal systems, like fasting hamsters and hibernating ground squirrels, observe declines in abundance at magnitudes comparable or larger than ours (Sonoyama et al., 2009; Stevenson et al., 2014). However, a study in *B. terrestris* queens found that microbial abundance in queen ileums fell approximately 50% during a four-month diapause (Bosmans, Pozo, Verreth, Crauwels, Wäckers, et al., 2018), a smaller decline than what we observed in our study. While this discrepancy could be due to the difference in study species, it may also be due to differences in the mating status of examined queens. Bosmans et al. used mated queens in their study, while our queens were unmated. In bumble bee queens, pre-diapause mating increases production of antimicrobial peptides (AMPs), which remain at elevated levels through diapause (Barribeau & Schmid-Hempel, 2017; Colgan et al., 2019). AMPs play an important role in regulating gut microbiotas (Ostaff et al., 2013), and therefore whether they are present at elevated levels (i.e., whether queens are mated or unmated) could influence gut microbiota abundance and community dynamics through diapause.

We believe there are two primary factors driving our observed decline in queen gut microbial abundance during diapause. The first is a decline in nutrients, especially carbohydrates from nectar and pollen, though possibly also host organic acids (Kwong & Moran, 2016; Quinn et al., 2024). Core social bee gut phylotypes (which are the dominant constituents in our microbiotas – see Taxonomic community structure shifts during diapause) primarily ferment sugars or metabolize organic acids (sugar fermentation products) to fuel growth (Hammer, Le, Martin, et al., 2021; Kwong & Moran, 2016; Martinson et al., 2014; Motta & Moran, 2023b; Quinn et al., 2024) and these substrates become increasing scarce as the duration of host fasting and metabolic depression lengthens. The second factor is a decline in temperature below microbial growth optima. The optimal *in vitro* growth temperatures for core social bee gut phylotypes fall within 35–40˚C (Engel et al., 2013; Hammer, Le, Moran, et al., 2021; Palmer-Young et al., 2023), close to average bumble bee colony and abdominal temperatures (Heinrich, 2004) and far above our diapause temperature of 4˚C. While some members of core genera can grow at temperatures as low as 2˚C, the lower thermal limits for growth of many core phylotype strains are considerably higher, ranging from 15–28˚C (Hammer, Le, Moran, et al., 2021; Hammes & Hertel, 2009; Shah, 2007). Together, a decline in temperature far below growth optima, and possibly below lower thermal limits, coupled with a decline in nutrients likely severely impedes the growth of many bumble bee gut microbes.

After diapause we observe an approximately eight-fold increase in average microbial abundance over the course of one week, though abundance remains more than six times lower than before diapause (Figure 4-1A). However, microbial abundance within recovery control queens varies substantially (range:

$2.3\times10^7 – 5.2\times10^8$). Gut microbial abundance during post-disturbance recovery also varies considerably in other systems, such as hibernating ground squirrels and mice exposed to antibiotics, in which full recovery to pre-disturbance abundances ranges from three to eight days (Ng et al., 2019; Stevenson et al., 2014). One potential reason why we do not observe microbial abundances return to pre-diapause levels within one week of recovery is the presence of a lag phase. When significant changes occur in a microbial population's environment, that population often enters a lag phase of up to a few days during which it adjusts to the new environment but does not grow (Bertranda, 2019; Vermeersch et al., 2019). After diapause is complete, bumble bee queens not only increase their body temperatures and resume food intake but also undergo a suite of physiological changes as they prepare to initiate colony development (Amsalem et al., 2015; Heinrich, 2004). Adjusting to this rapidly changing environment may slow the post-diapause regrowth of bee gut microbial populations.

Still, there is evidence from other studies that bumble bee queen gut microbial populations eventually return to pre-diapause abundance. Studies of wild and lab-reared queens found 16S rRNA gene copy counts comparable to our pre-diapause counts in queen gut microbiotas within a few weeks after diapause (Bosmans, Pozo, Verreth, Crauwels, Wilberts, et al., 2018; L. Wang et al., 2019). An interesting avenue for future studies would be to examine gut microbiota recovery at a fine time-scale for longer, and with larger sample sizes, than we did here, to attempt to capture the full regrowth of microbial populations after diapause.

The low microbial abundances in queen gut microbiotas during and after diapause caused some difficulties during sequencing and sequencing data analysis. As we extracted DNA from whole-gut samples without first enriching for prokaryotic cells, the ratio of host and plant DNA to microbiota DNA was higher in diapause and recovery samples than pre-diapause samples. Consequently, host and plant reads constitute more than 99% of reads in our sequencing libraries for most queen gut microbiotas sampled after the onset of diapause (Supplementary Table 4-1). Even after filtering out reads that mapped to host and plant genomes prior to assembly, most contigs in our metagenomic assemblies are taxonomically assigned to invertebrates and plants (Supplementary Figure 4-1, Supplementary Tables 4-2, 4-3, 4-4, 4-5). In host-microbiome systems, when samples contain a high proportion of host DNA and abundances of target microbial taxa are low, metagenomic sequencing can fail to detect target taxa (Pereira-Marques et al., 2019). Based on this information and our own data validations, which we discuss in subsequent sections, it is likely that many queen gut microbiotas in our study are not fully taxonomically or metabolically profiled. Our discussion takes this caveat into account, but there remain three key interpretations of our taxonomic and metabolic data that we believe stand despite this limitation: 1) Taxonomic community structure shifts during diapause and does not recover after one week, 2)

Metabolic potential remains constant through diapause stages, and 3) Glyphosate does not affect early post-diapause recovery of queen gut microbiotas.

4.5.2 Taxonomic community structure shifts during diapause

Taxonomic community structure in queen bumble bee gut microbiotas shifts after the onset of diapause and does not return to a pre-diapause structure after one week of recovery (Figure 4-2). Before diapause, queen gut microbiotas are dominated by five core social bee gut phylotypes: *Bifidobacterium* spp., *Bombilactobacillus* spp., *Lactobacillus* spp., *Schmidhempelia* spp., and *Snodgrassella* spp. After pre-diapause, core phylotypes continue to dominate, though some are lost in certain individual microbiotas (Figure 4-2). In reality, lost phylotypes are likely not extinct in those microbiotas; instead, phylotype abundance likely declined below the limit of detection for our taxonomic profiling. Generally, in our data, the fewer microbes present in a queen gut microbiota (i.e., the lower the 16S gene copy count), the lower the number of bacterial sequences present in that sample and, consequently, the lower the bacterial assembly quality and our ability to detect bacterial taxa (Supplementary Tables 4-1, 4-4, 4-5). Indeed, we fail to taxonomically profile any bacteria in nine of our samples, despite them containing positive, albeit low, 16S rRNA gene counts (Figure 4-1A). Additionally, we detect taxa in the coassemblies of low-abundance treatments that are absent in all constituent individual assemblies (Figure 4-2C, Supplementary Figure 4-6B). Taken together, these observations, alongside our small sample sizes, mean that the patterns we observe in taxonomic structure across treatments could be an artifact of inadequate microbiota sequencing.

Still, we believe that the taxa we detect in our microbiotas represent the dominant taxa in our queen gut microbiotas and therefore provide evidence of a shift in community structure, specifically a decline in the abundance of *Schmidhempelia* and *Snodgrassella* spp. In pre-diapause queen microbiotas, these two phylotypes consistently comprise a large proportion (often >75%) of the relative coverage (Figure 4-2A, Supplementary Figure 4-6A). If all microbial taxa declined at the same rate over diapause (i.e., microbial abundance changed but community structure was constant), then we would expect *Schmidhempelia* and *Snodgrassella* to remain among the most common taxa during and after diapause, and therefore among the most detected. However, we fail to detect these phylotypes at all past two months of diapause, and communities are instead dominated by *Bifidobacterium, Bombilactobacillus*, and *Lactobacillus*. When sequencing our microbiotas we used PCR-free library preparation and, excluding *Bifidobacterium* spp., GC content is similar between our detected core phylotypes, so it is unlikely that biased sequencing explains this pattern (Browne et al., 2020) (Supplementary Table 4-6). Likewise, bias towards Gram-negative microbes during DNA extraction would have increased detection of

Schmidhempelia and *Snodgrassella*, two Gram-negative phylotypes, relative to our other observed core phylotypes which are all are Gram-positive (Frostegård et al., 1999). For these reasons, we believe that *Schmidhempelia* and *Snodgrassella* abundance does decline during diapause and does not recover by one week after diapause ends. While these phylotypes are likely still present (Su et al., 2021), it is even possible that they become extinct during diapause, as can occur with *Gilliamella* spp., another core bee phylotype closely related to *Schmidhempelia* (Koch et al., 2013; Martinson et al., 2014). Future studies with larger sample sizes could use more sensitive bacterial taxonomic profiling methods (e.g., 16S rRNA gene amplicon sequencing, metagenomic sequencing on samples enriched for prokaryotes, qPCR with taxon-specific primers) to investigate this possibility. Interestingly, *Gilliamella* spp. are occasionally lacking in commercial *B. impatiens* colonies, as seen in this study and others (Hotchkiss et al., 2024; Motta & Moran, 2023a), and our observation of a severe decline in a related taxon coupled with Koch et al.'s (2013) culture-based work suggest that this absence could be due to loss during diapause.

It is unclear why *Schmidhempelia* and *Snodgrassella* would be more likely to decline or die off during diapause than other phylotypes. In other vertebrate guts, *Proteobacteria* (the phylum containing *Schmidhempelia* and *Snodgrassella*) tend to increase in relative abundance during hibernation and fasting (Greene et al., 2022; Maurice et al., 2015; Sonoyama et al., 2009; Stevenson et al., 2014; Xiao et al., 2019), though this pattern is less consistent in overwintering insects (Ferguson et al., 2018; Hou et al., 2021; J. Wang et al., 2017) and appears taxon-specific (Ferguson et al., 2018). In our system, the decrease in relative and absolute abundances of *Schmidhempelia* and *Snodgrassella* may be driven by differences in cold sensitivity between Gram-negative and Gram-positive bacteria (Schwab et al., 2014), higher lower thermal limits for growth in these taxa (Hammer, Le, Moran, et al., 2021; Hammes & Hertel, 2009; Shah, 2007), and/or higher sensitivity to changes in available exogenous nutrients and host metabolites during diapause (Quinn et al., 2024). The abundances of these two taxa may also be tightly linked if *Schmidhempelia* forms a biofilm with *Snodgrassella* like its relative *Gilliamella* (Hammer, Le, Martin, et al., 2021; Martinson et al., 2014), though this remains to be investigated.

Comparing our results to the only previous study that examined bumble bee queen gut microbiotas before and immediately after diapause, there are clear similarities and differences. Like us, Bosmans et al. (2018) saw a decrease in core phylotype abundance after a four-month diapause at 3˚C, a similar temperature to ours. However, they also observed an increase in non-core microbial abundance and diversity (Bosmans, Pozo, Verreth, Crauwels, Wäckers, et al., 2018), while we fail to detect any non-core microbes once diapause begins (Figure 4-2). It is possible that some microbiotas in our study may contain non-core phylotypes, but that abundances are too low for our method to detect; indeed, abundances of individual non-core taxa were low in the Bosmans et al. study, even if, collectively, these

taxa made up a large proportion of the microbiota. It should also be noted that both we and Bosmans et al. (2018) reared and incubated queens under sterile conditions, and so the patterns that we observe in non-core (i.e., environmental) microbial communities in these studies likely do not reflect what occurs in wild bumble bee queen gut microbiotas. Investigations of taxonomic shifts in queen gut microbiotas during diapause in more field-realistic conditions, such as non-sterile soil and leaf litter (Alford, 1969), should be a priority.

By one week after diapause, we find little evidence of community structure recovery in queen gut microbiotas (Figure 4-2). However, we believe that the taxonomic shifts that occur during diapause in bumble bee queen microbiotas are temporary, unlike the permanent changes observed in some other insect microbiotas (Ferguson et al., 2018). Studies surveying the gut microbiotas of lab-reared and wild bumble bee queens a few weeks after emergence from diapause found them dominated by core phylotypes, although dominance depended on environment (Bosmans, Pozo, Verreth, Crauwels, Wäckers, et al., 2018; L. Wang et al., 2019). It takes approximately four to five weeks for bumble bee workers to develop from egg to adult (Heinrich, 2004), so bumble bee queens theoretically have around one month for their gut microbiotas to recover (if they are to transmit a recovered microbiota to their workers), though their own health would likely benefit from faster recovery (Hammer, Le, Martin, et al., 2021). Future studies could serially examine bumble bee queen gut microbiotas from diapause end to eclosion of the first generation of workers to profile changes in taxonomic community structure during the entire recovery period and determine whether microbiotas consistently return to a pre-diapause state before worker eclosion.

4.5.3 Metabolic potential is consistent during and after diapause

We found that at the level of KEGG module categories, metabolic potential is consistent before, during, and after diapause (Figure 4-3, Supplementary Figure 4-7); regardless of treatment, the dominant metabolic categories are amino acid metabolism, carbohydrate metabolism, energy metabolism, and metabolism of vitamins and cofactors. This contrasts with hibernating mammal gut microbiotas, where a large metabolic turnover often occurs between active and non-active seasons as communities shift from being dominated by microbes that metabolize exogenous carbohydrates to those that metabolize host fats and nitrogenous wastes (Carey et al., 2013; Sommer et al., 2016; Xiao et al., 2019). But we do not observe large taxonomic turnover in bumble bee queen gut microbiotas between active and diapausing treatments – we consistently observe core bee gut phylotypes, just at varying abundances (Figure 4-2). As most core social bee gut phylotypes are sugar fermenters, high-level metabolic redundancy in the community is high (Hammer, Le, Martin, et al., 2021; Kwong & Moran, 2016). Thus, it is unsurprising

that carbohydrate metabolism remains dominant over lipid and nitrogen metabolisms, and that high-level metabolic potential is consistent through all stages of diapause. However, though metabolic potential (i.e., gene content) is consistent across diapause, it is unlikely the same is true of metabolic activity (i.e., gene expression). Future studies should investigate how transcriptomic, proteomic, and/or metabolomic profiles within bee gut microbiotas change as queens undergo diapause to further our understanding of how metabolism changes during this key life stage.

Meanwhile, when examining metabolism at higher resolution, we found that approximately 45% of all observed metabolic pathways were enriched in specific treatments (Figure 4-3C, Supplementary Figure 4-8). Here, we were particularly interested in modules that were enriched during diapause but not before or after, as these would provide evidence of metabolic shifts during diapause. However, we do not observe this pattern for any enriched module (Supplementary Figure 4-8). Instead, all enriched modules are enriched in pre-diapause queens, with 11 and 3 modules also enriched in recovery and diapause treatments, respectively. Additionally, some modules that are absent in all individual assemblies within a treatment are present in that treatment's coassembly (Supplementary Figures 4-8, 4-9). Together, these observations suggest that the enrichment patterns we observe are likely tied to assembly quality. While some enriched modules can be linked to genomic evidence and may truly be enriched in some treatments, such as the enrichment of vitamin and cofactor metabolism in microbiotas with *Schmidhempelia* and *Snodgrassella* (Kwong & Moran, 2016), many others are likely false positives and these data should be cautiously interpreted.

4.5.4 Glyphosate does not affect early post-diapause recovery of queen gut microbiotas

We found that glyphosate has a limited effect on bumble bee queen gut microbial abundance, taxonomic structure, and metabolic potential during a one-week post-diapause recovery period. There are no differences between the two recovery treatments in terms of microbial abundance, taxonomic structure, or high-level metabolic potential (Figures 4-1A, 4-2, 4-3A). Glyphosate also has no effect on post-diapause weight, weight gain, or sugar solution consumption (Supplementary Figures 4-3C,F, 4-4).

Notably, *Snodgrassella*, the phylotype which most consistently decreases in abundance after glyphosate exposure (Motta et al. 2018, 2020; Blot et al. 2019; Motta and Moran 2020, 2023b; Castelli et al. 2021; Helander et al. 2023; though see Cullen et al. 2023; Straw et al. 2023), is not detected in queen gut microbiotas from either recovery treatment (Figure 4-2, Supplementary Figure 4-6). Glyphosate has inconsistent and limited effects on other core social bee microbial phylotypes including *Bidobacterium* spp., *Bombilactobacillus* spp., and *Lactobacillus* spp. (Blot et al., 2019; Cullen et al., 2023; Helander et al., 2023; Motta et al., 2018, 2020; Motta & Moran, 2020, 2023a; Straw et al., 2023), so it is unsurprising

that we do not see an effect of glyphosate exposure on queen gut microbial abundance, taxonomic composition, or metabolic potential. As discussed previously, it is possible that *Snodgrassella* is present in recovery treatments in low abundance and that populations simply require more than one week to regrow to sizes that are detectable with our methods. An interesting direction for future research would be to conduct a similar recovery experiment but for a longer duration and with more sensitive methods to determine if and when *Snodgrassella* abundance recovers after diapause and if populations regrowth takes longer in queens exposed to glyphosate.

4.6 Conclusion

Our study is the first to investigate taxonomic and metabolic changes in queen bumble bee gut microbiotas before, during, and after diapause. We found that microbial abundance falls by an order of magnitude during diapause and does not recover by one week post-diapause. With this decline in abundance comes taxonomic shifts; while core social bee gut phylotypes dominate gut microbiotas in all treatments, *Schmidhempelia* and *Snodgrassella*, key constituents of pre-diapause gut microbiotas, are not detected past two months of diapause. Despite changes in taxonomic community structure, high-level metabolic potential is relatively consistent across all treatments. Lastly, we found that glyphosate exposure has a limited effect on early post-diapause queen gut microbiota recovery. These findings, coupled with those of previous studies (Bosmans, Pozo, Verreth, Crauwels, Wäckers, et al., 2018; Bosmans, Pozo, Verreth, Crauwels, Wilberts, et al., 2018; L. Wang et al., 2019), provide a foundation for future work on the gut microbiotas of diapausing and post-diapause bumble bee queens. Specifically, we recommend increasing the frequency at which queen gut microbiotas are sampled and the duration of post-diapause sampling to obtain a more complete picture of how abundance and community structure shift during diapause and afterwards during recovery. We also recommend that future studies use a combination of transcriptomics, metabolomics, and proteomics to clarify changes in metabolic activity in queen gut microbiotas during diapause. Finally, future studies should aim to investigate bumble bee queen gut microbiotas in more field-realistic, non-sterile environments to better understand what roles environmental microbes play in community dynamics through diapause and recovery.

Our results must be interpreted within the limitations of our study. Low microbial abundances caused poor bacterial metagenomic assembly quality for many queen gut microbiotas sampled after the onset of diapause, which resulted in low, uneven sample sizes and high limits of detection for taxa and metabolic pathways. As a result, we have interpreted results cautiously, and further studies with larger sample sizes are needed to verify the patterns we observe. Future metagenomic studies on the gut microbiotas of diapausing queens should use methods to increase the number of microbes present in

samples, such as sample pooling, alongside methods for prokaryotic enrichment, such as physical filtering (Ellegaard & Engel, 2019) and cell sorting (Dungan et al., 2023). Methods tailored to low-abundance taxa, such as single cell metagenomics, will also be useful (Xu & Zhao, 2018).

Nevertheless, despite limitations, our study provides valuable insight into how the gut microbiotas of bumble bees, important pollinators in both natural and agro-ecosystems, change during a key stage in the colony life cycle, and furthers our understanding of how host-associated microbial communities respond to varying environmental conditions.

4.7 Data Availability

Supplemental data is available at doi.org/10.6084/m9.figshare.c.7030340. All data and R scripts are available at github.com/michellehotch/Diapause2024. Sequences are available through the NCBI Sequence Read Archive PRJNA1066198. Note that six raw sequence files became corrupted during storage after analysis was complete, so we have provided host-filtered sequence files for all samples in addition to all uncorrupted raw sequence files.

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We declare no competing interests.

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4.10 Figures

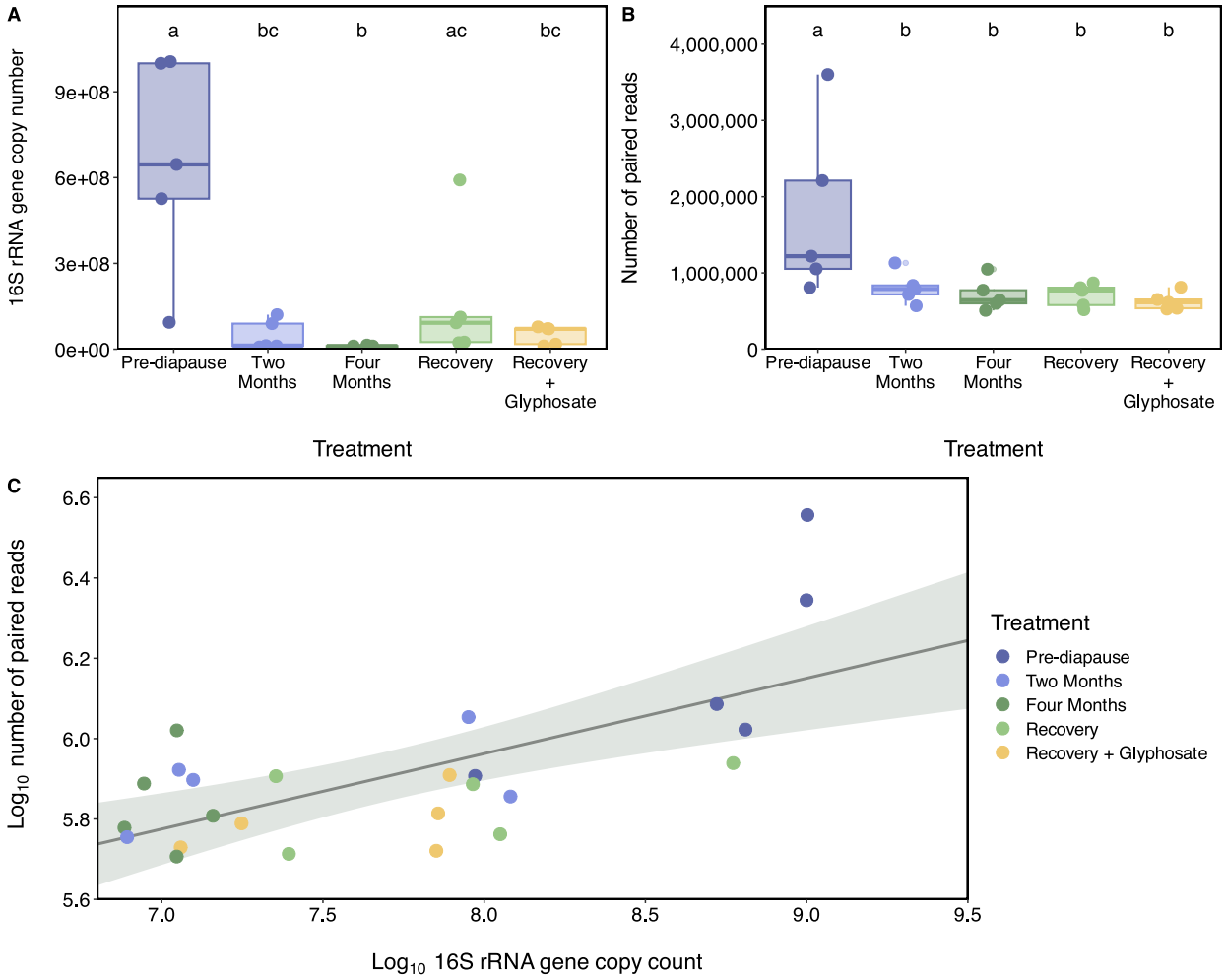


Figure 4-1: 16S rRNA gene copy counts and paired read counts in *B. impatiens* queen gut microbiotas.

A) Box plot of total 16S rRNA gene copy counts in queen *B. impatiens* gut microbiotas before ("pre-diapause"), during ("two months" and "four months"), and one week after the end of diapause ("recovery" and "recovery + glyphosate"). B) Box plot of the number of paired reads remaining after host and plant filtering in sequencing libraries of queen *B. impatiens* gut microbiotas sampled before, during, and after diapause. Boxes represent medians and interquartile ranges; whiskers extend to 1.5 × the interquartile range. Treatments with different letters are significantly different based on a Tukey post-hoc test. C) Correlation between log10 16S rRNA gene copy counts and log10 number of paired reads remaining after host and plant filtering for queen *B. impatiens* gut microbiotas. Trendline is drawn using the lm ("linear model") smoothing method in ggplot2. For all panels, n = 25 (n = 5 per treatment).

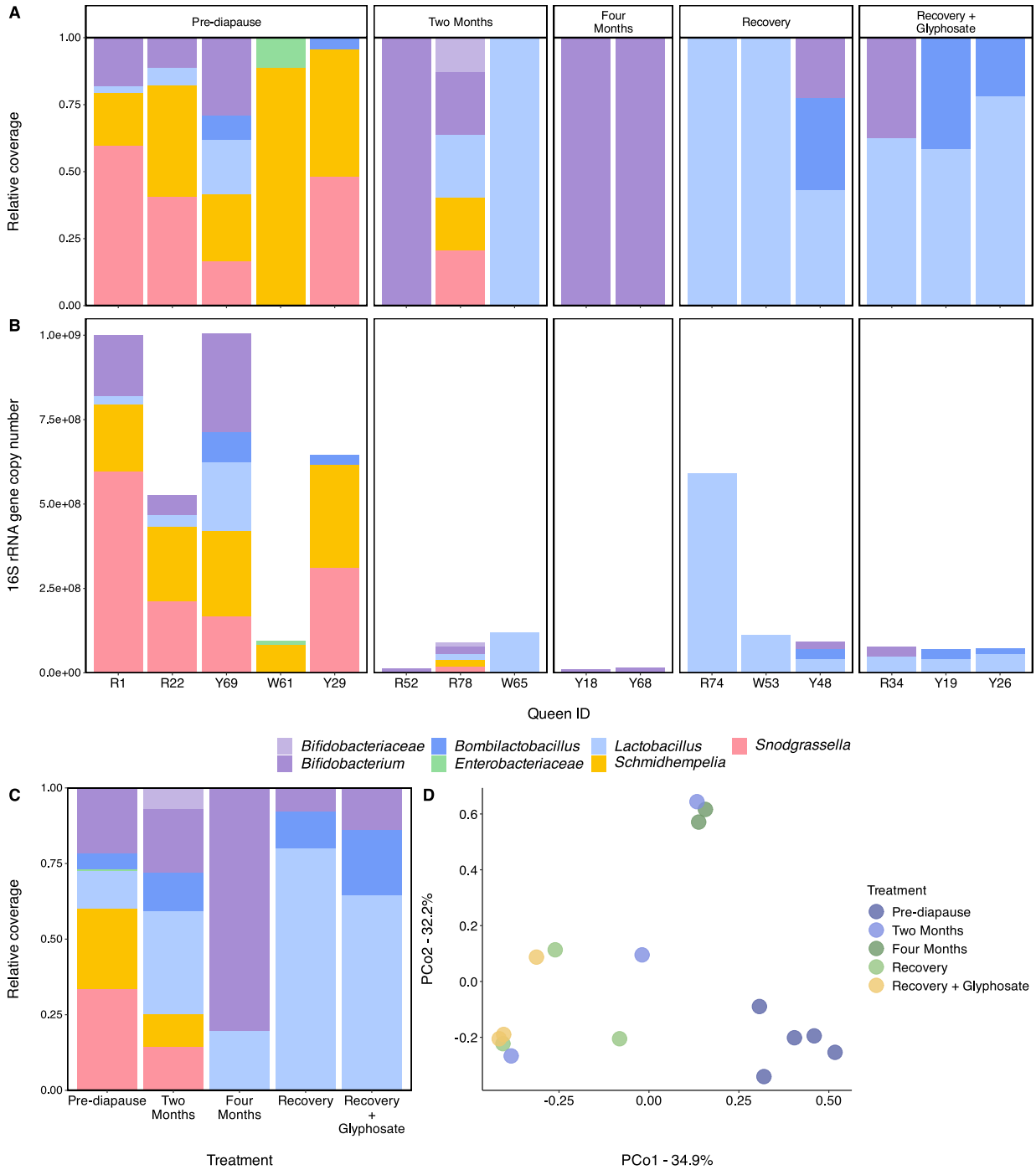


Figure 4-2: Taxonomic community structure of bumble bee (*B. impatiens*) queen gut microbiotas before, during, and after diapause.

A) Stacked bar plot of relative abundances of microbial phylotypes in microbiotas faceted by treatment (n = 16; n = 2-5 per treatment). B) Stacked bar plot of 16S rRNA gene copy counts of

microbial phylotypes in bumble bee queen gut microbiotas faceted by treatment (n = 16; n = 2-5 per treatment). Codes below bars are labels representing individual bees; letters (R, W, Y) represent different natal colonies. C) Stacked bar plot of relative abundances of microbial phylotypes in bumble bee queen gut microbiota coassemblies (coassembled by treatment) (n = 5; n = 1 per treatment). D) Principal coordinates analysis of bumble bee queen gut microbiotas (n = 16; n = 2-5 per treatment) using Bray-Curtis dissimilarities. For all panels, taxonomy was assigned in Anvi'o using using ribosomal protein S7. Community structure varies with treatment (PERMANOVA: $F_{4,9} = 4.4$, $p = 0.0003$) but not natal colony ID (PERMANOVA: $F_{2,9} = 1.8$, $p = 0.09$); pre-diapause gut microbiotas had significantly different community structure than all other treatments (all $F > 3.5$, all $p < 0.05$).

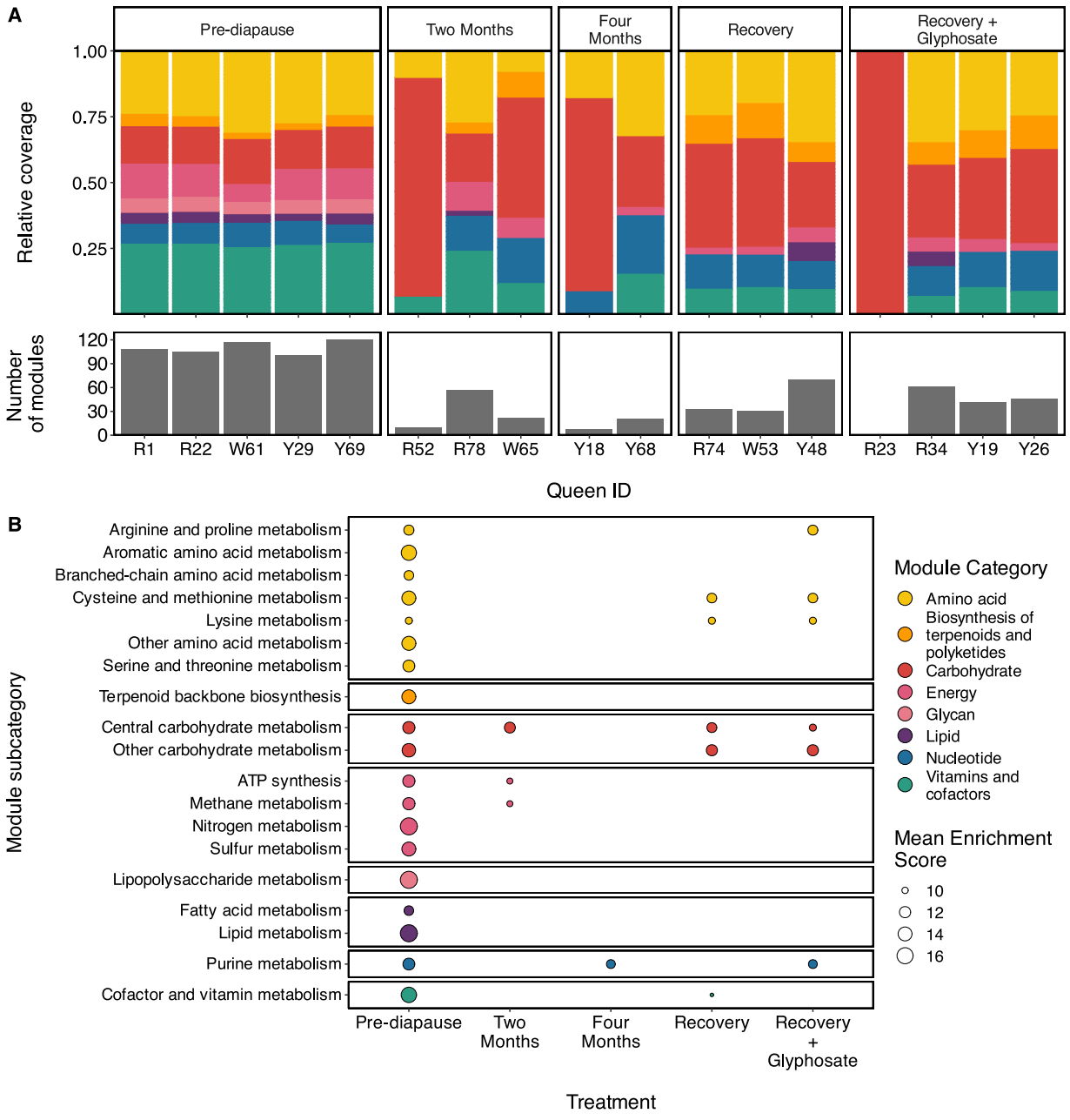


Figure 4-3: Metabolic potential of bumble bee (*B. impatiens*) queen gut microbiotas before, during, and after diapause.

A) Stacked bar plot of relative coverages of KEGG module categories (i.e., metabolism types) in individual queen gut microbiotas faceted by treatment (n = 16; n = 2-5 per treatment). Beneath is a bar plot of the number of KEGG modules identified in each microbiota; for R23, number of modules = 1. B) Plot of significantly enriched KEGG module subcategories, faceted by module category. Treatments have

a point for each module subcategory that contains enriched modules in that treatment. Point size corresponds to the mean enrichment score across all enriched modules in the module subcategory; the higher the enrichment score, the more strongly associated the given metabolism is with the treatment.

4.11 Supplementary Tables

Supplementary Table 4-1: Host and plant filtering statistics for paired-read sequence files of bumble bee queen gut microbiotas.

Queen ID	Treatment	# of raw reads	# reads post-quality filtering	# reads post-host filtering	# reads post-plant filtering	% raw reads remaining post-filtering
R15	Two mo.	99,265,244	97,881,894	1,102,202	836,359	0.84
R18	Recovery	81,369,165	80,340,533	1,467,402	806,605	0.99
R23	Rec. w/ G.	81,928,241	80,834,806	869,196	615,470	0.75
R34	Rec. w/ G.	85,897,067	84,608,912	1,048,842	811,919	0.95
W53	Recovery	79,306,396	78,347,287	803,532	578,302	0.73
W54	Four mo.	117,471,111	115,614,362	999,084	773,083	0.66
W596	Rec. w/ G.	77,479,236	76,498,166	716,331	536,223	0.69
W60	Four mo.	101,365,555	99,706,802	782,818	600,210	0.59
W61	Pre-diap.	91,551,732	90,273,670	1,008,462	807,523	0.88
W65	Two mo.	97,329,919	96,024,520	930,569	717,614	0.74
W66	Recovery	74,760,197	73,697,421	1,006,384	516,600	0.69
W68	Two mo.	94,455,902	92,875,992	736,109	568,683	0.60
R1	Pre-diap.	101,249,083	99,725,111	2,523,382	2,210,791	2.18
Y18	Four mo.	89,366,170	87,886,225	647,962	508,723	0.57
Y19	Rec. w/ G.	77,281,232	76,214,734	669,699	525,827	0.68
R22	Pre-diap.	76,834,433	75,758,902	1,416,804	1,219,545	1.59
Y26	Rec. w/ G.	83,236,115	81,987,870	1,406,222	651,534	0.78
Y29	Pre-diap.	94,004,688	92,651,450	1,258,235	1,053,440	1.12
R44	Four mo.	115,293,837	113,487,677	1,369,320	1,048,501	0.91
Y48	Recovery	99,132,233	97,497,367	1,011,681	770,207	0.78
Y52	Two mo.	88,520,320	86,964,863	1,037,977	789,496	0.89
Y68	Four mo.	102,356,290	100,970,866	824,777	642,682	0.63
Y69	Pre-diap.	99,955,029	98,573,968	3,852,131	3,601,528	3.60
R74	Recovery	78,401,255	77,346,140	1,074,890	869,212	1.11
R78	Two mo.	119,875,442	117,772,307	1,466,198	1,131,511	0.94

Supplementary Table 4-2: Average assembly statistics for individual metagenomic assemblies performed.

Average assembly statistics for metagenomic assemblies performed at the level of individual bumble bee queen gut microbiota, grouped by treatment (n=5 per treatment).

Treatment	Mean num. of contigs	Min num. of contigs	Max num. of contigs	Mean largest contig (Kbp)	Mean assem. length (Mbp)	Mean N50 (Kbp)	Mean L50 (K)
Pre-diapause	8,907.6	4,172	18,051	417.36	9.8	56.1	0.06
Two-month diapause	5,384.2	1,312	13,801	5.14	1	0.92	0.28
Four-month diapause	4,266.2	1,676	6,389	3.46	0.42	0.8	0.16
Recovery	14,713.2	3,996	31,382	97.72	2.42	17.8	0.16
Recovery w/ glyphosate	12,702.8	4,950	27,058	24.52	2.28	1.94	0.24

Supplementary Table 4-3: Assembly statistics for metagenomic coassemblies of bumble bee queen gut microbiotas by treatment.

Assembly statistics for metagenomic coassemblies of bumble bee queen gut microbiotas by treatment (n=1 per treatment).

Treatment	Number of contigs	Largest contig (Kbp)	Assembly length (Mbp)	N50 (Kbp)	L50 (K)
Pre-diapause	25,003	838.5	26.2	50	1.5
Two-month diapause	19,134	9.3	5.3	1.1	0.7
Four-month diapause	14,072	6.9	2.2	1.2	0.8
Recovery	62,228	185.1	8.3	7.5	1.4
Recovery w/ glyphosate	49,714	105.5	7.1	6.6	1.7

Supplementary Table 4-4: Average assembly statistics for individual metagenomic assemblies with bacterial contigs.

Average assembly statistics for metagenomic assemblies performed at the level of individual bumble bee queen gut microbiota after removing all non-bacterial contigs, grouped by treatment (n=5 per treatment).

Treatment	Mean num. of contigs	Min num. of contigs	Max num. of contigs	Mean largest contig (Kbp)	Mean assem. length (Mbp)	Mean N50 (Kbp)	Mean L50 (K)
Pre-diapause	2,299.8	963	4,934	417.36	8.2	75.42	0.02
Two-month diapause	885.2	4	2,189	3.92	0.48	1.16	0.12
Four-month diapause	290.2	4	797	1.84	0.1	1.08	0.04
Recovery	437.2	8	1,881	96.1	1.84	19.4	0.02
Recovery w/ glyphosate	1,025.2	21	2,061	23.56	1.64	2.6	0.12

Supplementary Table 4-5: Assembly statistics for metagenomic coassemblies by treatment with bacterial contigs.

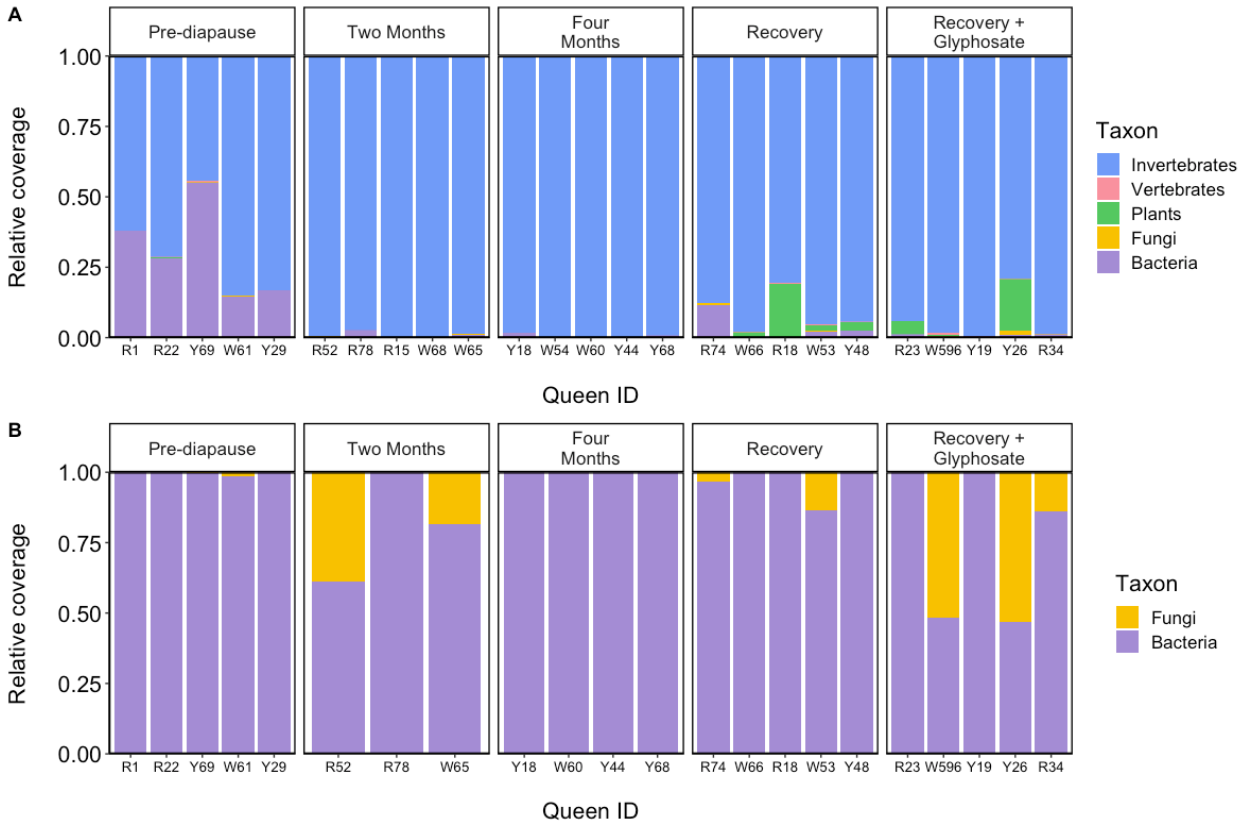
Assembly statistics for metagenomic coassemblies of bumble bee queen gut microbiotas by treatment after removing all non-bacterial contigs (n=1 per treatment).

Treatment	Number of contigs	Largest contig (Kbp)	Assembly length (Mbp)	N50 (Kbp)	L50 (K)
Pre-diapause	6,554	838.5	20.8	104.1	0
Two-month diapause	4,467	9.3	2.9	1.2	0.7
Four-month diapause	1,282	6.9	1	1.6	0.2
Recovery	1,555	185.1	6.1	25.3	0
Recovery w/ glyphosate	1,292	105.5	5.2	12.3	0.1

Supplementary Table 4-6: Lengths and GC content of a sample of core social bee gut microbiota reference genomes.

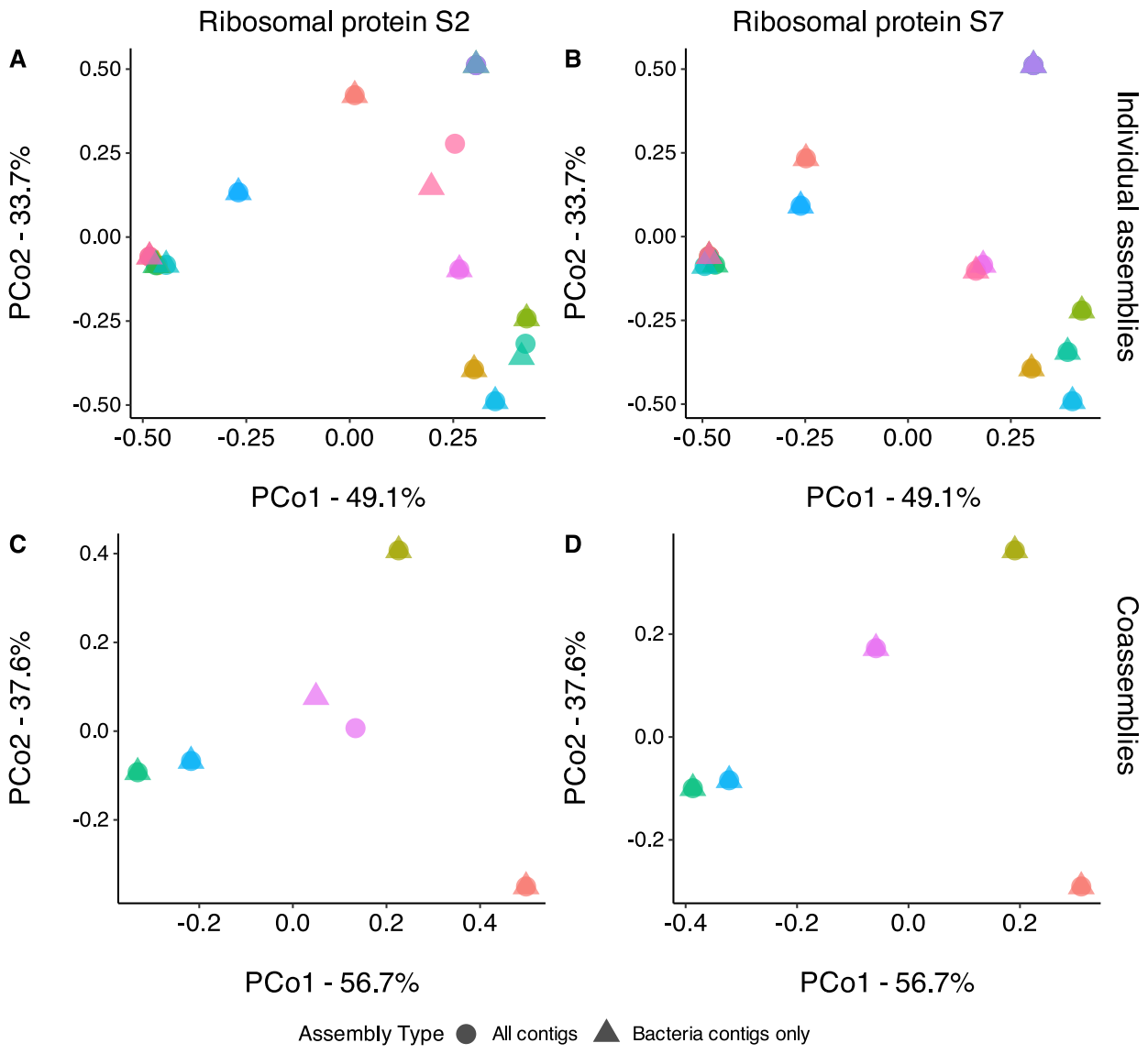
Genus	Species	NCBI Assembly ID	Length (Mbp)	GC content (%)
Bifidobacterium	*actinocoloniiforme*	ASM126339v1	1.8	62.5
	asteroides	ASM30421v1	2.2	60.0
	bohemicum	IMG-taxon 2616644833	2.0	57.5
	bombi	DSM-19703	1.9	56.0
	commune	IMG-taxon 2616644832	1.6	54.0
Bombilactobacillus	*apium*	ASM1338514v1	1.7	40.0
	bombi	ASM352296v1	1.8	34.5
	folatiphilus	ASM2338026v1	1.6	38.5
	mellifer	ASM97079v1	1.8	39.5
	mellis	ASM96724v1	1.8	36.0
	thymidiniphilus	ASM2338024v1	1.5	36.5
Lactobacillus	*apis*	ASM315093v1	1.7	37.5
	bombicola	ASM291691v1	1.7	34.5
	helsingborgensis	ASM317369v1	1.9	37.0
	helveticus	ASM305308v1	2.1	36.5
	kullabergensis	ASM315102v1	2.0	36.0
	melliventris	ASM97077v1	2.1	36.0
	panisapium	ASM1946926v1	2.2	38.0
Candidatus Schmidhempelia	*bombi*	BiG_1.2	2.2	36.5
Snodgrassella	*alvi*	ASM60000v1	2.5	41.5
	communis	LMG 28360	2.3	43.5
	gandavensis	LMG 30236	2.5	44.0

4.12 Supplementary Figures



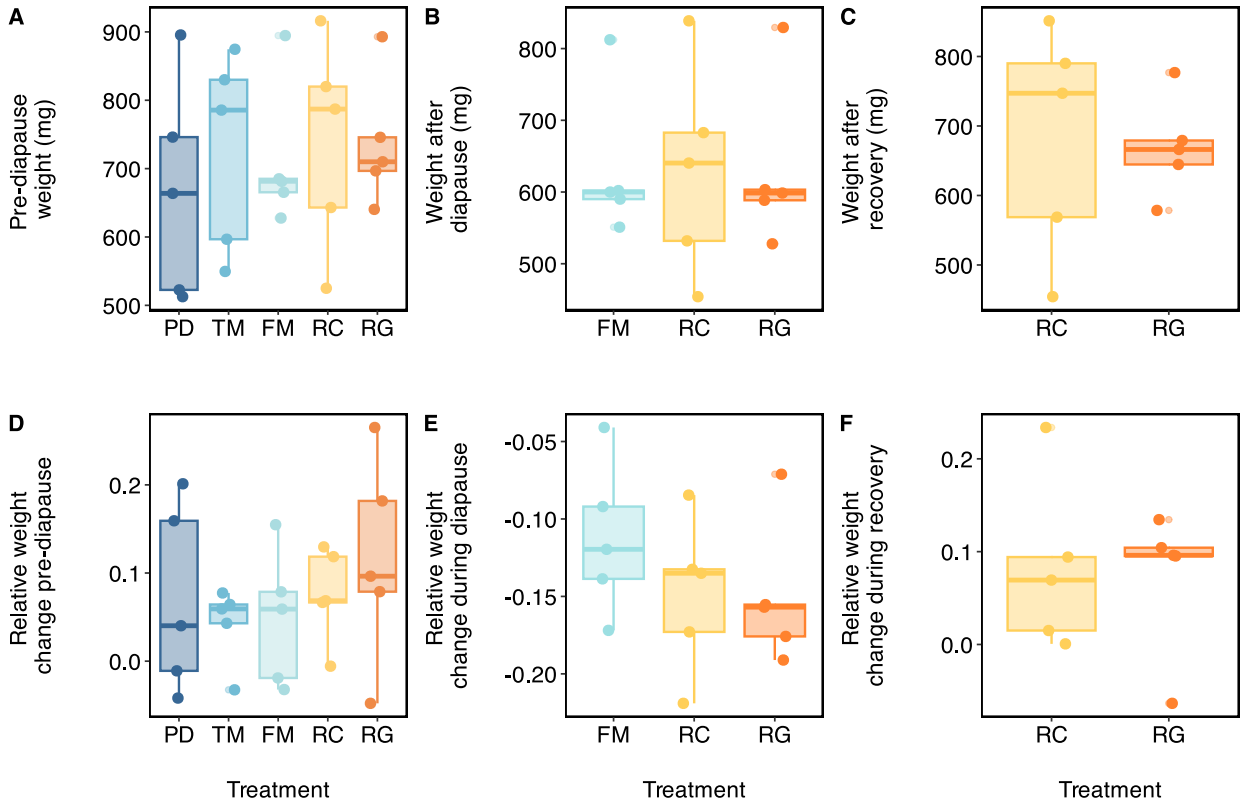
Supplementary Figure 4-1: Relative coverage of taxa in individual metagenomic assemblies based on assignment to the blast+ nt reference library.

Only contigs whose top taxonomic assignment had an e-value $\leq 1\times10^{-50}$ and a percent identity >90% were used. A) shows relative coverages for invertebrates, vertebrates, plants, fungi, and bacteria, while B) shows relative coverages when examining bacteria and fungi only.



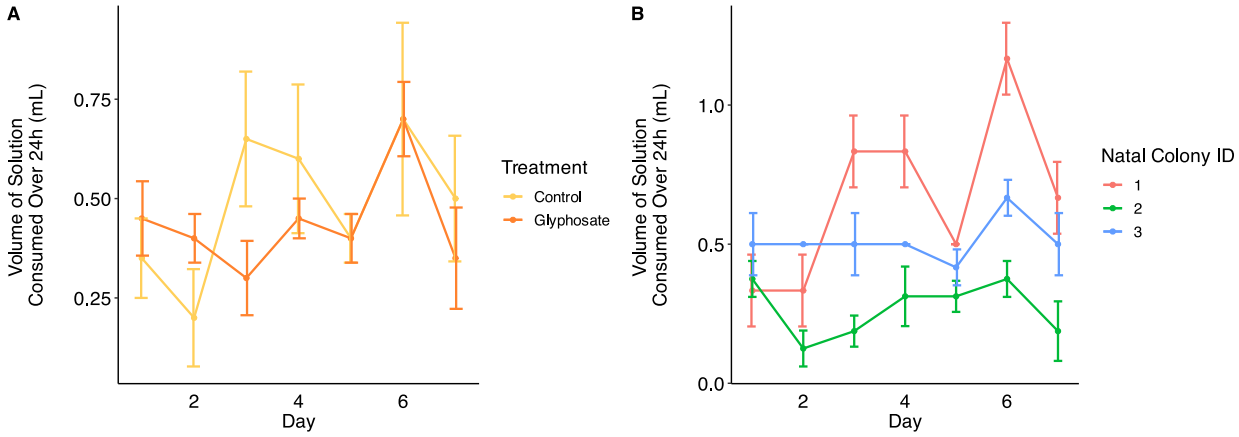
Supplementary Figure 4-2: Principal coordinates analyses of gut microbiota assemblies using Bray-Curtis dissimilarities.

Points are coloured by assembly ID and shaped by assembly type (i.e., all contigs included or bacterial contigs only). A and C) use taxonomies assigned with ribosomal protein S2, while B and D) use taxonomies assigned with ribosomal protein S7. A and B) display data for individual assemblies while C and D) display data for coassemblies. For both individual and coassemblies, taxonomic community structure does not vary by contig type (PERMANOVA with 9,999 permutations; individual assemblies: $F_{1,61} = 0.03$, $p = 0.99$; coassemblies: $F_{1,18} = 0.003$, $p = 0.99$).



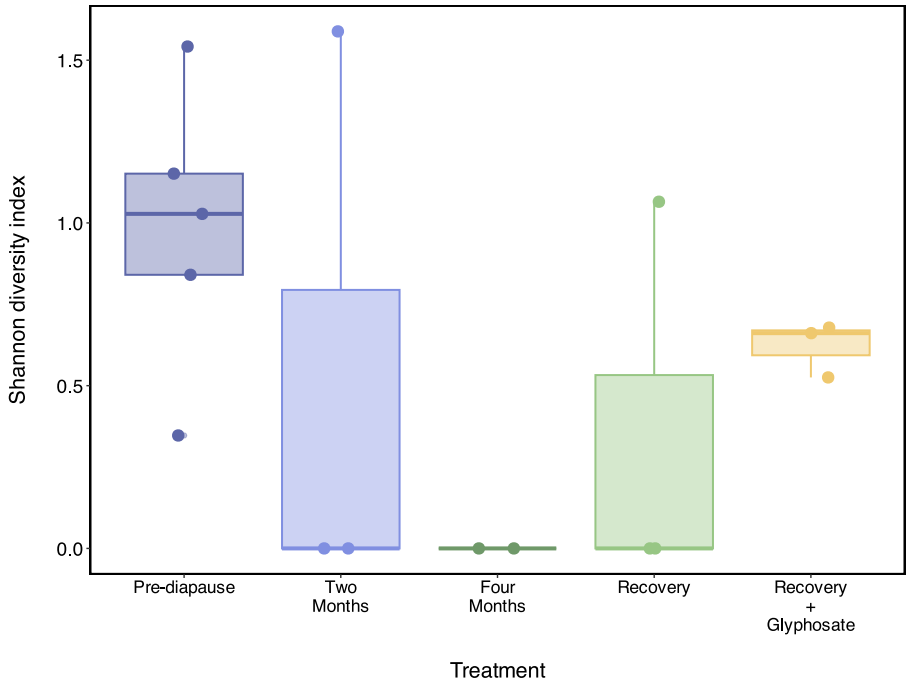
Supplementary Figure 4-3: Box plots of weights and relative weight changes of *Bombus impatiens* queens before, during, and after diapause.

A-C) Weights of queens before diapause, after a four-month diapause, and after a one-week recovery period (n=5 per treatment). D-F) Relative weight changes of queens before diapause, during a four-month diapause, and during a one-week recovery period (n=5 per treatment). PD = pre-diapause, TM = two months, FM = four months, RC = recovery control, RG = recovery + glyphosate. Boxes represent medians and interquartile ranges; the whiskers extend to 1.5 × the interquartile range. Treatment had no effect on weight or relative weight change during any stage of the experiment (all $F < 0.9$, all $p > 0.4$).



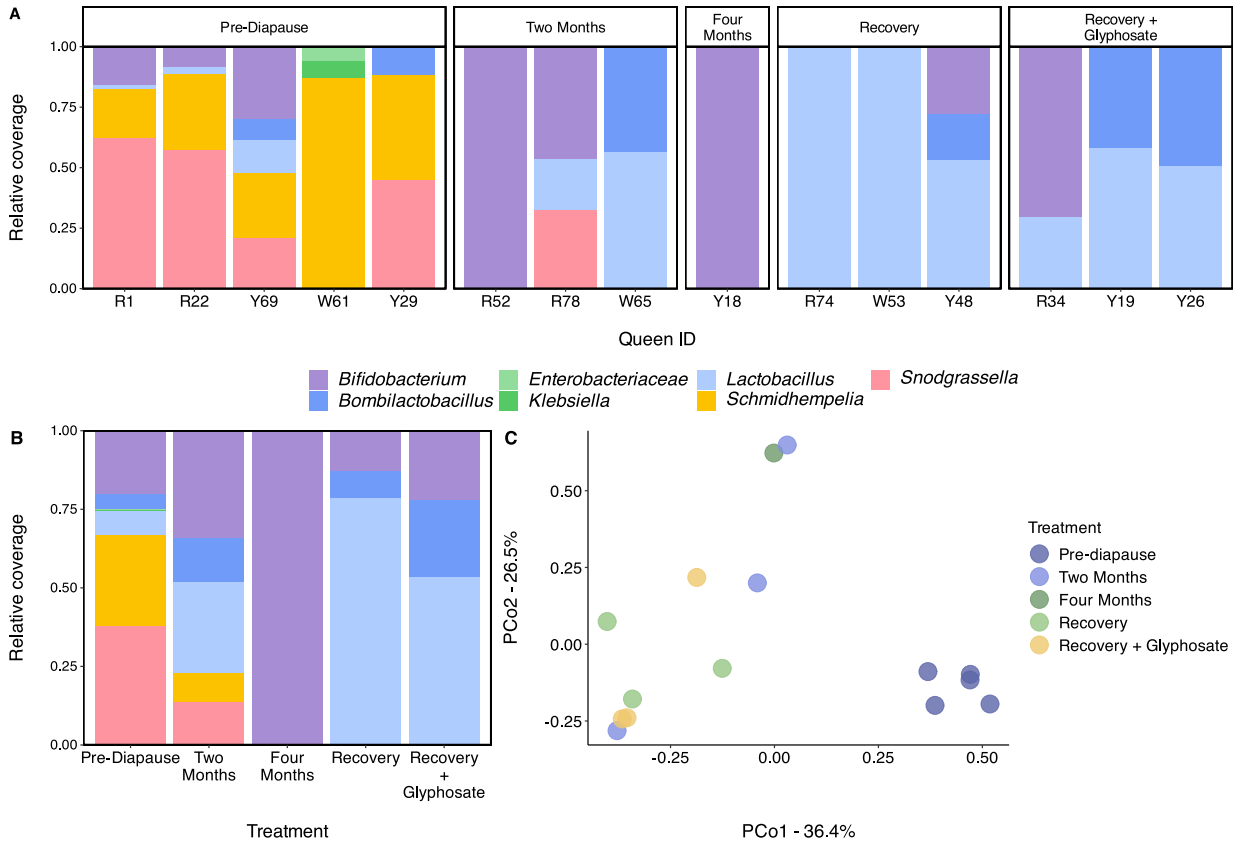
Supplementary Figure 4-4: Volume of sugar solution consumed by *B. impatiens* queens in the preceding 24 hours over the course of a one-week, post-diapause recovery.

In panel A data are separated by treatment (n = 5 per treatment) while in panel B data are separated by natal colony ID (n = 3–4 per natal colony). Dots represent means and bars extend to mean±SE. Solution consumption did not differ by treatment ($F_{1,60} = 0.84$, $p = 0.36$), but did by day ($F_{6,60} = 3.07$, $p = 0.011$) and natal colony ID ($F_{2,60} = 18.72$, $p < 0.001$); consumption was higher on Day 6 than Day 2 ($p = 0.004$) and was lower in queens from natal colony 2 overall (all $p < 0.001$).



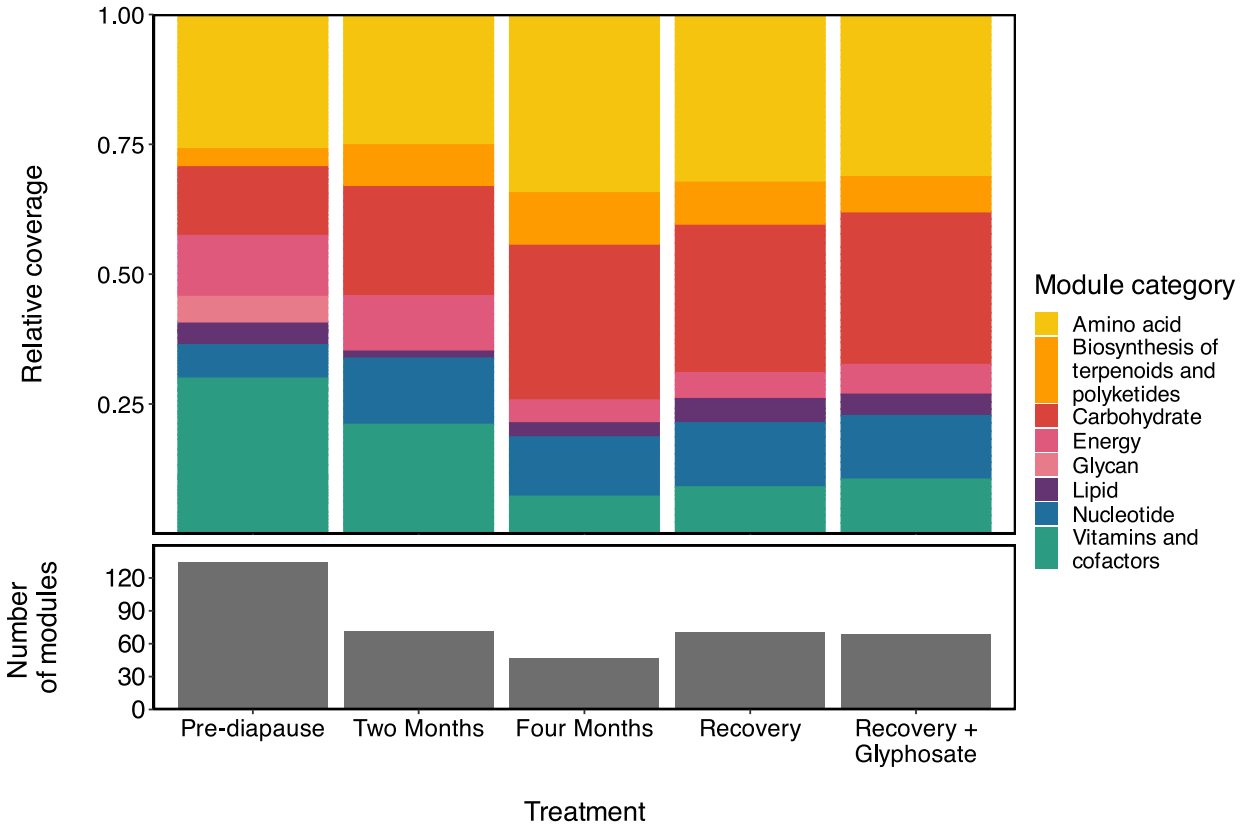
Supplementary Figure 4-5: Box plots of Shannon diversity indices of bumble bee queen gut microbiotas before, during, and after diapause.

Box plots of Shannon diversity indices of bumble bee queen gut microbiotas before, during, and after diapause (n = 2-5 per treatment). Boxes represent medians and interquartile ranges; the whiskers extend to 1.5 × the interquartile range. Alpha diversity did not vary with treatment, whether using an unranked ($F_{4,9} = 2.2$, $p = 0.16$) or ranked dependent variable ($F_{4,9} = 2.3$, $p = 0.14$).



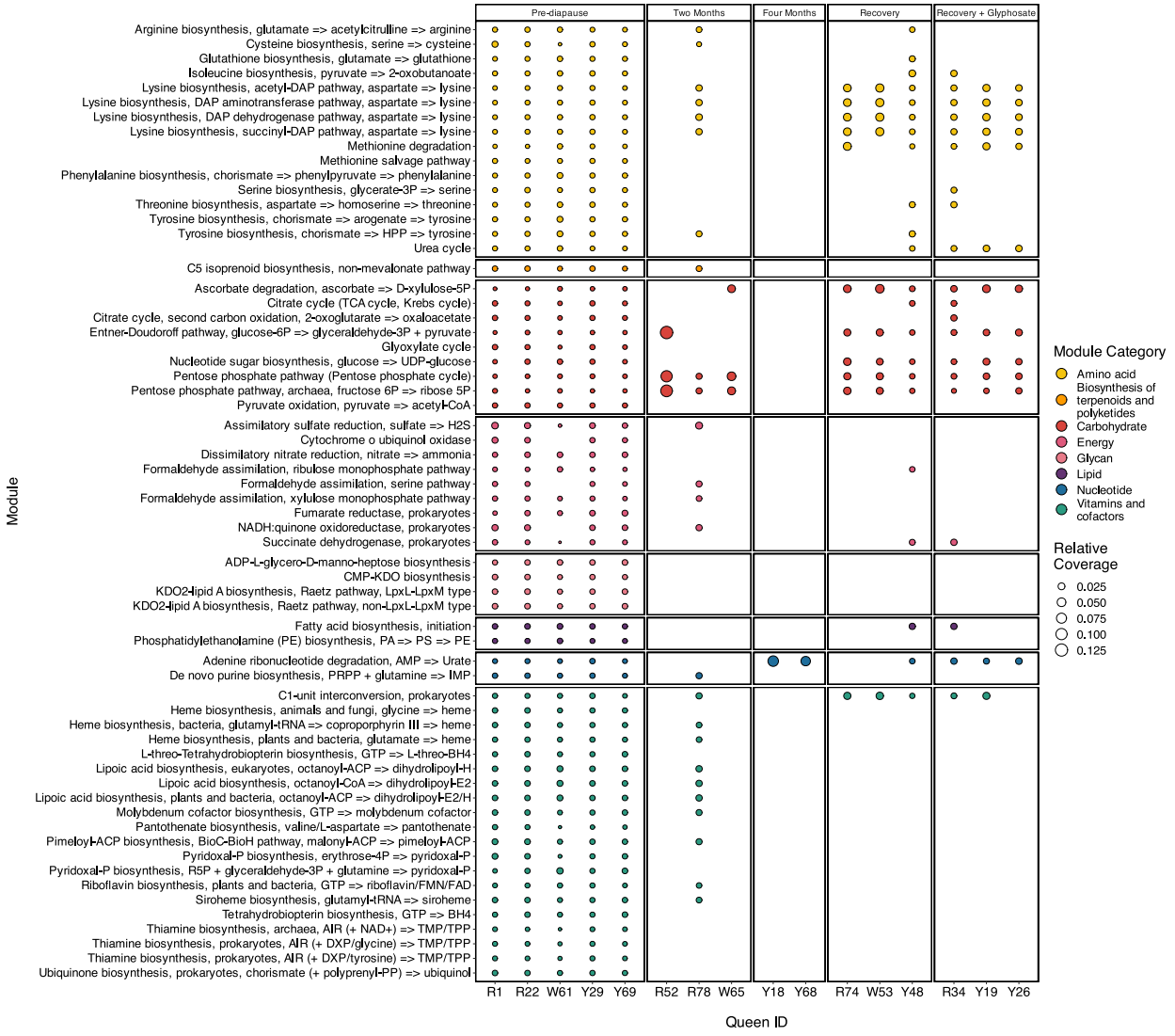
Supplementary Figure 4-6: Bumble bee queen gut microbiota taxonomic profiles assigned using ribosomal protein S2.

A) Stacked bar plot of relative abundances of microbial phylotypes in bumble bee (*B. impatiens*) queen gut microbiotas faceted by treatment (n = 15; n = 1-5 per treatment). B) Stacked bar plot of relative abundances of microbial phylotypes in bumble bee queen gut microbiota coassemblies (coassembled by treatment) (n = 5; n = 1 per treatment). C) Principal coordinates analysis of bumble bee queen gut microbiotas (n = 15; n = 1-5 per treatment) using Bray-Curtis dissimilarities. Community structure varies with treatment (PERMANOVA: $F_{4,8} = 3.5$, $p = 0.0001$) but not natal colony origin (PERMANOVA: $F_{2,8} = 1.8$, $p = 0.07$).



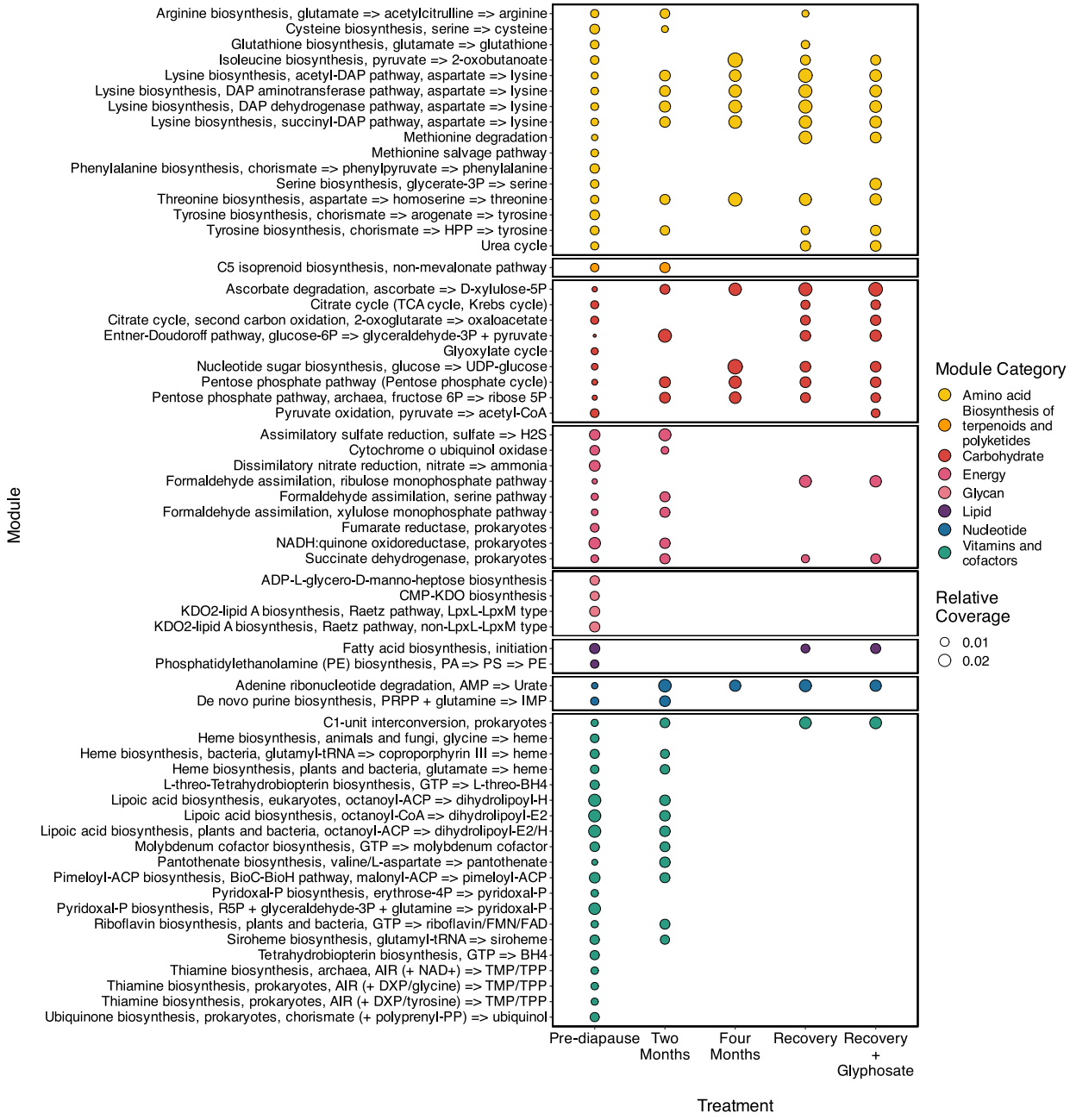
Supplementary Figure 4-7: Relative coverages of KEGG module categories in queen gut microbiota coassemblies by treatment.

Relative coverages of KEGG module categories (i.e., metabolism types) in queen gut microbiota coassemblies by treatment (n = 1 per treatment). Beneath is a bar plot of the number of KEGG modules identified in each coassembly.



Supplementary Figure 4-8: Relative coverage of enriched KEGG modules in individual queen gut microbiotas, faceted vertically by module category and horizontally by treatment.

Note that a module must be present in the majority of microbiotas within a treatment for that module to be considered enriched in the treatment (e.g., arginine biosynthesis is enriched in the pre-diapause treatment, but not the two-month diapause or recovery control treatment). Point size corresponds to relative coverage.



Supplementary Figure 4-9: A list of all enriched KEGG modules in analyses with individual assemblies and their coverages in queen gut microbiota coassemblies.

A list of all enriched KEGG modules in analyses with individual assemblies (Supplementary Figure 4-8), and their coverages in queen gut microbiota coassemblies. Plot is faceted by module category. Point size corresponds to relative coverage.

Chapter 5

Bumble bee gut microbial community structure differs between species and commercial suppliers, but metabolic potential remains largely consistent

5.1 Abstract

Bumble bees are key pollinators for natural and agricultural plant communities. Their health and performance are supported by the presence of a core gut microbiota composed of a few core bacterial taxa. However, bumble bee gut microbiota taxonomic composition and community structure can vary with species, environment, and origin (i.e., whether the colony comes from the wild or a commercial rearing facility), and it is unclear whether metabolic capabilities therefore vary as well. Here we used metagenomic sequencing to examine gut microbiota community composition, structure, and metabolic potential across bumble bees from two different commercial *Bombus impatiens* suppliers, wild *B. impatiens*, and three other wild bumble bee species sampled in a portion of their native eastern North American range. We found that gut microbiota taxonomic community structure varied between bumble bee species, between populations from different origins within the same species, and between commercial suppliers. Notably, we found that *Apibacter* is consistently present in some wild bumble bee species—suggesting it may be a previously unrecognized core phylotype of bumble bees—and that commercial *B. impatiens* colonies can lack core phylotypes consistently found in wild populations. However, despite variation in community structure, gut microbiota metabolic potential was largely consistent across all hosts, including for metabolic capabilities related to host performance, though metabolic activity remains to be investigated. Overall, these results provide new insight into bumble bee gut microbiota community structure and function, and will help researchers evaluate how well studies conducted in one bumble bee population will translate to other populations and species.

5.2 Introduction

Bumble bees (*Bombus* spp.; Hymenoptera: Apidae) are eusocial bees that are key pollinators in many ecosystems. Bumble bees not only pollinate many wild plants (Goulson, 2009), but also agricultural crops (Button & Elle, 2014; Eeraerts et al., 2020; Garibaldi et al., 2013; Lyu et al., 2023; Nayak et al., 2019; Roldán Serrano & Guerra-Sanz, 2006; Willmer et al., 1994), particularly those that require buzz pollination, a service that honey bees cannot perform. Understanding factors that influence bumble bee health is therefore important to maintain the viability and productivity of plant populations and communities, especially as some bumble bee species are experiencing considerable reductions in range and abundance (Cameron et al., 2011; Cameron & Sadd, 2020). One important facet of bumble bee health that has gained attention in recent years is the bumble bee gut microbiota (Hammer et al., 2021).

Social, corbiculate bees, a group which includes honey bees, bumble bees, and stingless bees, all have a highly conserved, core gut microbiota (Hammer et al., 2021; Kwong, Medina, et al., 2017; Kwong & Moran, 2016b). This microbial community primarily supports host health by inhibiting pathogen infection (Cariveau et al., 2014; Koch et al., 2022; Koch & Schmid-Hempel, 2011; Miller et al., 2021; Palmer-Young et al., 2019; Steele et al., 2021) and stimulating the host immune system (Horak et al., 2020; Kwong, Mancenido, et al., 2017; Lang et al., 2022; Steele et al., 2017), though a suite of other benefits has also been observed (Engel et al., 2012; Kešnerová et al., 2017; F. J. Lee et al., 2018; Leger & McFrederick, 2020; L. Li et al., 2021; Rothman et al., 2019; Wu et al., 2020; Zheng et al., 2017, 2019). Typically, social bee gut microbiotas are dominated by five core phylotypes (i.e., microbial taxa that are consistently present within a given host taxon) representing five bacterial taxa: *Bifidobacterium* spp. (*Bifidobacteriaceae*), *Bombilactobacillus* spp. (*Lactobacillaceae*), *Gilliamella* spp. (*Orbaceae*), *Lactobacillus* spp. near *melliventris* (*Lactobacillaceae*), and *Snodgrassella* spp. (*Neisseriaceae*) (Hammer et al., 2021; Kwong & Moran, 2016b). However, taxonomic composition can differ across host species and environments, particularly in stingless bees and bumble bees (Cerqueira et al., 2021; Kwong, Medina, et al., 2017).

Bumble bee gut microbiotas have been profiled across a variety of species and environments (e.g., Bosmans, Pozo, Verreth, Crauwels, Wilberts, et al., 2018; Cariveau et al., 2014; Kwong, Medina, et al., 2017; J. Li et al., 2015; Lim et al., 2015; A. Parmentier, Meeus, et al., 2018; Powell et al., 2016; Praet et al., 2018; Villabona et al., 2023), and patterns in community composition and structure have begun to emerge. Core social bee gut microbial phylotypes generally dominate bumble bee gut microbiotas, with *Gilliamella* and *Snodgrassella* being the most consistently present (e.g., Bosmans et al., 2018; Hammer et al., 2021; Krams et al., 2022; Kwong & Moran, 2016a; J. Li et al., 2015; Mockler et al., 2018; Newbold et

al., 2015; Praet et al., 2018; Villabona et al., 2023). However, the presence of some core phylotypes, particularly *Bombilactobacillus* and *Lactobacillus,* varies between host species (Kwong, Medina, et al., 2017; Praet et al., 2018). Furthermore, some *Bombus* species have gained the additional core phylotypes *Candidatus* Schmidhempelia (hereafter *Schmidhempelia*) (*Orbaceae*), and/or *Bombiscardovia* (*Bifidobacteriaceae*) (Killer et al., 2010; Kwong, Medina, et al., 2017; Martinson et al., 2014; Praet et al., 2018; Villabona et al., 2023). Regardless of species, wild bumble bees, which forage in outdoor environments, have more non-core phylotypes (i.e., microbial taxa that are sporadically present in a given host taxa) in their gut microbiotas than lab-reared bees. These non-core phylotypes include other corbiculate bee-associated microbes such as *Apibacter* (Mockler et al., 2018; Praet et al., 2016, 2018), and environmental microbes, like *Enterobacteriaceae*, *Fructobacillus*, and non-core *Lactobacillaceae* (Bosmans, Pozo, Verreth, Crauwels, Wilberts, et al., 2018; Krams et al., 2022; Kwong & Moran, 2016a; J. Li et al., 2015; Mockler et al., 2018; Newbold et al., 2015; Praet et al., 2016, 2018; Villabona et al., 2023). In some cases, these non-core phylotypes can displace core phylotypes and become dominant gut microbiota constituents (J. Li et al., 2015; Villabona et al., 2023), as is the case in many bumble bees collected from forest environments (Bosmans, Pozo, Verreth, Crauwels, Wilberts, et al., 2018; Krams et al., 2022). Meanwhile, lab-reared bees who are prevented from foraging outdoors tend to have few, if any, non-core phylotypes present; if present, non-core phylotypes tend to occur at low abundances (Billiet et al., 2016, 2017; Hammer et al., 2021, 2023; Hotchkiss et al., 2024; Motta & Moran, 2023).

In addition to species and rearing environment, whether bumble bees come from a commercial or wild population (i.e., bee origin) can also influence their gut microbiota taxonomic composition. Companies began to rear bumble bee colonies commercially in the late 1980s for agricultural pollination in fields and greenhouses (Velthuis & Van Doorn, 2006). Commercial stocks also present a quick and easy way for researchers to obtain fully-developed bumble bee colonies for experiments at any time of year, and lab studies on bumble bee gut microbiotas using commercial bees are becoming increasingly common (e.g., Cullen et al., 2023; Helander et al., 2023; Hotchkiss et al., 2024; L. Li et al., 2021; Motta & Moran, 2023; Seidenath et al., 2023; Straw et al., 2023). These stocks, which mainly comprise two species (the North American *Bombus impatiens* and the European *B. terrestris*), represent populations that have been reared in indoor facilities for generations with no access to the outside environment. Generally, commercial colonies harbour the same core phylotypes as wild bees (e.g., Hotchkiss et al., 2024; L. Li et al., 2021; Meeus et al., 2015; Straw et al., 2023). However, recent studies have observed that commercial *B. impatiens* colonies from some suppliers can lack *Gilliamella,* a core phylotype which is consistently present in wild *B. impatiens* populations (Hotchkiss et al., 2024; Motta & Moran, 2023).

The observation that gut microbiota community composition varies with bumble bee species, environment, and origin raises the question: Does this variation in taxonomic composition beget variation in gut microbial community function? The answer is unclear. Extensive work has been conducted to describe the metabolic capabilities of core social bee gut microbes. Multiple core microbial strains have been isolated from social bee species, including bumble bees, and had their genomes fully sequenced (e.g., Cornet et al., 2022; Dong et al., 2024; Ellegaard et al., 2015, 2020; Ellegaard & Engel, 2019; Engel et al., 2012; Kwong et al., 2014; Kwong & Moran, 2013; Martinson et al., 2014; Praet et al., 2017; Sun et al., 2021; Zhang et al., 2021; Zheng et al., 2019), providing extensive information on gene content and metabolic potential. *In vitro* growth experiments (e.g., Engel et al., 2012; Kešnerová et al., 2017; Palmer-Young et al., 2019; Zheng et al., 2016, 2019) and *in vivo* colonization experiments with these strains (e.g., Kešnerová et al., 2017; Quinn et al., 2024; Zheng et al., 2017) have experimentally validated the inferred metabolic functions. Based on this work, we know that *Bombilactobacillus*, *Bombiscardovia*, *Bifidobacterium*, *Gilliamella*, *Schmidhempelia,* and *Lactobacillus* all primarily ferment sugars, while *Snodgrassella* fuels its metabolism with fermentation products (i.e., organic acids) (Kwong & Moran, 2016b; Quinn et al., 2024). Many non-core taxa present in *Bombus* gut microbiotas also ferment sugars (Kwong et al., 2018; Mohamed et al., 2023). This functional redundancy present across bumble bee gut microbes may indicate that despite variation in taxonomic composition, metabolic potential remains somewhat consistent across *Bombus* populations, as is the case between *B. terrestris* and honey bees (Su et al., 2021). However, among social bee core phylotypes, phylotype-specific metabolic pathways still exist—for example, a complete citric acid cycle is present in *Snodgrassella* but lacking in all other core phylotypes (Engel et al., 2012; Kešnerová et al., 2017; Zheng et al., 2017). There is also additional intra-phylotype variation in metabolic capabilities (e.g., Cornet et al., 2022; Ellegaard et al., 2015; Zheng et al., 2016). Consequently, social bee gut microbiotas with similar phylotype composition can nevertheless differ in metabolic potential (Ellegaard et al., 2020).

Most bumble bee gut microbiota community profiling to date has used 16S rRNA gene amplicon sequencing, a technique which provides little information on intra-phylotype diversity and no information on functional gene content (Johnson et al., 2019; López-Aladid et al., 2023). Thus, we know little about sub-phylotype-level composition and metabolic potential of whole bumble bee gut microbiotas. If the phylotype-level taxonomic differences we observe between bumble bee gut microbiotas from different host species and populations correspond to differences in community function, then research conducted on a single host population could be of limited relevance to other populations. For example, understanding the degree to which community function in commercial bumble bee gut microbiotas

reflects that in wild gut microbiotas, and determining whether it is supplier-dependent, will be useful for designing experiments and interpreting results.

In this study, we use shotgun metagenomics to determine how genome-level taxonomic composition and metabolic potential in bumble bee gut microbiotas vary with host species and origin. Specifically, we examine the gut microbiotas of commercial *B. impatiens* from two different commercial suppliers, wild *B. impatiens*, and three other wild bumble bee species, *B. rufocinctus*, *B. ternarius,* and *B. vagans*, whose gut microbiotas have not been profiled before. These bumble bee populations sit at either end of the environment-origin spectrum—commercial bumble bees have been reared indoors and have never foraged outside, while wild bees come from colonies established by outdoor-foraging queens where there is abundant movement of workers between the hive and the outdoor environment. To our knowledge, this study is the first to compare genome-level taxonomic composition and metabolic potential of whole bumble bee gut microbial communities between commercial bumble bee colonies from different suppliers and between commercial and wild bumble bee populations.

5.3 Materials and Methods

5.3.1 Bumble bee gut microbiota sampling

In this study, we investigated the gut microbiotas of both commercial and wild bumble bees, all representing species native to our study area in eastern North America. In August 2023, we purchased three *Bombus impatiens* colonies from Biobest (Leamington, Ontario, Canada) and three *B. impatiens* colonies from Koppert (Scarborough, Ontario, Canada). Upon colony arrival, we removed two workers per colony and euthanized them by freezing at -20˚C. During the same month, we also collected wild bumble bees from six sites in Ottawa, Ontario, Canada: Bruce Pit, Chapman Mill's Conservation Area, Fletcher Wildlife Garden, Mer Bleue Bog, Mud Lake, and Petrie Island (Figure 5-1); all sites were at least 4 km apart. We opportunistically caught bumble bees at our sites using 50 mL Falcon tubes and identified them to morphospecies in the tubes. If we identified a bumble bee as a worker of an at-risk species (*B. bohemicus*, *B. pennsylvanicus*, or *B. terricola*), a queen, or a male, it was released. If not, we moved the tube containing the bee to a cooler filled with ice packs. At the end of each day, we returned to the lab and moved the bees from the cooler to a -20˚C freezer to be euthanized. We assigned all bees collected in this study a unique identifier ("bee ID") which included its species, site/colony, and replicate number.

Once the bees were dead, we removed them briefly from the freezer to identify them to species using the dichotomous keys and images in Williams et al., 2014. Ultimately, due to the number of individuals we had collected and their distribution across sites, we decided to investigate the gut

microbiotas of four of the wild species we caught: the common eastern bumble bee (*B. (Pyrobombus) impatiens*), the red-belted bumble bee (*B. (Cullumanobombus) rufocinctus*), the tri-coloured bumble bee (*B. (Pyrobombus) ternarius*), and the half-black bumble bee (*B. (Pyrobombus) vagans*). We collected *B. impatiens* from each of our six sites and collected the other bee species from three sites each; we collected all four species at Mud Lake (Figure 5-1).

In this study, we refer to Biobest *B. impatiens*, Koppert *B. impatiens*, wild *B. impatiens,* wild *B. rufocinctus,* wild *B. ternarius*, and wild *B. vagans* collectively as "bee hosts".

5.3.2 Gut dissections and enrichment for prokaryotic cells

We carried out all dissections in a sterile laminar flow hood. We dissected the guts of two bumble bee workers per bee host per site/commercial colony. We performed the dissections in sterile tissue culture dishes, using a new dish for each bee and sterilizing all dissection tools with 10% bleach and 70% ethanol between each dissection.

After gut dissections and prior to DNA extraction, we enriched our samples for prokaryotic cells relative to eukaryotic (i.e., host or pollen) cells using a protocol adapted from Ellegaard and Engel, 2019. We placed each dissected gut in a sterile 1.5 mL Eppendorf tube filled with 250 µL of sterile 1 mm glass beads and 500 µL of sterile 1M phosphate-buffered saline (PBS). We then homogenized guts with an Omni Bead Ruptor 4 Homogenizer (Gerogia, USA) for 30 seconds at speed 4 and subsequently centrifuged samples at 2500 rpm for five minutes and collected the supernatant in a new, sterile tube. Next, we centrifuged samples at 9000 rpm for 15 minutes, discarded the supernatant, and resuspended pellets in 800 µL of 1M PBS. We then centrifuged samples for a second time at 2500 rpm for five minutes. After this step, we passed the supernatant through 10 µM pluriStrainer Mini filters (California, USA) by centrifuging samples at 9000 rpm for 30 seconds. We finished by centrifuging at 10,000 rpm for 15 minutes, removing the supernatant, and resuspending the pellets in 750 µL of the bead-beating solution from the QIAGEN DNeasy PowerLyzer PowerSoil Kit (Hilden, Germany). Each 750 µL volume was then moved to a bead-beating tube from the QIAGEN kit to begin DNA extraction.

5.3.3 DNA extractions and sequencing

We extracted DNA from our samples using the QIAGEN DNeasy PowerLyzer PowerSoil Kit (Hilden, Germany), following protocol with the following modifications: 1) after adding 60 µL of solution C1 and vortexing samples, we incubated samples in a water bath for 10 minutes at 65˚C, 2) we conducted all centrifugation steps at 13,000 x *g*, and 3) we allowed solution C6 to sit on the membrane for 5 minutes before elution. We quantified DNA using the Qubit 2.0 and the Invitrogen Qubit dsDNA HS

Assay Kit, following protocol with the modification that the DNA was incubated in the dye/buffer mixture for five minutes. We sent extracted DNA to Genome Quebec for library preparation and metagenomic shotgun sequencing using the NovaSeq 6000 system; two *B. ternarius* samples, one *B. vagans* sample, and a negative DNA extraction control failed at the library creation step and so were excluded from further analysis.

5.3.4 qPCR for 16S rRNA gene copy number

We used qPCR to obtain 16S rRNA gene copy numbers from all gut samples. We generated a four-step standard curve of known 16S rRNA gene copy numbers using DNA extracted from NEB® 5-alpha competent *Escherichia coli* K-12 which we ran in triplicate on every plate. We diluted gut DNA extracts 1:150 in nuclease-free water and ran all samples in triplicate following the protocol in Motta et al. (2018). We used BioRad® SsoFast EvaGreen Supermix with a BioRad® CFX96 real-time system and C1000 thermocycler, and analyzed run data with Bio-Rad® CFX Maestro software (v.2.3).

5.3.5 Metagenomic bioinformatics

We had an average of 66 million paired-end raw reads per sample (range: 51.4 million – 79.9 million). We examined the quality of raw reads using FastQC v0.11.9 (Andrews, 2019), and trimmed and filtered out low-quality reads using fastp v0.23.2 with default settings (Chen et al., 2018). After this filtering step, we had an average of 65.6 million paired-end reads per sample (range: 50.9 million – 79.3 million). We then used Bowtie 2 v2.5.1 (Langmead & Salzberg, 2012) with the --very-sensitive flag to map reads against host genomes and retain the unmapped reads. For *B. impatiens* samples, we mapped reads against a *B. impatiens* genome (NCBI GCF_000188095.3) (Sadd et al., 2015). However, *B. rufocinctus*, *B. ternarius*, and *B. vagans* genomes have not been sequenced, so we used a published phylogeny to determine their closest relative with a sequenced genome (Cameron et al., 2007). Consequently, we mapped *B. rufocinctus* reads against a *B. cullumanus* genome (GenBank GCA_014737535.1), *B. ternarius* reads against a *B. bifarius* genome (NCBI GCF_011952205.1), and *B. vagans* against a *B. impatiens* genome (NCBI GCF_000188095.3) (Heraghty et al., 2020; Sadd et al., 2015; Sun et al., 2021). An average of 68% of paired reads per sample mapped to the host or host-related genome (range: 9.2 – 96.4%); after this filtering step, we were left with an average of 20.4 million paired-end reads per sample (range: 2.3 million – 57.2 million) (Supplementary Figures 5-1; 5-2A,B).

We assembled filtered reads for each sample using MEGAHIT v1.2.9 with the --no-mercy flag (D. Li et al., 2015). We assessed assembly quality using quast v5.0.2 and multiqc v1.9 (Ewels et al., 2016; Gurevich et al., 2013). We binned contigs from each sample into metagenome assembled genomes

(MAGs) using CONCOCT v1.1.0 with a minimum contig size of 1000 bp, MetaBAT 2 v2.15 with a minimum contig size of 1500 bp, and Maxbin 2.0 v2.2.7 with a minimum contig length of 500 bp. We refined MAGs within each sample using DAS Tool v1.1.6 (Sieber et al., 2018). We assessed the quality of all MAGs using CheckM2 v1.0.1 (Chklovski et al., 2022) and assigned taxonomy using GTDB-Tk and the Genome Taxonomy Database (GTDB) (Chaumeil et al., 2020; Parks et al., 2020).

Across all our bee gut microbiota samples, many MAGs were assigned to identical taxonomic groups and therefore presumably had high shared average nucleotide identities (ANIs). When mapping reads against a set of MAGs to determine the relative abundance of each MAG in a sample, the presence of many MAGs with high shared ANIs (i.e., >98%) can present issues for mapping tools (Evans & Denef, 2020). Therefore, we dereplicated MAGs using drep with a secondary ANI threshold of 98% to obtain a set of representative MAGs for mapping (Olm et al., 2017). After dereplication, we were left with 33 MAGs, all of which had >80% completeness and 31 of which had <10% contamination (i.e., redundancy). Both MAGs that had >10% contamination were assigned to *Fructobacillus tropaeola* and were the only MAGs for this taxon in our dereplicated set. Thus, we attempted to manually refine these MAGs so that this taxon would be not removed entirely. We used MMseqs2 to assign taxonomy to all contigs in each contaminated MAG using the GTDB and then removed any contigs that had been assigned to genera other than *Fructobacillus* (Parks et al., 2020; Steinegger & Söding, 2017). This technique decreased the contamination of one *F. tropaeola* MAG below 10% (10.14% to 7.75%), so we retained the refined version of the MAG in our representative set; we discarded the other MAG as contamination remained high even after manual refinement (24.19% to 19.62%). Thus, after dereplication and manual refinement, we had a set of 32 dereplicated MAGs, all with >80% completeness (median = 99.5%) and <10% contamination (median = 0.3%) (Supplementary Table 5-1). For each MAG, we created a taxonomic ID consisting of the lowest taxonomic assignment for that MAG and, when necessary, a number to distinguish between congeneric and conspecific MAGs. We used GToTree v1.8.4 to infer an approximate maximum-likelihood phylogenetic tree of the representative MAGs using 74 bacterial single copy genes (M. D. Lee, 2019).

We used CoverM v0.6.1 with a minimum percent identity of 95% and a minimum read aligned percent of 75% to map host-filtered reads from each bee gut microbiota against the set of representative MAGs to determine the coverage and relative abundance (i.e., relative reads per kilobase per million mapped reads) of each MAG in each bee gut microbiota (Woodcroft, 2021). The mean percentage of unmapped reads per was 42.5% (range: 3.1 – 96.7%), with *Bombus vagans* having the highest average unmapped percentage across all hosts (Supplementary Figure 5-2E). To investigate the reason for this high percentage of unmapped reads, we mapped host-filtered reads for each *B. vagans* sample against the

Kraken 2 nt database, which contains both eukaryotic and prokaryotic sequences, to determine which non-microbial taxa were present (Wood et al., 2019).

After determining the taxonomic community structure of our bee gut microbiotas, we wanted to examine whether gut microbiota metabolic potential varied with bee host and, for wild bees, by site. While the ultimate goal for the data collected in this study is to examine differential presence/absence of all genes predicted to be present in our MAGs, for the purposes of this thesis we have limited our metabolic investigation to the examination of differential presence/absence of modules from the Kyoto Encyclopedia of Genes and Genomes (KEGG) module database (Kanehisa et al., 2023). KEGG modules are gene sets that can be linked to particular metabolic functions. Having examined KEGG modules in unbinned contigs in a previous chapter (see Chapter 4), we had a KEGG module analysis pipeline that required little adjustment for working with MAGs, and so this seemed like a logical place to begin our metabolic investigation. However, we acknowledge that there are genes and gene sets from a variety of metabolic pathways that do not have corresponding KEGG modules (e.g., membrane transport proteins, secretion systems, etc.). Further investigation of genes using other databases, such as KEGG orthology database, the "evolutionary genealogy of genes: Non-supervised Orthologous Groups" (eggnog) database, or the Carbohydrate-Active enZYmes (CAZy) database, will be necessary to fully understand how the community-level functional potential of bee gut microbiotas changes with bee host (Drula et al., 2022; Huerta-Cepas et al., 2019; Kanehisa et al., 2023).

To begin investigating the variation in bee gut microbiota metabolic potential across bee hosts, we first had to determine the metabolic potential of each representative MAG. However, if we only examined the metabolic functions present in our representative MAGs, we could miss accessory functions found in closely related MAGs (i.e., shared ANIs >98%) that were removed during dereplication (Evans & Denef, 2020). To solve this issue, we took all MAGs refined by DAS Tool that were removed during dereplication and selected those with >80% completeness and <10% contamination. We then used shared ANI phylogenies produced by drep to assign each high-quality, DAS Tool-refined MAG to the representative MAG with which it shared the highest ANI (Olm et al., 2017); to avoid pseudoreplication, if two MAGs were from the same bee host and site/colony, had a shared ANI >99.5%, and were assigned to the same representative MAG, only the highest-quality MAG of the pair was retained for analysis. We called the resulting groups, which each consisted of a representative MAG and any related high-quality DAS Tool-refined MAGs, MAG clusters. We assigned these MAG clusters IDs identical to the taxonomic ID of the cluster's representative MAG. This clustering process resulted in 71 MAGs being assigned to MAG clusters for a total of 103 MAGs for subsequent metabolic analyses (71 high-quality DAS Tool MAGs + 32 representative MAGs); these MAGs had a median completeness of 95% and a

median contamination of 0.37% (Supplementary Table 5-2). To visualize relatedness between MAG clusters, we used GToTree v1.8.4 to infer an approximate maximum-likelihood phylogenetic tree using 74 bacterial single copy genes (M. D. Lee, 2019). We then annotated metabolic functions in all 103 clustered MAGs using the KEGG module database and Anvi'o, specifically the anvi-gen-contigs-database, anvi-run-kegg-kofams, and anvi-estimate-metabolism programs with the stepwise module completion threshold set at 0.75 (Eren et al., 2020; Kanehisa et al., 2023; Veseli et al., 2023).

After annotating the KEGG modules in each MAG cluster, the next step was to determine if the same KEGG modules were present across all bee gut microbiotas regardless of MAG cluster (i.e., taxonomic) composition. First, we calculated the relative presence of each KEGG module in each MAG cluster (i.e., number of MAGs in a MAG cluster with a given module / the total number of MAGs in that cluster). Second, we catalogued all KEGG modules that could be present in each bee gut microbiota based on which MAG clusters were present (i.e., which representative MAGs reads mapped to during coverage mapping); we considered a MAG cluster present in a bee gut microbiota if its representative MAG had a relative abundance >1%. Third, we calculated the minimum probability that each KEGG module was actually present in a given bee gut microbiota based on the relative presence of each metabolic pathway in all MAG clusters present in that gut microbiota. To provide a hypothetical example, if a gut microbiota contained the *Apibacter 1* and *Bifidobacterium 1* clusters, the relative presence of module A was 0.5 in *Apibacter 1* and 0.75 in *Bifidobacterium 1*, and we know that the gut microbiota must contain at minimum one MAG per MAG cluster, then the minimum probability that module A is present in the gut microbiota would be 1 - (probability that neither the *Apibacter 1* nor *Bifidobacterium 1* MAG contains module A), or 1 – ((1-0.5)*(1-0.75)), or 0.875. We performed this calculation for each KEGG module that could be present in each bee gut microbiota. Based on this information, for each module we also calculated the percent of individuals for each bee host whose gut microbiotas contained that module (i.e., the number of gut microbiotas for each bee host in which the minimum probability of module presence ≥0.75 / the total number of gut microbiotas sampled for that host).

5.3.6 Statistical analyses

We conducted all statistical analyses in R v4.3.1 (R Core Team, 2023). For many metrics that we measured in our study (e.g., microbial abundance, alpha diversity, community composition, etc.), we wanted to determine how these metrics varied between bee hosts (i.e., between Biobest *B. impatiens*, Koppert *B. impatiens*, wild *B. impatiens,* wild *B. rufocinctus,* wild *B. ternarius*, and wild *B. vagans*) and, for wild bees, between sites. Therefore, for each metric we typically ran two models: one using data from all bees with the metric of interest as the dependent variable and bee host as an independent variable with

six levels ("host model"), and another using data from only wild bees, with the metric of interest as the dependent variable and both site (six levels) and host (four levels) as independent variables ("site model"). The inclusion of bee host in the site model was to control for variation among hosts when testing for differences across sites; it had insufficient levels to be coded as random and was therefore coded as fixed. As differences between bee hosts were already tested in the species models, results for differences between species in site models were not reported.

We fit all linear and generalized linear models using the "lm", "glm" and "glm.nb" functions in the MASS and base R stats packages (R Core Team, 2023; Venables & Ripley, 2002). Often for linear models the assumptions of normality and homoscedasticity were violated; in these cases, we used non-parametric tests with ranked dependent variables. We determined significance of terms in linear and generalized linear models using Type 3 analyses of variance (ANOVAs) with the "Anova" function from the car package and examined model assumptions using the performance package (Fox & Weisberg, 2019; Lüdecke et al., 2021). We performed post-hoc tests using the "TukeyHSD" function in the stats package, and the "dunnTest" function in the FSD package and the emmeans package with Bonferroni corrections (Lenth, 2023; Ogle et al., 2023; R Core Team, 2023). We manipulated and visualized all data using the R packages dplyr, ggplot2, ggtree, patchwork, and leaflet (Graul, 2016; Pedersen, 2024; Wickham, 2016; Wickham et al., 2019, 2023; Xu et al., 2022).

We analyzed variation in host DNA filtering, microbial abundance, and representative MAG set mapping using linear host and site models with $\log_{10}$ number of reads post-host filtering, $\log_{10}$ 16S rRNA gene copy count, and percent of reads unmapped as the dependent variables, respectively. All initial linear models showed signs of heteroscedasticity, so we reran models with ranked dependent variables and conducted post-hoc analyses using Dunn's test (Ogle et al., 2023).

We conducted taxonomic analyses at two resolutions: phylotype (i.e., genus) and representative MAG. We used the vegan package to determine phylotype and representative MAG richness and Shannon diversity for each bee gut microbiota (Oksanen et al., 2022). We employed generalized linear models with Poisson distributions to analyze variation in phylotype and representative MAG richness with respect to bee host and site; the model analyzing MAG richness with respect to bee host was overdispersed, so we reran the model with a negative binomial distribution. Post-hoc tests for richness models were conducted with the emmeans package (Lenth, 2023). We used linear models with ranked dependent variables to analyze variation in phylotype and representative MAG Shannon indices as functions of bee host and site.

To analyze how phylotype and representative MAG community structure in bee gut microbiotas varied with bee host and site, we used the vegan and ape packages in R to generate Bray-Curtis

dissimilarity matrices of bee gut microbiota community structure (Oksanen et al., 2022; Paradis & Schliep, 2019). We then used the "adonis2" function in the vegan package to conduct permutational analyses of variance (PERMANOVAs) with 9,999 permutations (Oksanen et al., 2022). We evaluated homogeneity of group dispersions using the "betadisper" function in the vegan package with type set to "median" (Oksanen et al., 2022). We conducted post-hoc comparisons using the pairwiseAdonis package for PERMANOVA models and the "TukeyHSD" function for differences in group dispersions (Martinez Arbizu, 2020). We also visualized differences in principal coordinates analyses (PCoAs). To identify which phylotypes were differentially abundant across bee hosts, we used a series of linear models with ranked dependent variables. For each model, the relative abundance of a given phylotype was the dependent variable and bee host was the independent variable. If the species term in a model was significant based on a Type 3 ANOVA, we conducted a post-hoc analysis, using Dunn's test to determine which bee species were significantly different (Ogle et al., 2023). As we had over 30 representative MAGs and many of their relative abundances clearly varied with bee host (i.e., most MAGs were completely absent from at least one host; see Results), we elected not to formally analyze which representative MAGs were differentially abundant across bee hosts.

To visualize differences in the metabolic potential of MAG clusters, we used the vegan and ape packages in R to generate a Jaccard distance matrix of KEGG module presence and absence in MAG clusters (Oksanen et al., 2022; Paradis & Schliep, 2019), which we then used for PCoA. To examine if any KEGG modules were differentially enriched across MAG clusters (i.e., if any modules were consistently present in some MAG clusters but consistently absent in others), we used the Anvi'o program anvi-compute-metabolic-enrichment with the stepwise module completion threshold set to 0.75 (Eren et al., 2020; Shaiber et al., 2020). This analysis provides two values: an unadjusted p-value, and a q-value adjusted for false detection rates. In our case, adjusted q-values were lower than unadjusted p-values; to be conservative, we report results using unadjusted p-values.

Finally, to investigate if the metabolic potential of bee gut microbiotas varied with bee host or, for wild bees, by site, we used the vegan and ape packages in R to generate Jaccard distance matrices of KEGG module presence and absence in the gut microbiotas of all bees and wild bees only (Oksanen et al., 2022; Paradis & Schliep, 2019); a KEGG module was considered "present" in a bee gut microbiota if its minimum probability of presence was ≥0.75. We visualized these data using PCoAs. We then used the "adonis2" function in the vegan package to conduct permutational analyses of variance (PERMANOVAs) with 9,999 permutations to analyze how the metabolic potential of bee gut microbiotas varied with bee host and, for wild bees, with site (Oksanen et al., 2022). We evaluated homogeneity of group dispersions and conducted post-hoc comparisons as described above for community structure. To identify which

KEGG modules were differentially enriched across bee hosts, we manually compiled a file with the same structure as the "modules" mode output file from the Anvi'o program "anvi-estimate-metabolism" (Veseli et al., 2023). In this file, we listed a module as being present in a bee gut microbiota (i.e., stepwise_module_is_complete = TRUE) if its minimum probability of presence was ≥0.75. We then used that file with the Anvi'o program anvi-compute-metabolic-enrichment (Shaiber et al., 2020) to calculate enrichment scores, unadjusted p-values, and adjusted q-values. In this case, the adjusted q-values were greater than the p-values, and so we used q-values to determine module enrichment significance.

5.4 Results

5.4.1 Read mapping and 16S rRNA gene copy counts

The number of reads that remained after mapping to host or host-related genomes did not vary with bee host ($F_{5,33} = 1.35$, $P = 0.27$) or with site for wild bees ($F_{5,18} = 1.24$, $P = 0.33$) (Supplementary Figures 5-1, 5-2), though the average number of reads post-host filtering for *B. ternarius* was an order of magnitude lower than that for all other hosts (10^6 vs. 10^7).

Bumble bee gut microbiota 16S rRNA gene copy counts, a proxy for microbial abundance, varied with bee host ($F_{5,33} = 4.00$, $P = 0.006$) but not site ($F_{5,18} = 2.14$, $P = 0.11$) (Supplementary Figure 5-2). Specifically, gene copy counts were significantly lower in *B. vagans* gut microbiotas than in those of Biobest *B. impatiens* ($|Z| = 3.06$, $P = 0.03$) (Supplementary Figure 5-2A).

After mapping reads against the set of representative MAGs, the percentage of unmapped reads varied with bee host ($F_{5,33} = 11.5$, $P < 0.0001$) but not site ($F_{5,18} = 2.65$, $P = 0.06$) (Supplemental Figure 5-2). The percentage of unmapped reads was higher in *B. vagans* gut microbiotas than in Koppert *B. impatiens* or wild *B. impatiens* microbiotas (all $|Z| > 3.69$, all $P < 0.01$). Subsequent mapping of host-filtered reads from *B. vagans* microbiotas against a database containing eukaryotic and prokaryotic sequences revealed that an average of 91.3% of reads mapped to the genus *Bombus*.

5.4.2 Taxonomic community structure

We identified 10 phylotypes across all bee gut microbiotas: six core bumble bee gut phylotypes (*Bifidobacterium*, *Bombilactobacillus*, *Gilliamella*, *Lactobacillus*, *Schmidhempelia*, and *Snodgrassella*) and four non-core phylotypes (*Apibacter, Arsenophonus, Fructobacillus,* and *Lactobacillaceae CALYQJ01*) (Figure 5-2A). The non-core phylotypes we observed were still known constituents of social bee gut microbiotas (Hammer et al. 2021; NCBI GCA_945273735.1), though it is worth noting that this is only the second recorded observation of *Arsenophonus* spp. in bumble bee guts and the first of

Arsenophonus apicola (Newbold et al., 2015). In four of the 13 of the gut microbiotas containing *Arsenophonus*, this taxon dominated the microbial community, and these *Arsenophonus*-dominated microbiotas had higher 16S rRNA gene counts (average gene count in *Arsenophonus*-dominated *B. impatiens* and *B. rufocinctus* microbiotas: 9.9×10^8; average gene count in all other *B. impatiens* and *B. rufocinctus* microbiotas: 7.4×10^7).

This study is the first to characterize gut microbiotas of *B. rufocinctus, B. ternarius,* and *B. vagans* (Figure 5-2A). Core phylotype constituents of these species' microbiotas included *Bifidobacterium* and *Gilliamella* in all three host species, *Schmidhempelia* in *B. vagans*, and *Apibacter* in *B. rufocinctus* and *B. vagans. Lactobacillus* was only observed in one *B. rufocinctus* individual and *Bombilactobacillus* was not observed in any species. *Snodgrassella* was also largely absent.

For *B. impatiens* hosts, major core phylotype constituents were *Bifidobacterium*, *Lactobacillus*, and *Schmidhempelia* (Figure 5-2A). *Bombilactobacillus* was absent from wild *B. impatiens* but present in commercial colonies, while *Snodgrassella* was absent from all Biobest bees. *Bombilactobacillus* and *Gilliamella* were also missing in some individual Biobest and Koppert colonies.

Across all bee gut microbiotas sampled in this study, phylotype richness, but not Shannon diversity, varied with bee host (richness: likelihood ratio $\chi^2 = 20.6$, df = 5, $P < 0.001$; Shannon diversity: $F_{5,33} = 1.57$, $P = 0.19$) (Figure 5-2B; Supplementary Figure 5-3); neither metric varied with site for wild bee gut microbiotas (richness: likelihood ratio $\chi^2 = 3.95$, df = 5, $P = 0.56$; Shannon diversity: $F_{5,18} = 0.75$, $P = 0.54$) (Supplementary Figure 5-3). Specifically, wild *B. impatiens* gut microbiotas had higher phylotype richness than those of *B. rufocinctus* and *B. ternarius* (all $|Z| > 3.00$, all $P < 0.05$).

Phylotype-level community structure varied across bee hosts ($F_{5,33} = 4.82$, $P < 0.001$) (Figure 5-2), with all hosts having significantly different community structures from one another (all $F > 2.49$, all $P < 0.05$), except that *B. ternarius* community structure did not differ significantly from that of *B. rufocinctus* or wild *B. impatiens* (both $F < 2.48$, $P > 0.08$). Community structure of wild bee gut microbiotas did not vary with site ($F_{5,18} = 1.12$, $P = 0.32$), and group dispersions were similar across sites and hosts ($F < 1.1$, $P > 0.3$) (Supplementary Figure 5-4). Linear models revealed that the relative abundances of all phylotypes except *Bifidobacterium* varied with bee host (all $F_{5,33} > 4.50$, all $P < 0.01$), though post-hoc analyses also showed no significant pairwise differences between bee hosts for the *Lactobacillaceae CALYQJ01* phylotype (Figure 5-4; Supplementary Figure 5-5).

The 10 phylotypes we identified comprised 32 representative MAGs; the number of MAGs in each phylotype ranged from 1–7 (Supplementary Figure 5-6). MAG richness varied with bee host (likelihood ratio $\chi^2 = 54.2$, df = 5, $P < 0.0001$) and, in wild bees, with site (likelihood ratio $\chi^2 = 31.1$, df = 5, $P < 0.0001$) (Figure 5-3). Specifically, for all bee hosts, wild *B. impatiens* gut microbiotas had higher

MAG richness than all other hosts except Koppert *B. impatiens* (all $|Z| > 4.10$, all $P < 0.001$), and Koppert *B.* impatiens gut microbiotas had higher MAG richness than those of *B. ternarius* and *B. vagans* (all $|Z| > 3.10$, all $P < 0.05$) (Figure 5-3A). For wild bee hosts only, MAG richness was lower at Petrie Island than any other site (all $|Z| > 3.30$, all $P < 0.01$) (Figure 5-3B). MAG Shannon diversity did not vary with bee host or site (host: $F_{5,33} = 2.39$, $P = 0.06$; site: $F_{5,18} = 1.00$, $P = 0.45$) (Supplementary Figure 5-7).

MAG-level community structure varied across bee hosts ($F_{5,33} = 6.94$, $P < 0.001$) (Figure 5-3C, 5-4), but did not vary with site in wild bee gut microbiotas ($F_{5,18} = 1.24$, $P = 0.21$) (Supplementary Figure 5-8); group dispersions did not vary for either variable (all $F < 1.5$, all $P > 0.2$). Specifically, all hosts had significantly different community compositions from one another (all $F > 2.8$, all $P < 0.05$). Though not statistically analyzed, visually the relative abundance of all representative MAGs varied strongly with bee host, with all MAGs being completely absent from at least one bee host (Figure 5-4). This pattern held true even for MAGs of *Bifidobacterium*, which was consistently present across hosts at the phylotype level (Supplementary Figure 5-5C).

5.4.3 Metabolic potential of MAG clusters

We had 103 high-quality MAGs (i.e., >80% completeness and <10% contamination) comprising 32 MAG clusters (Supplementary Figure 5-8), with the number of MAGs in each MAG cluster ranging from 1–14. Half of these MAGs (52) were generated from wild *B. impatiens* gut microbiotas, 18 from Koppert *B. impatiens*, 13 from Biobest *B. impatiens*, 13 from *B. rufocinctus*, 5 from *B. ternarius* and 2 from *B. vagans* (Supplementary Figure 5-9). Across all MAG clusters, we identified 104 KEGG modules belonging to eight module categories. The presence of these KEGG modules varied significantly by MAG cluster ($F_{31,71} = 31.4$, $P < 0.001$) (Supplementary Figure 5-10); MAG cluster group dispersions did not vary ($F_{31,71} = 1.14$, $P = 0.33$). Enrichment analysis revealed that of the 104 identified modules, 100 were differentially enriched across MAG clusters (all $P < 0.05$) (Supplementary Figure 5-11). Almost all modules were enriched in multiple MAG clusters that belonged to at least two different phylotypes, showing a high level of functional redundancy across phylotypes. Of the non-differentially enriched modules, two had consistently low prevalence (heme biosynthesis in animals and fungi, and NAD biosynthesis beginning with tryptophan, both modules within cofactor metabolism) and two were consistently present (PRPP biosynthesis, a carbohydrate metabolism module, and adenine ribonucleotide biosynthesis, a nucleotide metabolism module).

5.4.4 Metabolic potential of bee gut microbiotas

We identified 97 KEGG modules that had at least a 75% probability of being present in at least one bee. The number of modules expected to be present in each bee gut microbiota ranged from 57-99 and varied with bee host (likelihood ratio $\chi^2 = 16.9$, df = 5, $P = 0.005$) (Figure 5-5A). Specifically, wild *B. impatiens* gut microbiotas contained higher numbers of unique KEGG modules than Biobest *B. impatiens* gut microbiotas ($|Z| = 3.31$, $P = 0.01$). Additionally, bee gut microbiota KEGG module composition varied with bee host ($F_{5,33} = 6.02$, $P < 0.001$) (Figure 5-5B), though not to the same extent as phylotype or MAG-level taxonomic community structure. Neither the number of KEGG modules nor KEGG module composition varied by site for wild bee gut microbiotas (number: likelihood ratio $\chi^2 = 1.52$, df = 5, $P = 0.91$; composition: $F_{5,18} = 1.54$, $P = 0.13$) (Supplementary Figure 5-12).

Enrichment analysis revealed that of the 97 KEGG modules present across bee hosts, only 20 (21%) were differentially enriched (all adjusted $q < 0.05$); the other 77 had similar prevalence across all bee hosts (Figure 5-5C). Of those 77 modules, 61 were consistently present and 16 had consistently low prevalence (all adjusted $q > 0.05$) (Supplementary Figures 5-13, 5-14). Of the 61 modules that were consistently present, 44 (72%) were present in every individual in every host. All differentially enriched modules were enriched in at least two bee hosts (Supplementary Figure 5-15). Koppert and wild *B. impatiens* gut microbiotas contained the greatest number of enriched modules, 17 each; *B. vagans* had 11, *B. rufocinctus* had nine, *B. ternarius* had seven, and Biobest *B. impatiens* had six.

5.5 Discussion

5.5.1 Gut microbiota community structure varies with species in wild bumble bees

We found that gut microbial community composition and structure varied between wild bumble bee species sampled from the same geographic area. At phylotype resolution, composition and community structure were relatively consistent within, but clearly differed between, species (Figure 5-2A,C). Many of these phylotypes were composed of multiple sequence-discrete populations (i.e., representative MAGs) whose prevalence and abundance also varied with species (Figure 5-4); thus, gut microbiotas were even more distinct between species at the MAG level (Figure 5-3C). Intra-phylotype variation is often not fully captured using standard 16S rRNA gene amplicon sequencing (Johnson et al., 2019; López-Aladid et al., 2023), and our results highlight the loss of information that results from examining gut microbiotas at lower taxonomic resolutions. Overall, our results suggest that the wild bumble bee species surveyed in our study have species-specific core gut microbiotas composed of unique

combinations of bee-associated microbial phylotypes and phylotype populations, as seen in other bumble bee communities (Dong et al., 2024; Villabona et al., 2023).

Profiled for the first time in this study, *B. rufocinctus*, *B. ternarius*, and *B. vagans* gut microbiotas were quite different from those typically observed in bumble bees (Hammer, Le, Martin, et al., 2021; Kwong, Medina, et al., 2017; Villabona et al., 2023). Notably, most *B. rufocinctus* and *B. ternarius* individuals and all *B. vagans* individuals lacked *Snodgrassella*, a striking result given this phylotype is highly prevalent in most if not all other profiled bumble bee species (Dong et al., 2024; Hammer, Le, Martin, et al., 2021; Kwong, Medina, et al., 2017; Praet et al., 2018; Villabona et al., 2023). *Snodgrassella* has been isolated from *B. vagans* previously (Kwong & Moran, 2013), so its absence from this species in our study may be population-specific; more extensive sampling would provide clarification. Another previous study (Hammer et al., 2023) documented an absence of Snodgrassella in bumble bees that had recently eclosed (i.e., emerged from pupation). However, newly-eclosed workers do not forage until a few days post-eclosion (Heinrich, 2004), and we surveyed foraging bees during peak colony activity when older workers were abundant. Thus, it is unlikely that young worker age explains the lack of *Snodgrassella* in our study. *Lactobacillaceae* phylotypes were also rare in all three species (Figure 5-2), though this is a more common observation, especially for *Bombilactobacillus* (Kwong, Medina, et al., 2017; Villabona et al., 2023). In fact, a lack of *Bombilactobacillus* in these species lends support to a hypothesis that *Bombilactobacillus* was lost in a common ancestor of many "short-faced" bumble bees (Cameron et al., 2007; Kwong, Medina, et al., 2017). Meanwhile, two *Apibacter* MAGs were present in all *B. vagans* and *B. rufocinctus* individuals, and at higher relative abundances than is typically seen in most bumble bee gut microbiotas (Kwong, Medina, et al., 2017; Powell et al., 2016). *Apibacter* is a genus of corbiculate-bee-associated microbes whose presence has been associated with decreased pathogen infection in *B. impatiens* (Kwong & Moran, 2016a; Mockler et al., 2018; Praet et al., 2016; W. Zhang et al., 2022). While currently considered a rare, non-core member of bumble bee microbiotas (Praet et al., 2016), the prevalence of *Apibacter* in *B. rufocinctus*, *B. vagans*, and the recently surveyed *B. friseanus* (Dong et al., 2024) suggests this phylotype may constitute part of the core gut microbiota for some bumble bee species or populations, joining the likes of *Schmidhempelia* and *Bombiscardovia*. However, we sampled a small number of individuals from these species over a limited geographic area and time period; more extensive sampling would be required to support this hypothesis.

Another interesting phenomenon we observed was the presence of *Arsenophonus apicola*-dominated gut microbiotas in *B. impatiens* and *B. rufocinctus* (Figure 5-2A). While other *Arsenophonus* species have been found in bumble bee guts (A. Parmentier, Billiet, et al., 2018), this is the first recorded instance of *Arsenophonus apicola* (Nadal-Jimenez et al., 2022). *Arsenophonus* is an environmental

phylotype found in flowers (McFrederick et al., 2017), solitary bees (Gerth et al., 2015), and social bee midguts (Drew et al., 2021; Kwong, Medina, et al., 2017) that shows few signs of vertical transmission in bee hosts (Drew et al., 2021). Thus, it appears that *Arsenophonus*-dominated microbiotas represent a new variation of a recurring gut enterotype in bumble bees wherein environmental microbes constitute most of the gut microbiota (J. Li et al., 2015; Villabona et al., 2023)—a shift that may adversely affect host health (Budge et al., 2016; L. Parmentier et al., 2016). However, typically in these environmental enterotypes, core phylotypes are mostly or completely displaced (J. Li et al., 2015; Villabona et al., 2023). We found that core gut phylotypes were still present in *Arsenophonus*-dominated gut microbiotas, while overall microbial abundances were an order of magnitude higher than in non-*Arsenophonus*-dominated microbiotas within the same host species. These observations imply that a large *Arsenophonus* population is present in addition to, not instead of, core phylotypes, perhaps growing in a gut compartment other than the core-dominated hindgut (Drew et al., 2021). Further research is needed to determine where *Arsenophonus* resides in the bumble bee gut and the effect, if any, of such a large population on host health and performance.

5.5.2 Commercial and wild *B. impatiens* have different gut microbial communities

Gut microbiota community structure differed not only among species but also with bee origin (i.e., commercial or wild) within the same species, and between commercial suppliers. Consistent with previous studies, the gut microbiotas of wild *B. impatiens* were generally dominated by *Bifidobacterium*, *Gilliamella*, *Lactobacillus*, *Schmidhempelia*, and *Snodgrassella*, with environmental bacteria as minor constituents (Cariveau et al., 2014; Powell et al., 2016). As expected, commercial colonies did not contain any non-core or environmental phylotypes (Figure 5-2A). Instead, all but one commercial colony contained *Bombilactobacillus*, a phylotype which is often present in commercial colonies and absent in wild *B. impatiens* (Hotchkiss et al., 2024; Mockler et al., 2018; Powell et al., 2016). Beyond phylotype differences, some *Bifidobacterium*, *Gilliamella*, and *Lactobacillus* MAGs were restricted to either commercial or wild *B. impatiens*, and, in some cases, were largely restricted to specific suppliers, as with *Bifidobacterium sp024104275* and *Lactobacillus apium* in Biobest bees.

However, the most striking difference in phylotype composition was the complete lack of *Snodgrassella* in all Biobest bees (Figure 5-2A). *Snodgrassella* is typically prevalent at high relative abundances in *B. impatiens* gut microbiotas (Powell et al., 2016), including those sampled from Biobest colonies (Hotchkiss et al., 2024; Motta & Moran, 2023). While an absence of *Snodgrassella* has been observed once before in a Biobest colony (Hotchkiss et al., 2024), it has never been observed in multiple colonies purchased together. Still, we believe this result, while unusual, to be real: all colonies were well-

established so it is unlikely all six individuals were newly-eclosed workers with still-assembling gut microbiotas, and no high-quality *Snodgrassella* MAGs were generated from Biobest sequence data (Supplementary Figure 5-9).

We also found that individual commercial colonies could lack additional core phylotypes. One colony from each supplier did not contain *Gilliamella*, and even in the colonies where it was present, within-phylotype diversity was much lower than in wild *B. impatiens* (Figures 5-2A, 5-4). In some bumble bee colonies, *Gilliamella* can take up to two weeks to establish in bee guts after eclosion (Hammer et al., 2023), therefore these absences could be due to age effects. However, *Gilliamella* can also establish much earlier (Su et al., 2021) and a consistent lack of *Gilliamella* over multiple weeks has been seen in other colonies (Hotchkiss et al., 2024). While noted before in Biobest colonies (Hotchkiss et al., 2024; Motta & Moran, 2023), this is the first observed *Gilliamella* absence in Koppert colonies. The same Koppert colony missing *Gilliamella* was also missing *Bombilactobacillus*, a phylotype that was found in all other commercial bees.

The complete absence of common core phylotypes in some commercial colonies is concerning. Core social bee gut phylotypes vary in their metabolisms (Engel et al., 2012; Kešnerová et al., 2017; Zheng et al., 2017) and sensitivity to external stimuli (Hammer, Le, & Moran, 2021; Hotchkiss et al., 2022; Raymann et al., 2017). As such, the presence of certain phylotypes is important for investigating certain topics, such as the presence of *Snodgrassella* for investigating impacts of glyphosate exposure (Motta et al., 2018, 2020; Motta & Moran, 2020, 2023). The fact that multiple commercial colonies could be ordered for an experiment and some, possibly all, could lack a crucial phylotype is troubling. The absence of core phylotypes could also alter microbial community dynamics, limiting the relevance of certain studies in commercial *B. impatiens* colonies to wild *B. impatiens* populations. To mitigate this risk, we recommend that, if possible, researchers purchase commercial colonies for experiments in multiple batches at different time points, and, if a specific phylotype is required for an experiment, its presence be confirmed using culturing or molecular techniques prior to experimentation. Additionally, we recommend that future research be dedicated to identifying factors driving core phylotype loss in commercial bees. Core phylotypes can decline dramatically in abundance and even be lost completely in diapausing queen bumble bees (Chapter 4; Bosmans, Pozo, Verreth, Crauwels, Wäckers, et al., 2018; Koch et al., 2013), who seed the gut microbiotas of their workers (Su et al., 2021); thus, diapause may be a good place to begin these investigations.

Overall, although wild *B. impatiens* and bees from the two commercial suppliers all harboured distinct gut microbiotas, gut microbiotas from Koppert colony bees were the most like those of wild *B. impatiens* (Figure 5-2B, 5-3C). However, we sampled a small number of workers from only a few

colonies purchased at one time, and some of the differences that made Biobest bee gut microbiotas so distinct in this study (i.e., the lack of *Snodgrassella*) are rare. More extensive sampling of commercial colonies purchased at multiple time points is required to verify and clarify the patterns we observed.

5.5.3 Metabolic potential is largely consistent across bee hosts

We found that taxonomic differences in bumble bee gut microbiotas between bee hosts did not correspond to differences in metabolic potential. Despite significant differences in gut microbiota taxonomic richness and community structure between bee hosts, almost 80% (77/97) of all metabolic modules identified in bee gut microbiotas had either consistently high or low prevalence across all hosts (Figure 5-5C; Supplementary Figures 5-13, 5-14). In fact, of the 61 modules that were consistently present, more than two thirds were present in all surveyed individuals (Supplementary Figure 5-13), demonstrating a high degree of stability in metabolic function both within and across hosts. Our observations match those from a wide variety of environmental and host-associated microbiotas in which community function remains stable within environments despite fluctuations in taxonomic composition and structure (Jiao et al., 2022; Louca et al., 2018; Moya & Ferrer, 2016; Su et al., 2021); this stability is due to metabolic redundancy across microbial taxa, which we also observed in our phylotypes and MAG clusters (Supplementary Figure 5-11).

The variable prevalence of core phylotypes but largely consistent metabolic potential support the idea of a flexible bumble bee core gut microbiota in which multiple combinations of phylotypes can create communities capable of similar function and support of host health. In fact, in bumble bees, gut microbial contributions to host performance are primarily associated with fundamental microbial metabolisms, such as organic acid and glycerophospholipid biosynthesis (L. Li et al., 2021; Palmer-Young et al., 2019). Modules which produce important precursors in these biosynthetic pathways, such as glycolysis, pyruvate oxidation, and phosphatidylethanolamine biosynthesis, were—unsurprisingly—consistently present across all hosts (Supplementary Figure 5-13). Consistent metabolic potential across bumble bee hosts also implies that for certain types of research, such as fundamental work describing how the presence or absence of a gut microbiota affects bumble bee function and performance, research conducted on one host population is likely relevant to many other bumble bee populations. However, relevance could still be limited by phylotype-specific responses to experimental conditions as discussed previously.

Though metabolic potential was largely consistent across bee hosts, there were metabolic modules that were differentially enriched (Figure 5-5D). Some of these modules, like those for isoleucine, lysine, C5 isoprenoid, and pyrimidine biosynthesis, were functionally redundant with other non-

differentially enriched modules; the same metabolic process could be achieved through multiple pathways (Supplementary Figure 5-13). Thus, their differential presence may have little effect on community function. However, the differential enrichment of other modules may have implications for gut microbiota and/or host performance. For example, microbiotas that can synthesize their own cysteine, a key amino acid, or pantothenate, an essential B-vitamin, may better tolerate environmental fluctuations in the availability of these organic molecules, as may their hosts (Hu et al., 2022; Jeannerod et al., 2022; Parish et al., 2022; Pearson, 1942; Serrato-Salas & Gendrin, 2023; Stabler et al., 2015). Still, these effects remain purely speculative; further studies are required to determine whether and to what degree the presence or absence of these differentially-enriched modules affects gut microbiota performance and bee host health.

Our study presents an initial look at metabolic potential across bumble bee gut microbiotas, but there are limitations to our methodology. The KEGG module database that we used to compare metabolic potential is composed of a limited number of genes and gene sets. Thus, a wide variety of enzymes and metabolic pathways were excluded from our analysis. Further investigation of metabolic potential using other gene databases will provide a more comprehensive understanding of how community-level functional potential of bumble bee gut microbiotas changes with bee host (Drula et al., 2022; Huerta-Cepas et al., 2019; Kanehisa et al., 2023). However, even with more extensive gene and pathway annotation, all discussion of microbiota metabolism would remain speculative. First, the genomes we analyzed were metagenome-assembled genomes and may not represent real genomes of microbes from our sampled communities (Meziti et al., 2021). Furthermore, we analyzed changes in gene presence and absence, not gene activity, and so we do not know if the genes we observed were expressed. Future experiments should employ metatranscriptomics, metabolomics, and metaproteomics to determine whether the consistency we observed in metabolic potential corresponds to consistency in gene expression and community function.

5.6 Conclusion

We found that gut microbiota community composition and structure varied with bumble bee species and origin (i.e., commercial vs. wild), and between commercial suppliers. However, despite these differences, metabolic potential was largely consistent across bee hosts. Our observations support the idea of a flexible bumble bee core gut microbiota in which multiple combinations of core phylotypes can create communities that function, and thereby support host health and performance, in similar ways. Still, our study surveyed a small number of bee gut microbiotas from a limited number of sites and commercial colonies, and only examined metabolic potential. Further research sampling bees over a more extensive

geographic area and time period, and incorporating a broader array of 'omics approaches, would complement and enrich our results.

Altogether, our results provide new insights into the structure and function of bumble bee gut microbiotas. Bumble bees are key pollinators, and their gut microbiotas are important components of their health and performance. As we continue to research this symbiosis, the results presented here will be useful for evaluating how knowledge gained from studying one bumble bee population may translate to other host populations and species.

5.7 Data availability

All data, R scripts, and MAGs are available at github.com/michellehotch/Chapter5. Raw sequences are available through the NCBI Sequence Read Archive PRJNA1044797.

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We declare no competing interests.

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5.10 Figures

Figure 5-1: Map of wild bumble bee collection sites and species collected.

Grey dots represent the locations of field sites (BP: Bruce Pit, CM: Chapman Mills Conservation Area, FG: Fletcher Wildlife Garden, MB: Mer Bleue Bog, ML: Mud Lake, PI: Petrie Island). Bumble bee icons above each site represent the species from that site from which we sampled gut microbiotas.

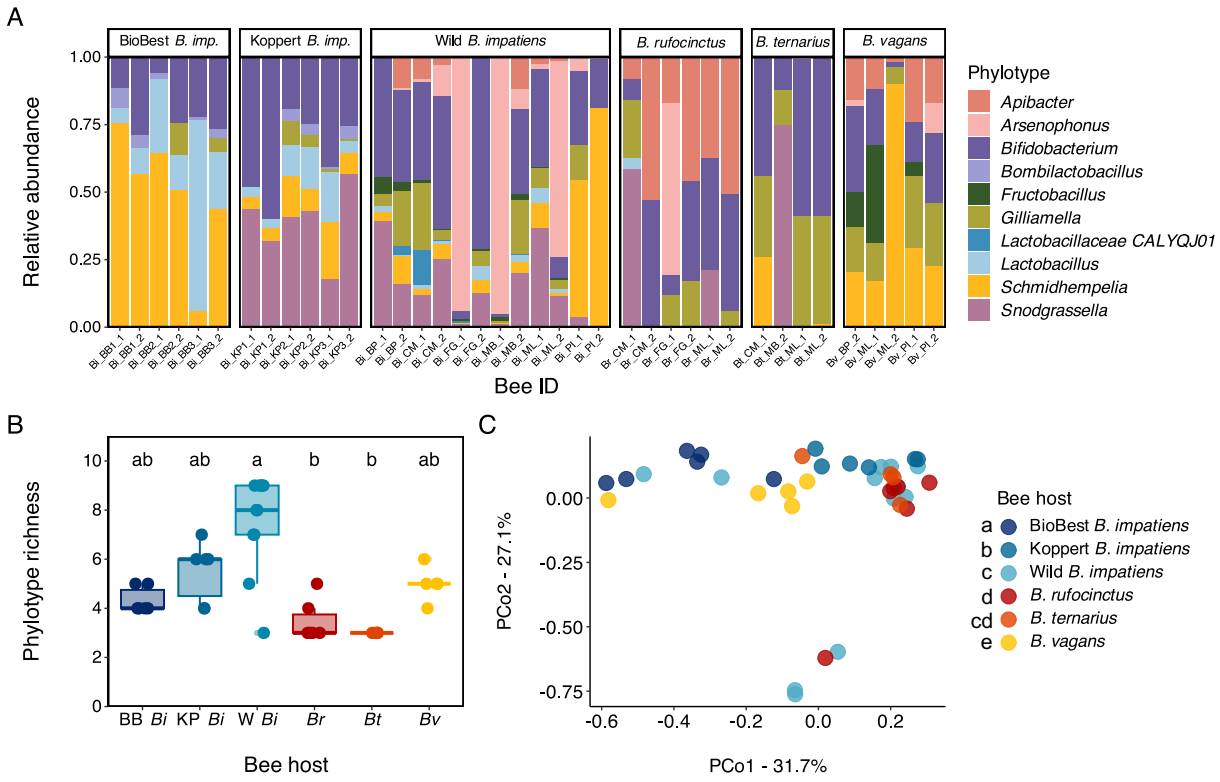


Figure 5-2: Bee gut microbiota diversity at the phylotype level.

A) Stacked bar plot of relative abundances of microbial phylotypes in gut microbiotas faceted by bee host. B) Box plot of phylotype richness by bee host. Boxes represent medians and interquartile ranges; whiskers extend to 1.5 × the interquartile range. BB Bi = Biobest B. impatiens, KP Bi = Koppert B. impatiens, W Bi = Wild B. impatiens, Br = B. rufocinctus, Bt = B. ternarius, and Bv = B. vagans. Hosts with different letters are significantly different based on pairwise comparisons of estimated marginal means. C) Principal coordinates analysis of phylotype community structure in bee gut microbiotas using Bray-Curtis dissimilarities. Bee hosts in the legend with different letters have significantly different community structures based on pairwise adonis analysis. For each plot, n = 39 individual bees and microbiotas; n = 4-12 per host.

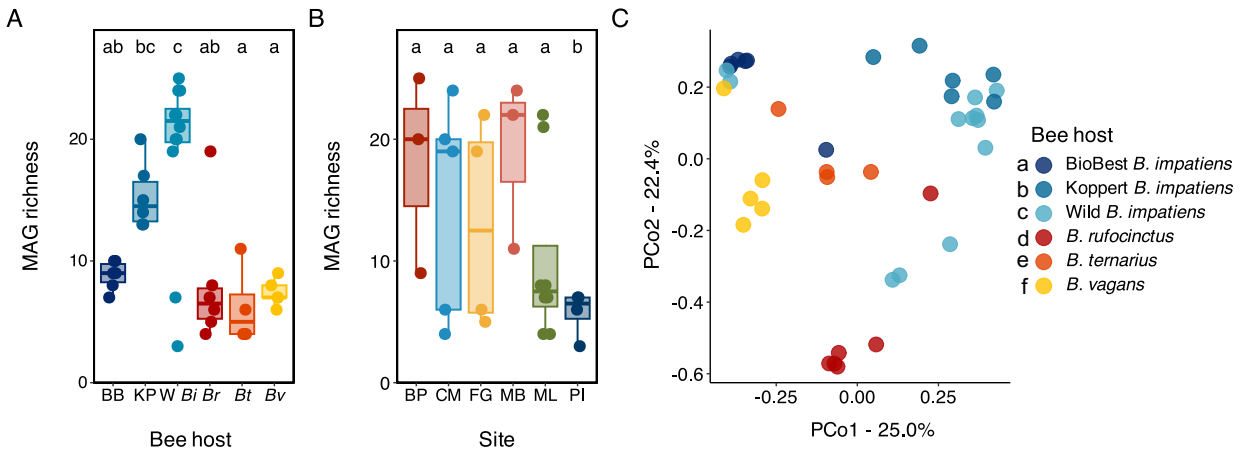


Figure 5-3: MAG richness and community structure in *Bombus* gut microbiotas.

A) Box plot of MAG richness by bee host in all bee gut microbiotas (n = 39; n = 4-12 per bee host). BB = BioBest *B. impatiens*, KP = Koppert *B. impatiens*, W *Bi* = Wild *B. impatiens*, *Br* = *B. rufocinctus, Bt* = *B. ternarius,* and *Bv* = *B. vagans*. B) Box plot of MAG richness by site in wild bee gut microbiotas (n = 27; n = 3–8 per site). BP = Bruce Pit, CM = Chapman Mills Conservation Area, FG = Fletcher Wildlife Garden, MB = Mer Bleue Bog, ML = Mud Lake, PI = Petrie Island. For both box plots, boxes represent medians and interquartile ranges; whiskers extend to 1.5 × the interquartile range. Within each box plot, bee hosts and sites with different letters are significantly different based on pairwise comparisons of estimate marginal means. C) Principal coordinates analysis of MAG community structure in all bee gut microbiotas using Bray-Curtis dissimilarities (n = 39; n = 4-12 per bee host). Bee hosts in the legend with different letters have significantly different community structures based on pairwise adonis analysis.

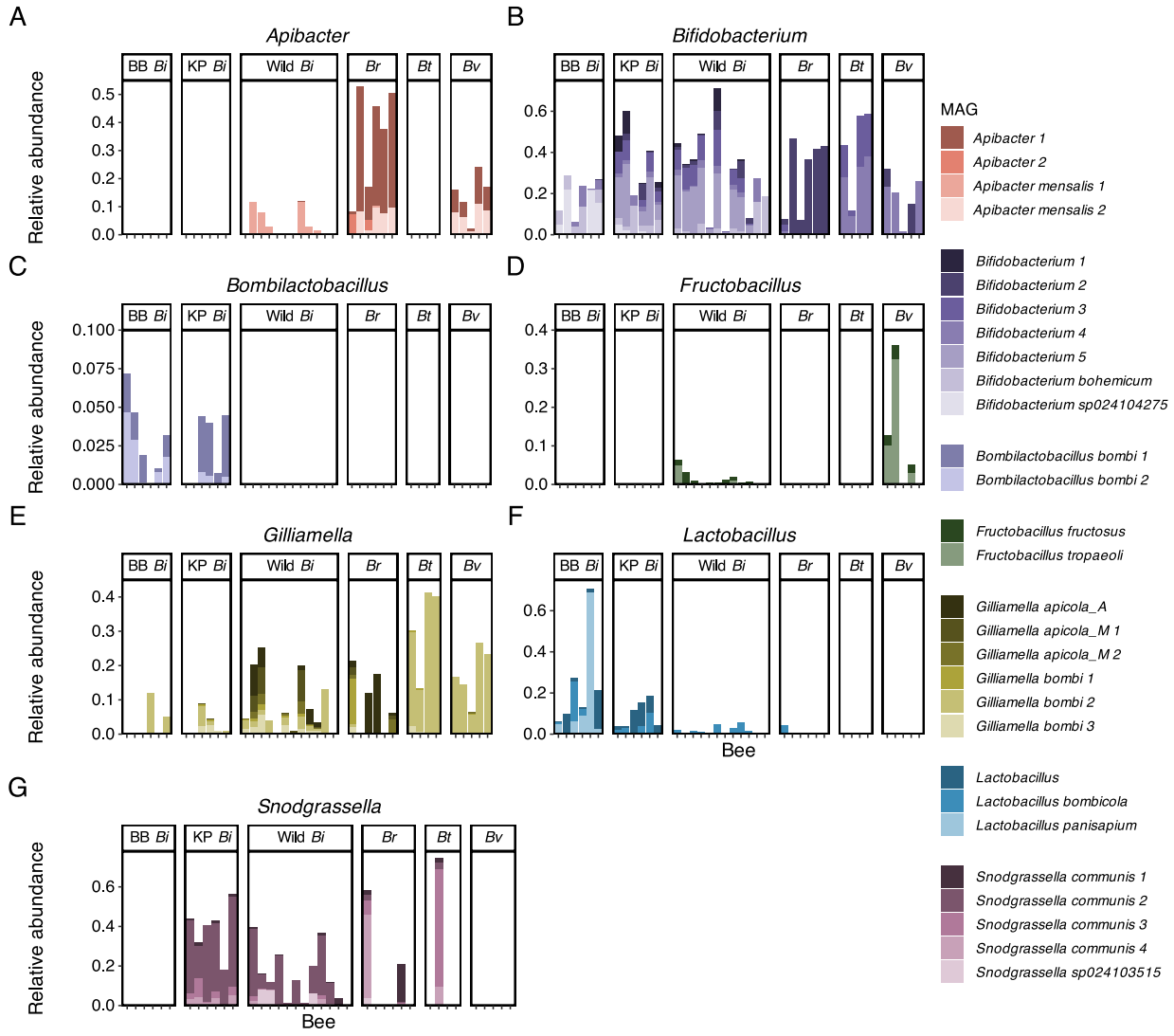


Figure 5-4: Stacked bar plots of relative abundances of representative MAGs in *Bombus* gut microbiotas.

BB *Bi* = Biobest *B. impatiens*, KP *Bi* = Koppert *B. impatiens*, Wild *Bi* = Wild *B. impatiens*, *Br* = *B. rufocinctus, Bt* = *B. ternarius,* and *Bv* = *B. vagans*. As *Arsenophonus*, *Lactobacillaceae CALYQJ01*, and *Schmidhempelia* were all represented by only one MAG, the variation in MAG relative abundance was the same as the variation in phylotype relative abundance (see Supplementary Figure 5-5).

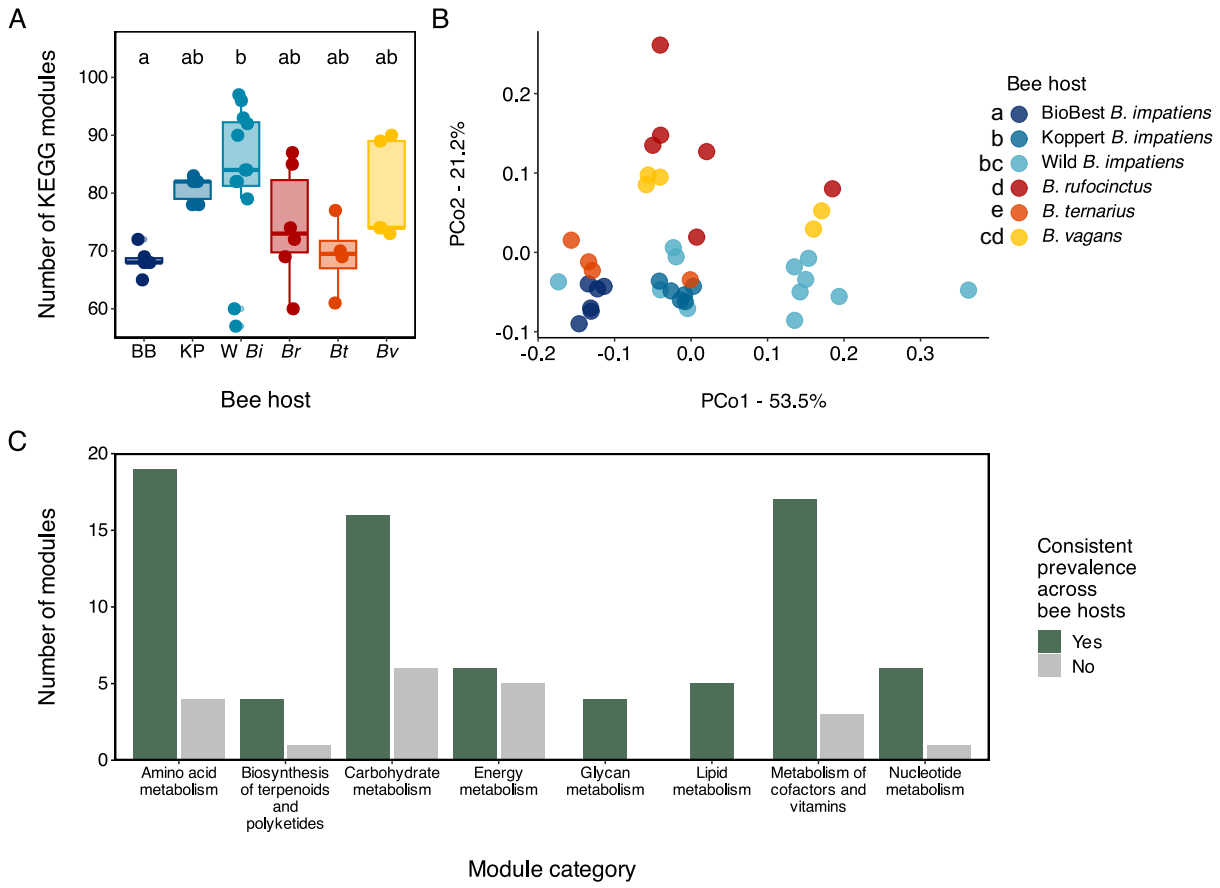


Figure 5-5: Whole community metabolism of bumble bee gut microbiotas.

A) Box plot of the number of KEGG modules expected to be present in bee gut microbiotas by bee host (n = 39; n = 4-12 per bee host). BB = Biobest *B. impatiens*, KP = Koppert *B. impatiens*, W *Bi* = Wild *B. impatiens*, *Br* = *B. rufocinctus, Bt* = *B. ternarius,* and *Bv* = *B. vagans*. Boxes represent medians and interquartile ranges; whiskers extend to 1.5 × the interquartile range. Bee hosts with different letters are significantly different based on pairwise comparisons of estimate marginal means. B) Principal coordinates analysis of KEGG module presence/absence in all bee gut microbiotas using Jaccard distances (n = 39; n = 4-12 per bee host). Points are jittered for visibility. Bee hosts with different letters in the legend have significantly different community structures based on pairwise adonis analysis. C) Bar plot of the number of KEGG modules in all bee gut microbiotas that are and are not differentially enriched in bee hosts.

5.11 Supplementary Tables

Supplementary Table 5-1: Representative metagenome assembled genomes from commercial and wild *Bombus* species.

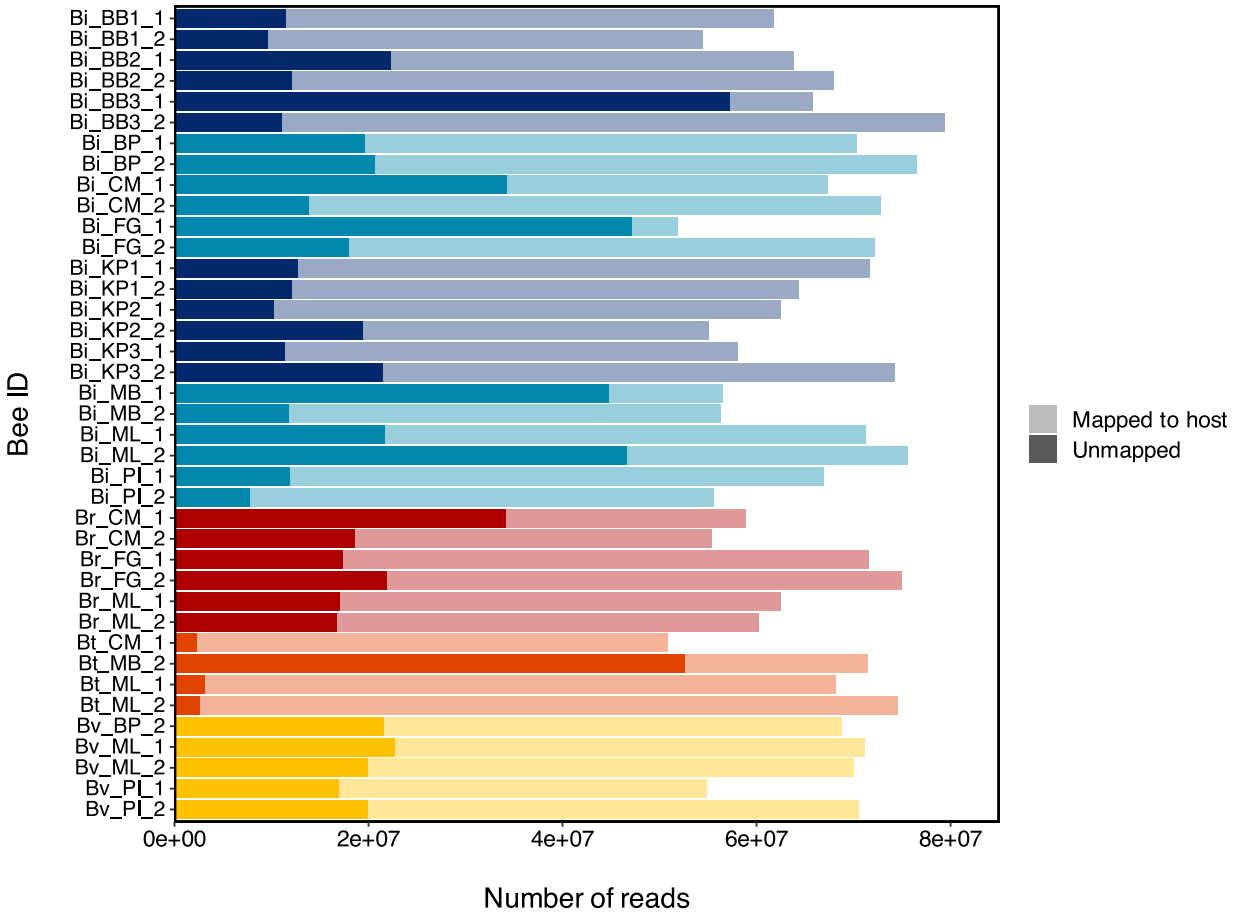
MAG name	Taxonomic ID	Completeness	Contamination
concoct_Bi_BP_1_bin_25_sub.fa	*Fructobacillus tropaeoli*	94.54	7.75
concoct_Bi_FG_1_bin_16_sub.fa	*Fructobacillus fructosus*	89.79	5.34
concoct_Bi_KP3_2_bin_22_sub.fa	*Bombilactobacillus bombi 1*	92.61	3.36
concoct_Bi_ML_1_bin_41.fa	*Arsenophonus apicola*	99.93	0.55
concoct_Br_ML_2_bin_77.fa	*Gilliamella apicola_M 2*	80.61	5.31
maxbin2_Bi_BB3_1_bin.002.fa	*Lactobacillus panisapium*	99.97	4.00
maxbin2_Bi_FG_1_bin.007.fa	*Bifidobacterium 5*	98.73	0.09
maxbin2_Bi_KP1_2_bin.006.fa	*Bifidobacterium 1*	93.23	8.07
maxbin2_Bi_KP2_1_bin.009.fa	*Lactobacillus*	88.65	0.00
maxbin2_Bi_KP3_2_bin.010.fa	*Bifidobacterium bohemicum*	96.53	0.84
maxbin2_Bi_ML_2_bin.006.fa	*Snodgrassella communis 2*	100	0.12
maxbin2_Br_CM_1_bin.004.fa	*Snodgrassella communis 4*	100	0.37
maxbin2_Br_CM_1_bin.008.fa	*Gilliamella bombi 1*	89.27	3.14
maxbin2_Br_CM_1_bin.015.fa	*Apibacter 2*	100	0.06
maxbin2_Br_FG_1_bin.002_sub.fa	*Apibacter 1*	100	1.79
maxbin2_Br_FG_2_bin.004.fa	*Apibacter mensalis 2*	100	0.19
maxbin2_Br_ML_1_bin.004.fa	*Bifidobacterium 2*	99.88	0.34
maxbin2_Br_ML_1_bin.005.fa	*Snodgrassella communis 1*	100	0.31
maxbin2_Bt_MB_2_bin.001.fa	*Bifidobacterium 4*	95.39	0.07
maxbin2_Bt_MB_2_bin.002.fa	*Gilliamella bombi 2*	100	0.46
maxbin2_Bt_MB_2_bin.003.fa	*Snodgrassella communis 3*	100	0.79
maxbin2_Bt_MB_2_bin.004.fa	*Bifidobacterium 3*	99.68	0.15
maxbin2_Bv_ML_2_bin.003.fa	*Schmidhempelia bombi*	99.94	0.01
metabat2_Bi_BB1_1_bin_.6_sub.fa	*Bombilactobacillus bombi 2*	95.36	1.36
metabat2_Bi_BB3_2_bin_.3.fa	*Bifidobacterium sp024104275*	95.63	0.04
metabat2_Bi_FG_1_bin_.11.fa	*Lactobacillaceae* CALYQJ01	83.13	0.02
metabat2_Bi_FG_2_bin_.9.fa	*Gilliamella bombi 3*	98.48	1.89
metabat2_Bi_MB_1_bin_.5.fa	*Gilliamella apicola_M 1*	99.91	0.22
metabat2_Bi_MB_2_bin_.1.fa	*Snodgrassella sp024103515*	88.67	0.04
metabat2_Bi_ML_2_bin_.3.fa	*Apibacter mensalis 1*	99.99	0.04
metabat2_Bi_ML_2_bin_.9.fa	*Lactobacillus bombicola*	99.37	0.18
metabat2_Br_FG_1_bin_.6.fa	*Gilliamella apicola_A*	100	0.01

Supplementary Table 5-2: All metagenome-assembled genomes from commercial and wild *Bombus* species used for metabolic potential analyses.

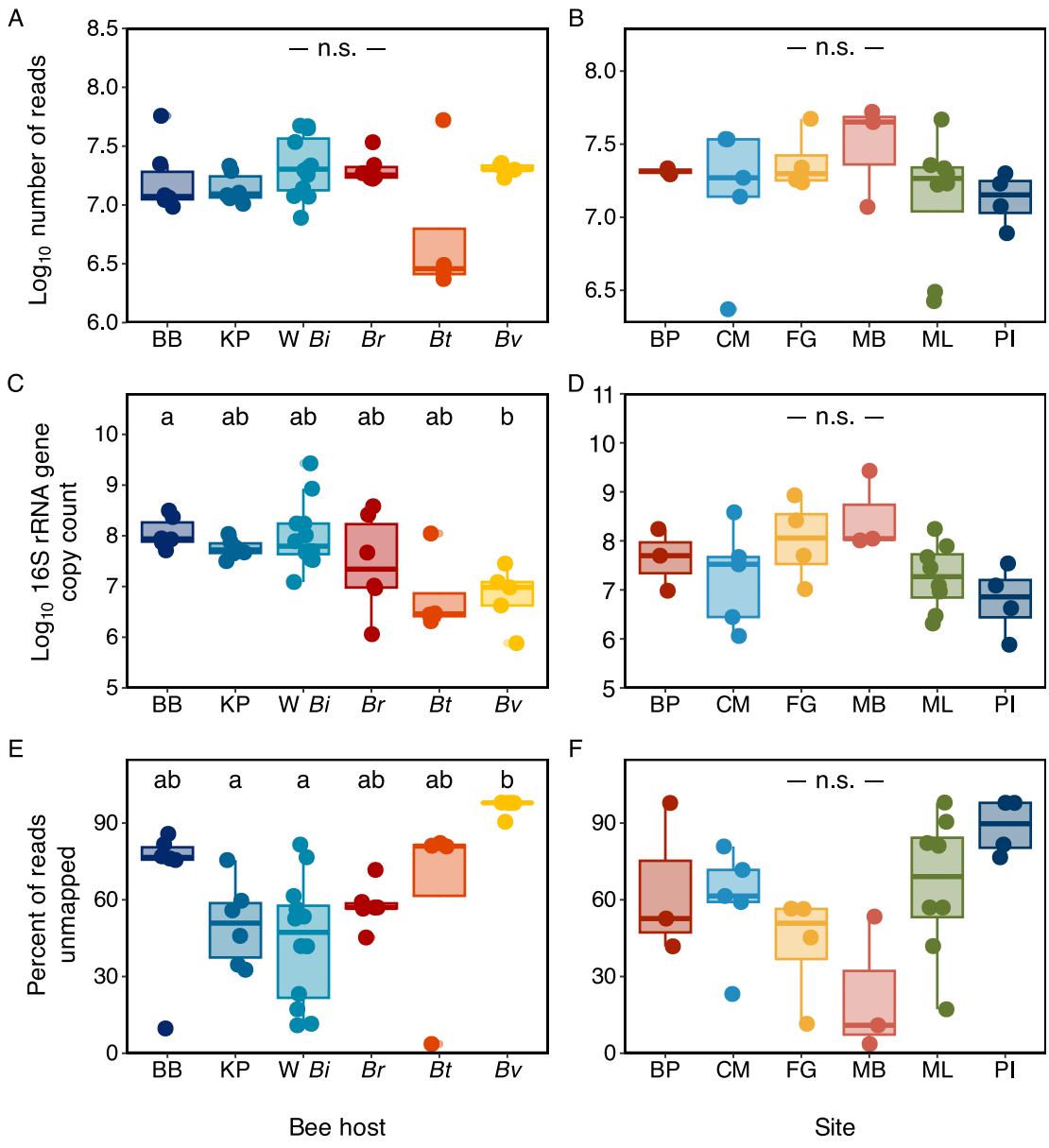
MAG name	MAG cluster ID	Completeness	Contamination
concoct_Bi_BB1_1_bin_47	*Schmidhempelia bombi*	99.94	0.21
concoct_Bi_BB1_1_bin_8_sub	*Lactobacillus panisapium*	89.10	9.25
concoct_Bi_BB1_2_bin_34	*Bifidobacterium sp024104275*	97.17	2.90
concoct_Bi_BB3_1_bin_27_sub	*Bifidobacterium bohemicum*	87.47	1.44
concoct_Bi_BP_1_bin_25_sub	*Fructobacillus tropaeoli*	94.54	7.75
concoct_Bi_BP_2_bin_2_sub	*Snodgrassella communis 2*	87.98	5.31
concoct_Bi_CM_1_bin_37	*Bifidobacterium 5*	86.41	3.07
concoct_Bi_FG_1_bin_1	*Arsenophonus apicola*	81.95	0.37
concoct_Bi_FG_1_bin_16_sub	*Fructobacillus fructosus*	89.79	5.34
concoct_Bi_KP2_1_bin_9	*Bifidobacterium bohemicum*	90.31	0.24
concoct_Bi_KP3_2_bin_22_sub	*Bombilactobacillus bombi 1*	92.61	3.36
concoct_Bi_MB_2_bin_57	*Arsenophonus apicola*	85.31	0.46
concoct_Bi_ML_1_bin_41	*Arsenophonus apicola*	99.93	0.55
concoct_Bi_PI_1_bin_38	*Schmidhempelia bombi*	81.39	0.02
concoct_Br_ML_2_bin_77	*Gilliamella apicola_M 2*	80.61	5.31
concoct_Bv_ML_2_bin_24	*Gilliamella bombi 2*	89.63	6.73
maxbin2_Bi_BB1_2_bin.004_sub	*Schmidhempelia bombi*	99.50	0.04
maxbin2_Bi_BB1_2_bin.005_sub	*Lactobacillus*	88.57	0.39
maxbin2_Bi_BB1_2_bin.006	*Bombilactobacillus bombi 2*	85.40	3.65
maxbin2_Bi_BB3_1_bin.002	*Lactobacillus panisapium*	99.97	4.00
maxbin2_Bi_BB3_1_bin.003	*Bifidobacterium sp024104275*	95.68	0.05
maxbin2_Bi_BB3_2_bin.003	*Schmidhempelia bombi*	99.94	2.17
maxbin2_Bi_BP_1_bin.004	*Snodgrassella communis 2*	100	0.65
maxbin2_Bi_BP_1_bin.007	*Bifidobacterium 5*	95.22	3.26
maxbin2_Bi_BP_1_bin.009	*Bifidobacterium 2*	96.64	0.16
maxbin2_Bi_BP_1_bin.011_sub	*Schmidhempelia bombi*	96.18	0.03
maxbin2_Bi_BP_2_bin.010_sub	*Snodgrassella sp024103515*	88.24	3.70
maxbin2_Bi_BP_2_bin.017_sub	*Bifidobacterium 2*	81.28	5.97
maxbin2_Bi_CM_1_bin.013_sub	*Bifidobacterium 2*	84.38	1.87
maxbin2_Bi_CM_1_bin.015_sub	*Schmidhempelia bombi*	99.08	3.16
maxbin2_Bi_CM_1_bin.016_sub	*Snodgrassella communis 2*	89.45	0.19
maxbin2_Bi_CM_1_bin.017	*Lactobacillus bombicola*	99.97	4.14
maxbin2_Bi_CM_2_bin.005_sub	*Arsenophonus apicola*	98.51	2.67
maxbin2_Bi_FG_1_bin.006_sub	*Bifidobacterium 2*	98.70	2.74
maxbin2_Bi_FG_1_bin.007	*Bifidobacterium 5*	98.73	0.09
maxbin2_Bi_FG_2_bin.002	*Snodgrassella communis 2*	89.49	6.72
maxbin2_Bi_KP1_1_bin.005	*Snodgrassella communis 2*	100	2.98
maxbin2_Bi_KP1_2_bin.004	*Bifidobacterium 2*	96.19	3.04
maxbin2_Bi_KP1_2_bin.006	*Bifidobacterium 1*	93.23	8.07
maxbin2_Bi_KP2_1_bin.008	*Schmidhempelia bombi*	99.84	2.45
maxbin2_Bi_KP2_1_bin.009	*Lactobacillus*	88.65	0.00
maxbin2_Bi_KP2_1_bin.010_sub	*Snodgrassella communis 2*	100	0.16
maxbin2_Bi_KP3_2_bin.003	*Snodgrassella communis 2*	100	3.12
maxbin2_Bi_KP3_2_bin.009	*Schmidhempelia bombi*	99.99	0.85
maxbin2_Bi_KP3_2_bin.010	*Bifidobacterium bohemicum*	96.53	0.84
maxbin2_Bi_MB_2_bin.006_sub	*Gilliamella apicola_M 1*	89.27	3.74
maxbin2_Bi_ML_1_bin.014_sub	*Apibacter mensalis 1*	100	2.16
maxbin2_Bi_ML_2_bin.006	*Snodgrassella communis 2*	100	0.12
maxbin2_Bi_ML_2_bin.007	*Bifidobacterium 2*	97.7	0.28
maxbin2_Br_CM_1_bin.004	*Snodgrassella communis 4*	100	0.37
maxbin2_Br_CM_1_bin.008	*Gilliamella bombi 1*	89.27	3.14

maxbin2_Br_CM_1_bin.015	*Apibacter 2*	100	0.06
maxbin2_Br_CM_1_bin.017_sub	*Snodgrassella sp024103515*	86.61	5.62
maxbin2_Br_FG_1_bin.002_sub	*Apibacter 1*	100	1.79
maxbin2_Br_FG_1_bin.006_sub	*Arsenophonus apicola*	92.52	0.16
maxbin2_Br_FG_2_bin.003	*Gilliamella apicola_A*	94.45	0.02
maxbin2_Br_FG_2_bin.004	*Apibacter mensalis 2*	100	0.19
maxbin2_Br_ML_1_bin.004	*Bifidobacterium 2*	99.88	0.34
maxbin2_Br_ML_1_bin.005	*Snodgrassella communis 1*	100	0.31
maxbin2_Bt_MB_2_bin.001	*Bifidobacterium 4*	95.39	0.07
maxbin2_Bt_MB_2_bin.002	*Gilliamella bombi 2*	100	0.46
maxbin2_Bt_MB_2_bin.003	*Snodgrassella communis 3*	100	0.79
maxbin2_Bt_MB_2_bin.004	*Bifidobacterium 3*	99.68	0.15
maxbin2_Bv_ML_2_bin.003	*Schmidhempelia bombi*	99.94	0.01
metabat2_Bi_BB1_1_bin_.6_sub	*Bombilactobacillus bombi 2*	95.36	1.36
metabat2_Bi_BB3_2_bin_.3	*Bifidobacterium sp024104275*	95.63	0.04
metabat2_Bi_BB3_2_bin_.5_sub	*Lactobacillus*	80.68	0.18
metabat2_Bi_BP_1_bin_.18	*Gilliamella bombi 3*	99.98	3.24
metabat2_Bi_BP_1_bin_.6	*Lactobacillus bombicola*	84.48	0.11
metabat2_Bi_BP_2_bin_.2	*Lactobacillaceae CALYQJ01*	82.94	0.03
metabat2_Bi_BP_2_bin_.7	*Lactobacillus bombicola*	86.53	3.32
metabat2_Bi_CM_2_bin_.4	*Schmidhempelia bombi*	84.15	0.02
metabat2_Bi_CM_2_bin_.6	*Snodgrassella communis 2*	80.65	0.05
metabat2_Bi_FG_1_bin_.11	*Lactobacillaceae CALYQJ01*	83.13	0.02
metabat2_Bi_FG_1_bin_.8	*Snodgrassella communis 2*	88.26	0.18
metabat2_Bi_FG_2_bin_.11	*Lactobacillus bombicola*	84.02	0.05
metabat2_Bi_FG_2_bin_.14	*Bifidobacterium 2*	95.62	0.02
metabat2_Bi_FG_2_bin_.9	*Gilliamella bombi 3*	98.48	1.89
metabat2_Bi_KP1_1_bin_.5	*Lactobacillus bombicola*	93.40	0.02
metabat2_Bi_KP1_2_bin_.3	*Snodgrassella communis 3*	85.52	1.43
metabat2_Bi_KP1_2_bin_.4	*Bifidobacterium sp024104275*	88.03	0.08
metabat2_Bi_KP1_2_bin_.6	*Schmidhempelia bombi*	86.86	0.01
metabat2_Bi_KP1_2_bin_.7	*Lactobacillus bombicola*	92.26	0.09
metabat2_Bi_KP2_2_bin_.15	*Bifidobacterium 2*	93.02	0.19
metabat2_Bi_KP3_2_bin_.19	*Bifidobacterium 2*	92.93	0.19
metabat2_Bi_MB_1_bin_.2	*Apibacter mensalis 1*	95.35	2.14
metabat2_Bi_MB_1_bin_.4	*Snodgrassella sp024103515*	84.57	4.12
metabat2_Bi_MB_1_bin_.5	*Gilliamella apicola_M 1*	99.91	0.22
metabat2_Bi_MB_2_bin_.1	*Snodgrassella sp024103515*	88.67	0.04
metabat2_Bi_MB_2_bin_.11	*Apibacter mensalis 1*	99.99	0.04
metabat2_Bi_MB_2_bin_.2	*Schmidhempelia bombi*	99.66	0.12
metabat2_Bi_MB_2_bin_.3	*Lactobacillus bombicola*	96.09	0.97
metabat2_Bi_MB_2_bin_.4	*Bifidobacterium 2*	81.59	0.06
metabat2_Bi_ML_1_bin_.10	*Gilliamella apicola_A*	99.96	1.61
metabat2_Bi_ML_1_bin_.13	*Bifidobacterium 2*	96.36	0.14
metabat2_Bi_ML_1_bin_.3	*Lactobacillus bombicola*	96.40	0.03
metabat2_Bi_ML_2_bin_.12_sub	*Gilliamella apicola_A*	80.29	0.91
metabat2_Bi_ML_2_bin_.3	*Apibacter mensalis 1*	99.99	0.04
metabat2_Bi_ML_2_bin_.9	*Lactobacillus bombicola*	99.37	0.18
metabat2_Bi_PI_2_bin_.1	*Schmidhempelia bombi*	81.41	0.02
metabat2_Br_CM_1_bin_.1	*Bifidobacterium 2*	95.37	0.13
metabat2_Br_FG_1_bin_.6	*Gilliamella apicola_A*	100	0.01
metabat2_Bt_CM_1_bin_.3	*Schmidhempelia bombi*	99.22	0.61

5.12 Supplementary Figures



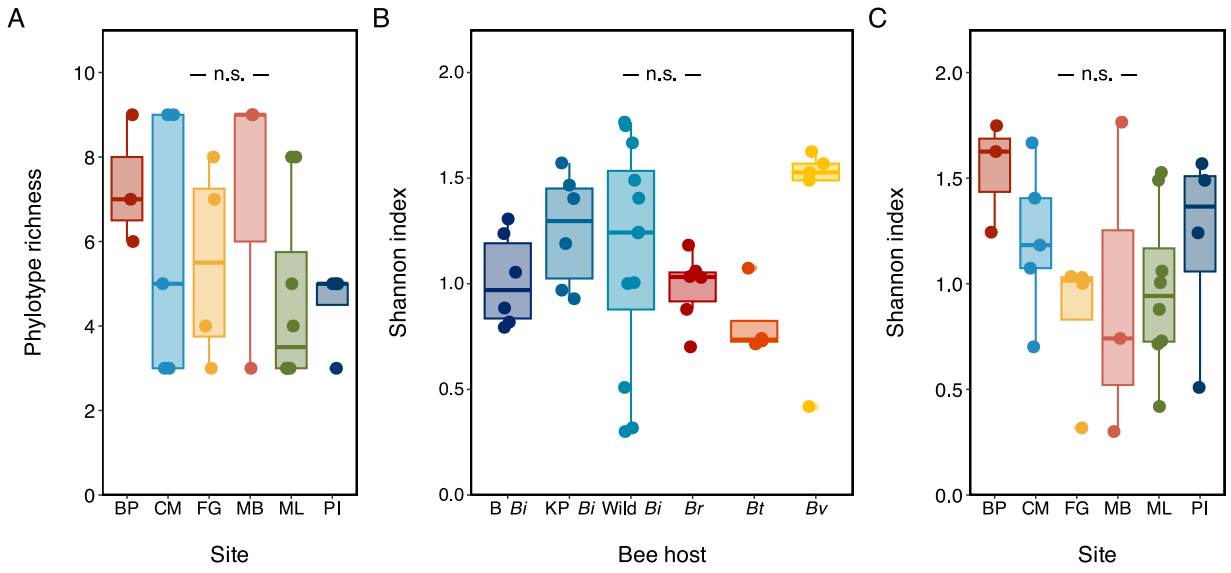
Supplementary Figure 5-1: Number of reads in *Bombus* gut microbiotas that did and did not map to host and host-related genomes.



Supplementary Figure 5-2: Number of reads post-host filtering, 16S rRNA gene copy counts, and percent of reads not mapped to representative MAGs for *Bombus* gut microbiotas.

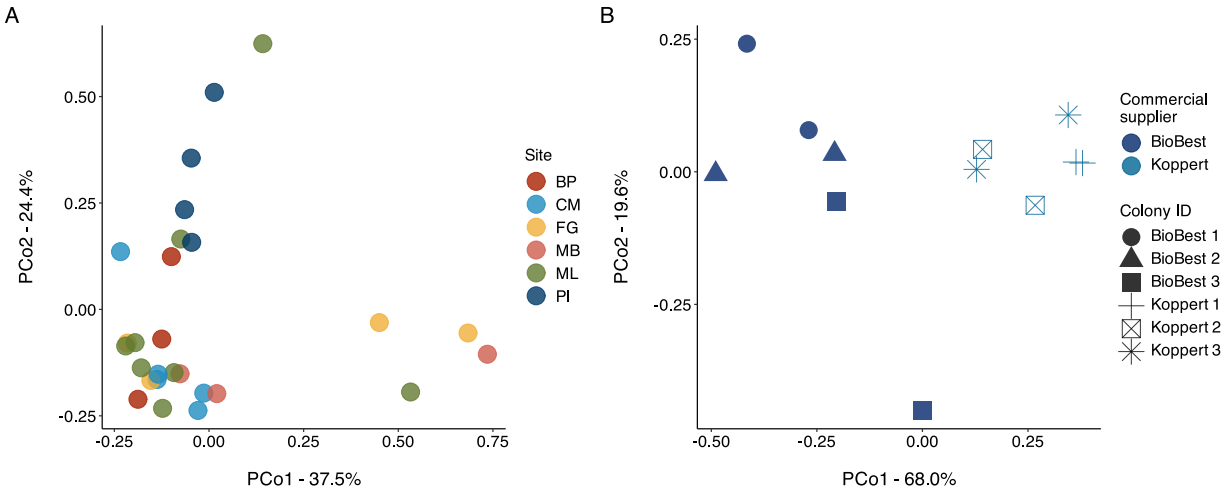
A,B) Number of reads after filtering out host reads for A) all bee gut microbiotas by host, and B) wild bee gut microbiotas by site. C,D) Total 16S rRNA gene copy counts in C) all *Bombus* gut microbiotas by bee host, and D) wild bee gut microbiotas by site. E,F) Percent of reads that did not map to any representative MAGs during taxonomic profiling for E) all bee gut microbiotas by host, and F) wild bee gut microbiotas by site. For A,C,E) BB = Biobest *B. impatiens*, KP = Koppert *B. impatiens*, W *Bi* = Wild *B. impatiens*, *Br*

= *B. rufocinctus, Bt* = *B. ternarius,* and *Bv* = *B. vagans* (n = 39; n = 4–12 per host). For B,D,F) BP = Bruce Pit, CM = Chapman Mills Conservation Area, FG = Fletcher Wildlife Garden, MB = Mer Bleue Bog, ML = Mud Lake, PI = Petrie Island (n = 27; n = 3–8 per site). Boxes represent medians and interquartile ranges; whiskers extend to 1.5 × the interquartile range. Hosts with different letters are significantly different based on Dunn's test. n.s. = non-significant.



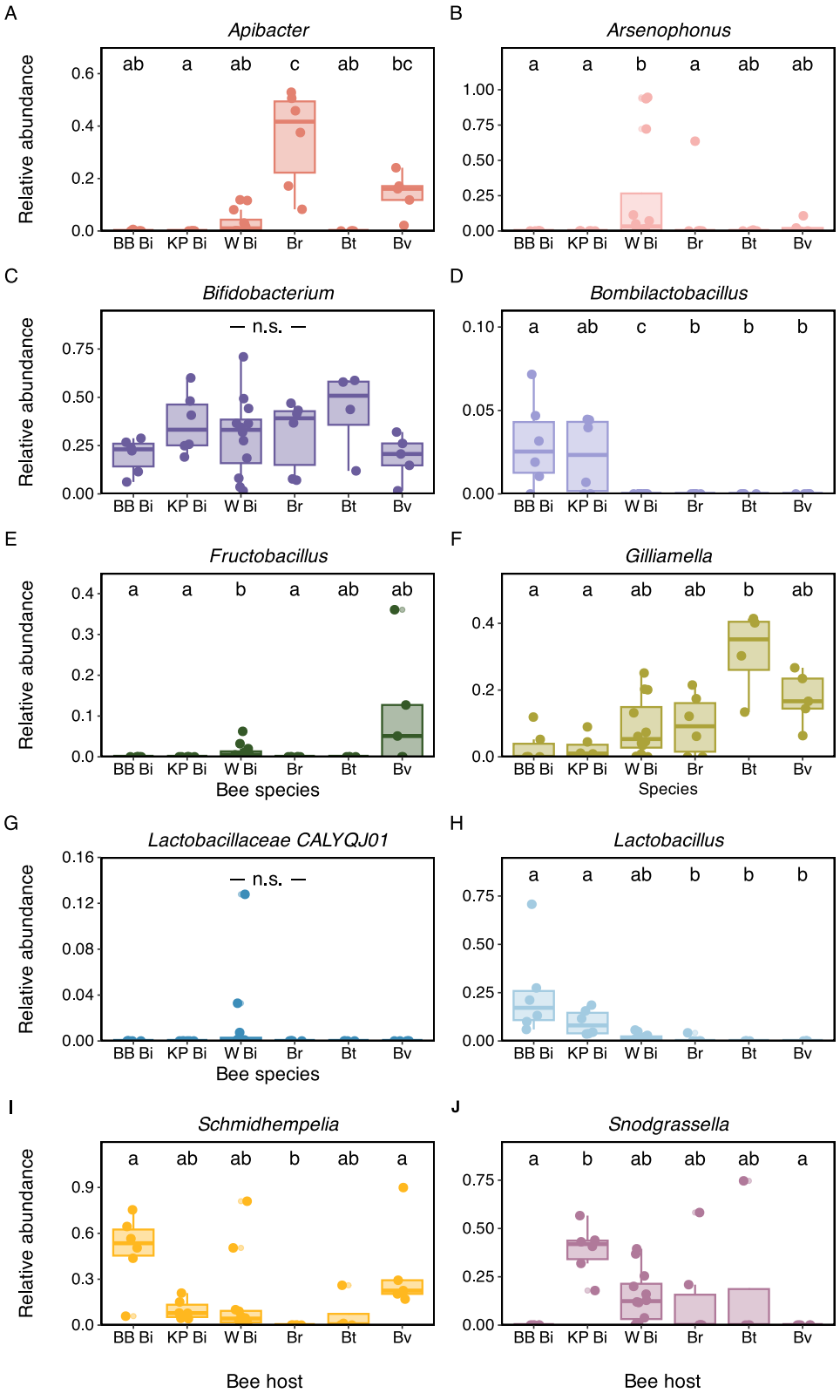
Supplementary Figure 5-3: Box plots of phylotype richness and Shannon diversity in *Bombus* gut microbiotas.

A) Box plot of phylotype richness by site in wild bee gut microbiotas (n=27; n=3-8 per site). B) Box plot of Shannon index by bee host for all bee gut microbiotas (n=39; n=4-12 per host). C) Box plot of Shannon index by site in wild bee gut microbiotas (n=27; n=3-8 per site). Boxes represent medians and interquartile ranges; whiskers extend to 1.5 × the interquartile range. BB *Bi* = Biobest *B. impatiens*, K *Bi* = Koppert *B. impatiens*, Wild *Bi* = Wild *B. impatiens*, *Br* = *B. rufocinctus, Bt* = *B. ternarius,* and *Bv* = *B. vagans*. n.s. = non-significant. BP = Bruce Pit, CM = Chapman Mills Conservation Area, FG = Fletcher Wildlife Garden, MB = Mer Bleue Bog, ML = Mud Lake, PI = Petrie Island. n.s. = non-significant.



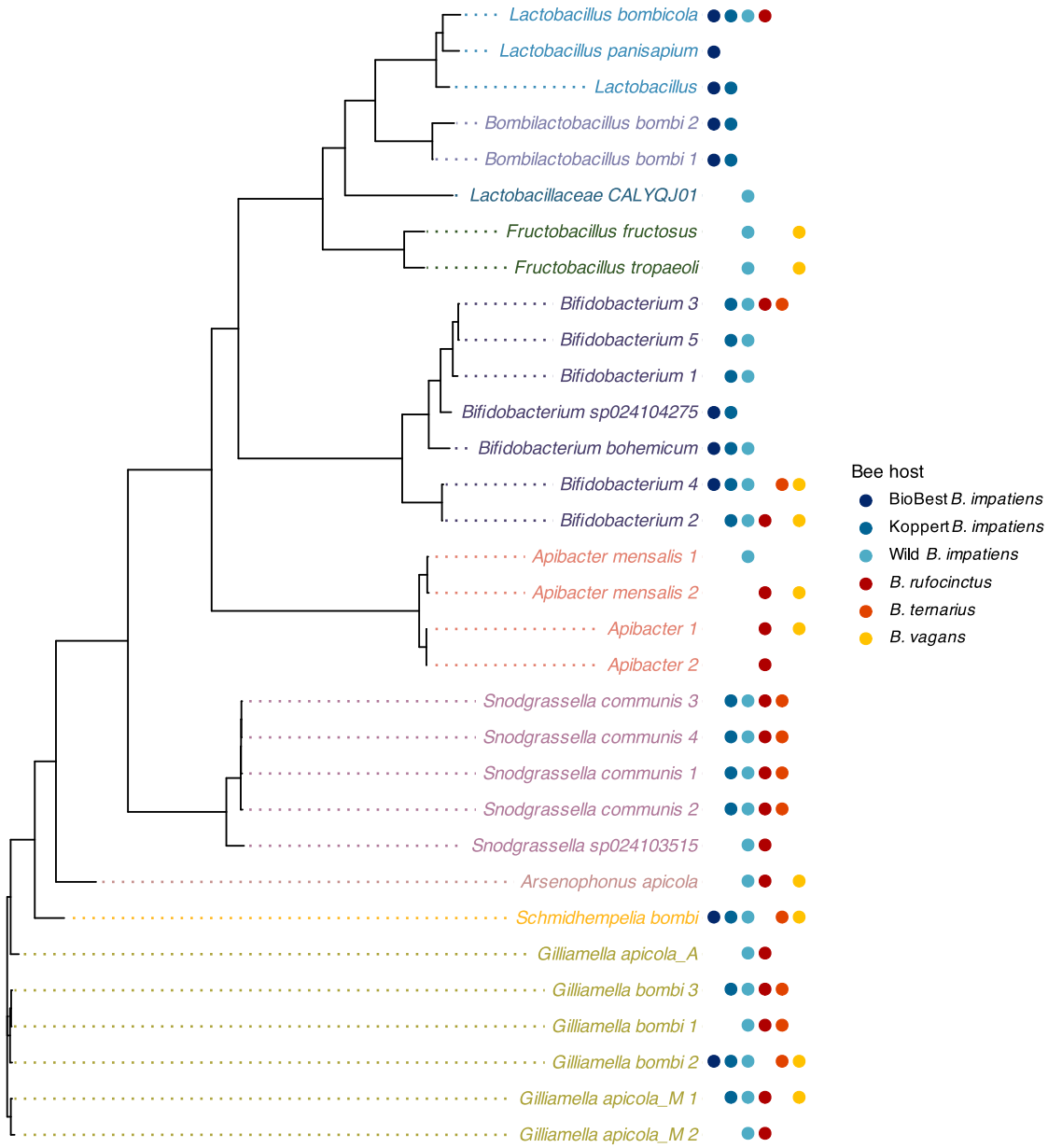
Supplementary Figure 5-4: Principal coordinates analyses of phylotype community structure in wild and commercial bee gut microbiotas.

A) Principal coordinates analysis of phylotype community structure in wild bee gut microbiotas using Bray-Curtis dissimilarities (n = 27; n = 3-8 per site). B) Principal coordinates analysis of phylotype community structure in commercial bee gut microbiotas using Bray-Curtis dissimilarities (n = 12; n = 6 per supplier; n = 2 per colony ID).


A
Apibacter
ab a ab c ab bc
B
Arsenophonus
a a b a ab ab
C
Bifidobacterium
— n.s. —
D
Bombilactobacillus
a ab c b b b
E
Fructobacillus
a a b a ab ab
F
Gilliamella
a a ab ab b ab
G
Lactobacillaceae CALYQJ01
— n.s. —
H
Lactobacillus
a a ab b b b
I
Schmidhempelia
a ab ab b ab a
J
Snodgrassella
a b ab ab ab a
Relative abundance
BB Bi KP Bi W Bi Br Bt Bv
Bee species
Species
Bee host

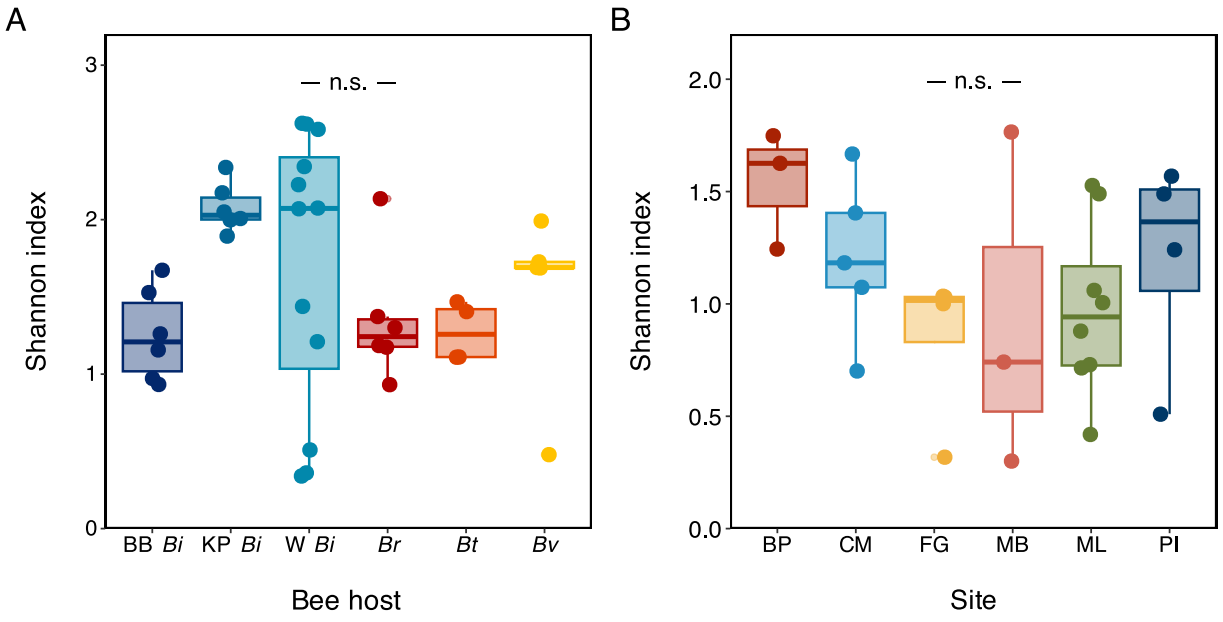
Supplementary Figure 5-5: Box plots of phylotype relative abundance across bee hosts.

Boxes represent medians and interquartile ranges; whiskers extend to 1.5 × the interquartile range. BB *Bi* = Biobest *B. impatiens*, KP *Bi* = Koppert *B. impatiens*, W *Bi* = Wild *B. impatiens*, *Br* = *B. rufocinctus, Bt* = *B. ternarius,* and *Bv* = *B. vagans*. Bee hosts with different letters are significantly different based on pairwise comparisons with Dunn's test. For each plot, n = 39; n = 4-12 per bee host.



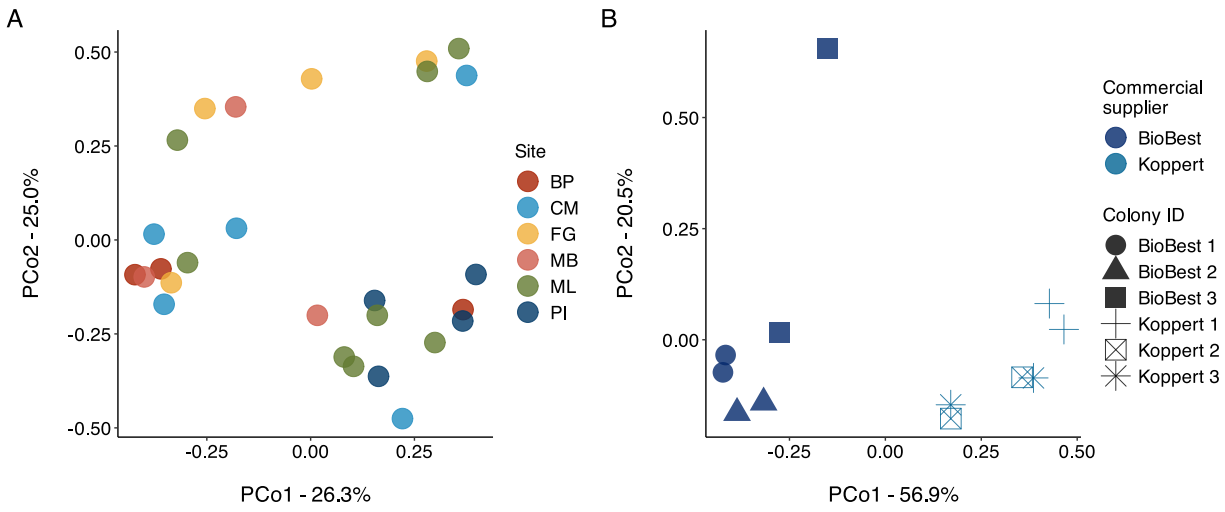
Supplementary Figure 5-6: Unrooted phylogenetic tree of representative MAGs in bee gut microbiotas.

Branches are labelled with representative MAG IDs, and MAG IDs are coloured by phylotype. Beside each MAG ID are dots coloured by the bee hosts in which each representative MAG was observed.



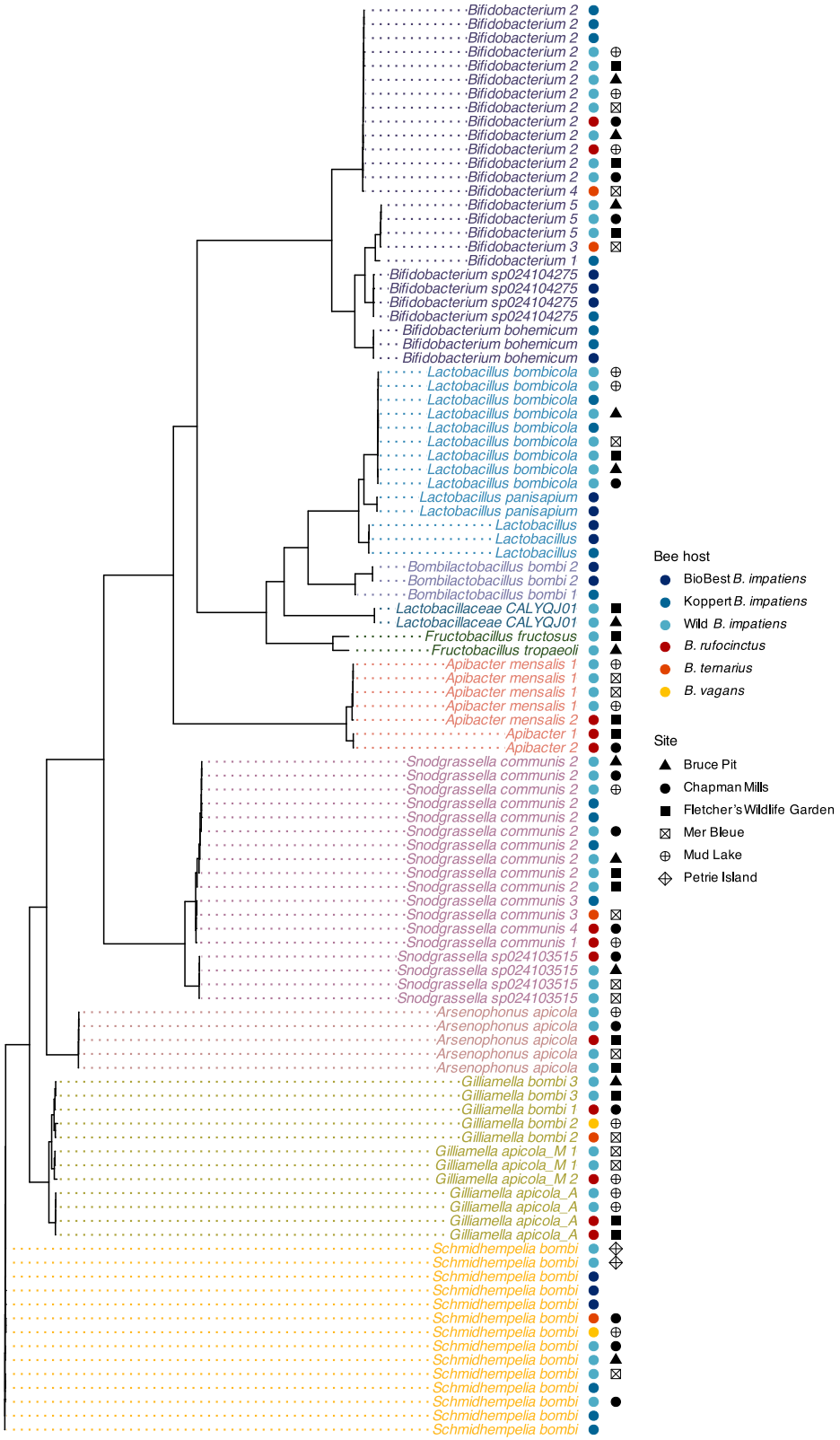
Supplementary Figure 5-7: Box plots of MAG-level Shannon diversity in *Bombus* gut microbiotas.

A) Box plot of Shannon index by bee host for all bee gut microbiotas (n=39; n=4-12 per host). B) Box plot of Shannon index by site in wild bee gut microbiotas (n=27; n=3-8 per site). Boxes represent medians and interquartile ranges; whiskers extend to 1.5 × the interquartile range. B *Bi* = Biobest *B. impatiens*, K *Bi* = Koppert *B. impatiens*, W *Bi* = Wild *B. impatiens*, *Br* = *B. rufocinctus, Bt* = *B. ternarius,* and *Bv* = *B. vagans*. BP = Bruce Pit, CM = Chapman Mills Conservation Area, FG = Fletcher Wildlife Garden, MB = Mer Bleue Bog, ML = Mud Lake, PI = Petrie Island. n.s. = non-significant.



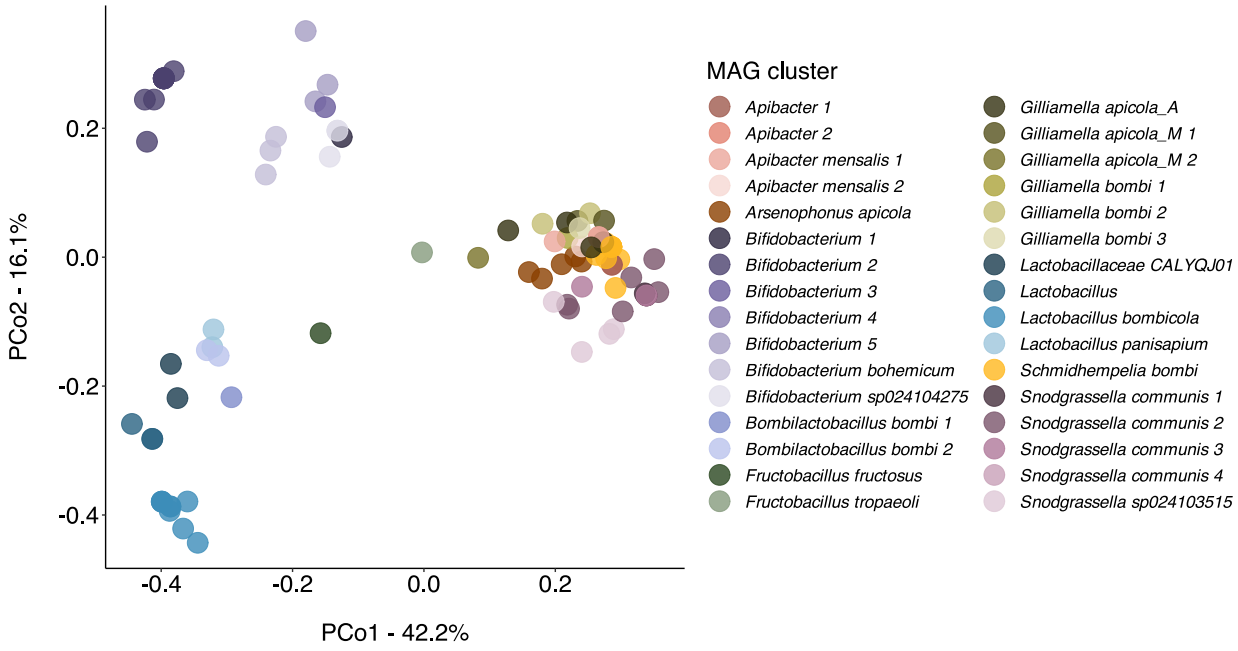
Supplementary Figure 5-8: Principal coordinates analyses of MAG-level community structure in wild and commercial bee gut microbiotas.

A) Principal coordinates analysis of MAG community structure in wild bee gut microbiotas using Bray-Curtis dissimilarities (n = 27; n = 3-8 per site). B) Principal coordinates analysis of MAG community structure in commercial bee gut microbiotas using Bray-Curtis dissimilarities (n = 12; n = 6 per supplier; n = 2 per colony ID).


Bifidobacterium 2
Bifidobacterium 2
Bifidobacterium 2
Bifidobacterium 2
Bifidobacterium 2
Bifidobacterium 2
Bifidobacterium 2
Bifidobacterium 2
Bifidobacterium 2
Bifidobacterium 2
Bifidobacterium 2
Bifidobacterium 2
Bifidobacterium 2
Bifidobacterium 2
Bifidobacterium 4
Bifidobacterium 5
Bifidobacterium 5
Bifidobacterium 5
Bifidobacterium 3
Bifidobacterium 1
Bifidobacterium sp024104275
Bifidobacterium sp024104275
Bifidobacterium sp024104275
Bifidobacterium sp024104275
Bifidobacterium bohemicum
Bifidobacterium bohemicum
Bifidobacterium bohemicum
Lactobacillus bombicola
Lactobacillus bombicola
Lactobacillus bombicola
Lactobacillus bombicola
Lactobacillus bombicola
Lactobacillus bombicola
Lactobacillus bombicola
Lactobacillus bombicola
Lactobacillus bombicola
Lactobacillus panisapium
Lactobacillus panisapium
Lactobacillus
Lactobacillus
Lactobacillus
Bombilactobacillus bombi 2
Bombilactobacillus bombi 2
Bombilactobacillus bombi 1
Lactobacillaceae CALYQJ01
Lactobacillaceae CALYQJ01
Fructobacillus fructosus
Fructobacillus tropaeoli
Apibacter mensalis 1
Apibacter mensalis 1
Apibacter mensalis 1
Apibacter mensalis 1
Apibacter mensalis 2
Apibacter 1
Apibacter 2
Snodgrassella communis 2
Snodgrassella communis 2
Snodgrassella communis 2
Snodgrassella communis 2
Snodgrassella communis 2
Snodgrassella communis 2
Snodgrassella communis 2
Snodgrassella communis 2
Snodgrassella communis 2
Snodgrassella communis 2
Snodgrassella communis 2
Snodgrassella communis 3
Snodgrassella communis 3
Snodgrassella communis 4
Snodgrassella communis 1
Snodgrassella sp024103515
Snodgrassella sp024103515
Snodgrassella sp024103515
Snodgrassella sp024103515
Arsenophonus apicola
Arsenophonus apicola
Arsenophonus apicola
Arsenophonus apicola
Arsenophonus apicola
Gilliamella bombi 3
Gilliamella bombi 3
Gilliamella bombi 1
Gilliamella bombi 2
Gilliamella bombi 2
Gilliamella apicola_M 1
Gilliamella apicola_M 1
Gilliamella apicola_M 2
Gilliamella apicola_A
Gilliamella apicola_A
Gilliamella apicola_A
Gilliamella apicola_A
Schmidhempelia bombi
Schmidhempelia bombi
Schmidhempelia bombi
Schmidhempelia bombi
Schmidhempelia bombi
Schmidhempelia bombi
Schmidhempelia bombi
Schmidhempelia bombi
Schmidhempelia bombi
Schmidhempelia bombi
Schmidhempelia bombi
Schmidhempelia bombi
Schmidhempelia bombi
Schmidhempelia bombi
Bee host
BioBest B. impatiens
Koppert B. impatiens
Wild B. impatiens
B. rufocinctus
B. ternarius
B. vagans
Site
Bruce Pit
Chapman Mills
Fletcher's Wildlife Garden
Mer Bleue
Mud Lake
Petrie Island

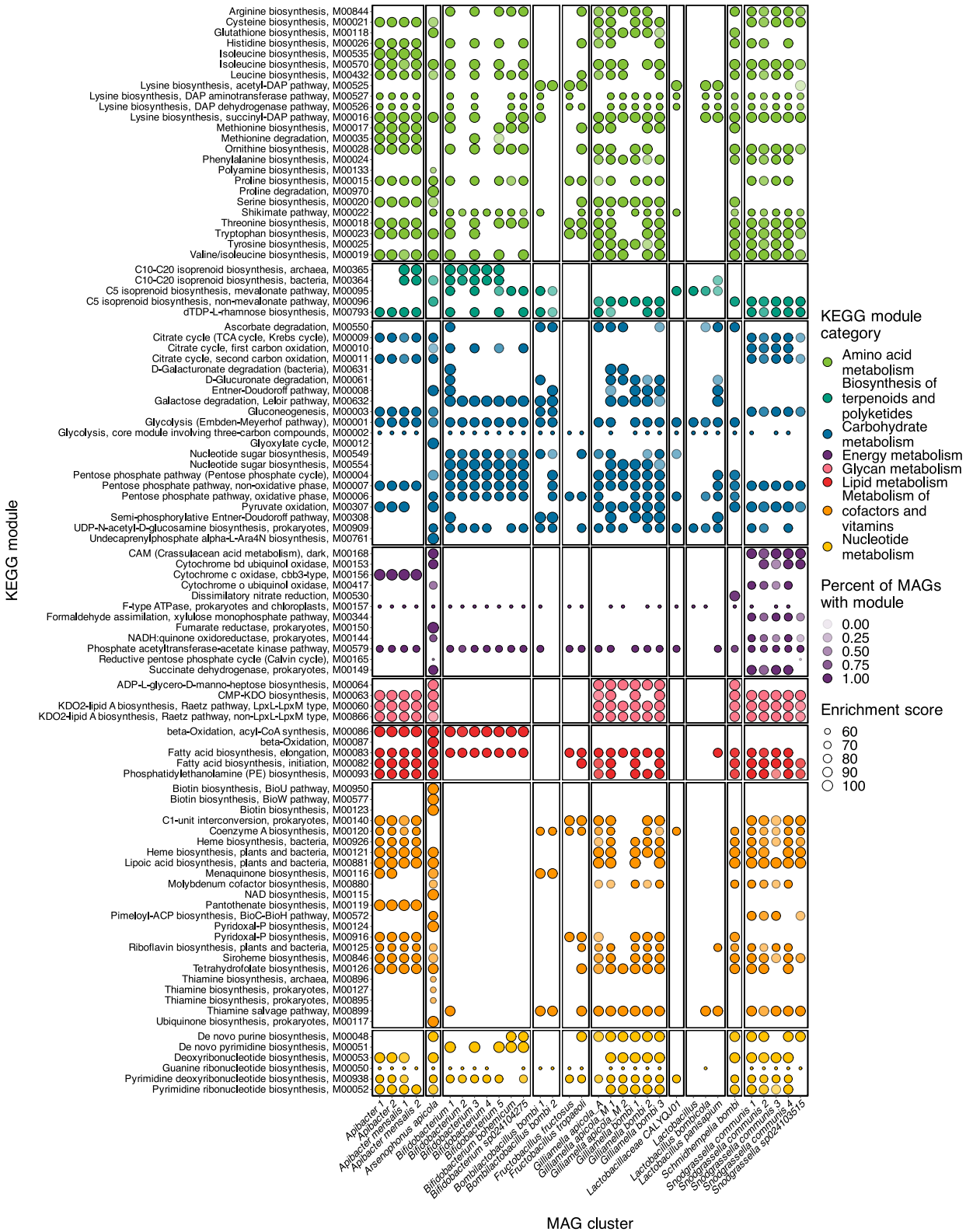
Supplementary Figure 5-9: Unrooted phylogenetic tree of all high-quality MAGs in bee gut microbiotas.

MAGs are labelled and coloured by MAG cluster. Points to the right of each MAG represent the bee host and site from which the MAG was generated.



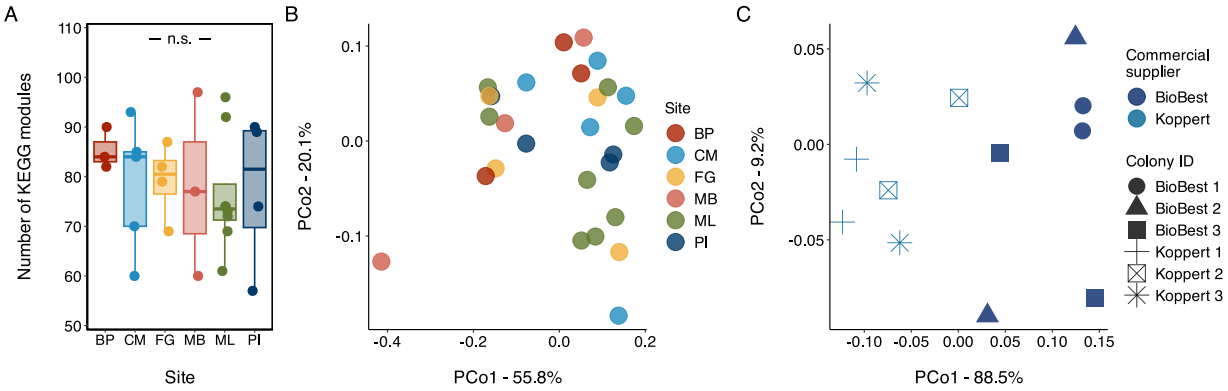
Supplementary Figure 5-10: Principal coordinates analysis of KEGG module presence/absence in MAG clusters.

Principal coordinates analysis of KEGG module presence/absence in MAG clusters from *Bombus* gut microbiotas using Jaccard distances (n = 103; n = 1-14 per cluster).


KEGG module
Arginine biosynthesis, M00844
Cysteine biosynthesis, M00021
Glutathione biosynthesis, M00118
Histidine biosynthesis, M00026
Isoleucine biosynthesis, M00535
Isoleucine biosynthesis, M00570
Leucine biosynthesis, M00432
Lysine biosynthesis, acetyl-DAP pathway, M00525
Lysine biosynthesis, DAP aminotransferase pathway, M00527
Lysine biosynthesis, DAP dehydrogenase pathway, M00526
Lysine biosynthesis, succinyl-DAP pathway, M00016
Methionine biosynthesis, M00017
Methionine degradation, M00035
Ornithine biosynthesis, M00028
Phenylalanine biosynthesis, M00024
Polyamine biosynthesis, M00133
Proline biosynthesis, M00015
Proline degradation, M00970
Serine biosynthesis, M00020
Shikimate pathway, M00022
Threonine biosynthesis, M00018
Tryptophan biosynthesis, M00023
Tyrosine biosynthesis, M00025
Valine/isoleucine biosynthesis, M00019
C10-C20 isoprenoid biosynthesis, archaea, M00365
C10-C20 isoprenoid biosynthesis, bacteria, M00364
C5 isoprenoid biosynthesis, mevalonate pathway, M00095
C5 isoprenoid biosynthesis, non-mevalonate pathway, M00096
dTDP-L-rhamnose biosynthesis, M00793
Ascorbate degradation, M00550
Citrate cycle (TCA cycle, Krebs cycle), M00009
Citrate cycle, first carbon oxidation, M00010
Citrate cycle, second carbon oxidation, M00011
D-Galacturonate degradation (bacteria), M00631
D-Glucuronate degradation, M00061
Entner-Doudoroff pathway, M00008
Galactose degradation, Leloir pathway, M00632
Gluconeogenesis, M00003
Glycolysis (Embden-Meyerhof pathway), M00001
Glycolysis, core module involving three-carbon compounds, M00002
Glyoxylate cycle, M00012
Nucleotide sugar biosynthesis, M00549
Nucleotide sugar biosynthesis, M00554
Pentose phosphate pathway (Pentose phosphate cycle), M00004
Pentose phosphate pathway, non-oxidative phase, M00007
Pentose phosphate pathway, oxidative phase, M00006
Pyruvate oxidation, M00307
Semi-phosphorylative Entner-Doudoroff pathway, M00308
UDP-N-acetyl-D-glucosamine biosynthesis, prokaryotes, M00909
Undecaprenylphosphate alpha-L-Ara4N biosynthesis, M00761
CAM (Crassulacean acid metabolism), dark, M00168
Cytochrome bd ubiquinol oxidase, M00153
Cytochrome c oxidase, cbb3-type, M00156
Cytochrome o ubiquinol oxidase, M00417
Dissimilatory nitrate reduction, M00530
F-type ATPase, prokaryotes and chloroplasts, M00157
Formaldehyde assimilation, xylulose monophosphate pathway, M00344
Fumarate reductase, prokaryotes, M00150
NADH:quinone oxidoreductase, prokaryotes, M00144
Phosphate acetyltransferase-acetate kinase pathway, M00579
Reductive pentose phosphate cycle (Calvin cycle), M00165
Succinate dehydrogenase, prokaryotes, M00149
ADP-L-glycero-D-manno-heptose biosynthesis, M00064
CMP-KDO biosynthesis, M00063
KDO2-lipid A biosynthesis, Raetz pathway, LpxL-LpxM type, M00060
KDO2-lipid A biosynthesis, Raetz pathway, non-LpxL-LpxM type, M00866
beta-Oxidation, acyl-CoA synthesis, M00086
beta-Oxidation, M00087
Fatty acid biosynthesis, elongation, M00083
Fatty acid biosynthesis, initiation, M00082
Phosphatidylethanolamine (PE) biosynthesis, M00093
Biotin biosynthesis, BioU pathway, M00950
Biotin biosynthesis, BioW pathway, M00577
Biotin biosynthesis, M00123
C1-unit interconversion, prokaryotes, M00140
Coenzyme A biosynthesis, M00120
Heme biosynthesis, bacteria, M00926
Heme biosynthesis, plants and bacteria, M00121
Lipoic acid biosynthesis, plants and bacteria, M00881
Menaquinone biosynthesis, M00116
Molybdenum cofactor biosynthesis, M00880
NAD biosynthesis, M00115
Pantothenate biosynthesis, M00119
Pimeloyl-ACP biosynthesis, BioC-BioH pathway, M00572
Pyridoxal-P biosynthesis, M00124
Pyridoxal-P biosynthesis, M00916
Riboflavin biosynthesis, plants and bacteria, M00125
Siroheme biosynthesis, M00846
Tetrahydrofolate biosynthesis, M00126
Thiamine biosynthesis, archaea, M00896
Thiamine biosynthesis, prokaryotes, M00127
Thiamine biosynthesis, prokaryotes, M00895
Thiamine salvage pathway, M00899
Ubiquinone biosynthesis, prokaryotes, M00117
De novo purine biosynthesis, M00048
De novo pyrimidine biosynthesis, M00051
Deoxyribonucleotide biosynthesis, M00053
Guanine ribonucleotide biosynthesis, M00050
Pyrimidine deoxyribonucleotide biosynthesis, M00938
Pyrimidine ribonucleotide biosynthesis, M00052
Aplacter1_2
Aplacter_2
Aplacter mensalis_1_2
Aplacter mensalis_2
Arsenophonus apicola
Bifidobacterium_1
Bifidobacterium_2
Bifidobacterium_3
Bifidobacterium_4
Bifidobacterium_5
Bifidobacterium bohemicum
Bifidobacterium sp024104275
Bombilactobacillus bombi_1
Bombilactobacillus bombi_2
Fructobacillus fructosus
Fructobacillus tropaeoli
Gilliamella apicola_A_1
Gilliamella apicola_M_1_2
Gilliamella bombi_1
Gilliamella bombi_2
Gilliamella bombi_3
Lactobacillaceae CALYQJ01
Lactobacillus
Lactobacillus bombicola
Lactobacillus panisapium
Schmidhempelia bombi
Snodgrassella communis_1_2
Snodgrassella communis_3
Snodgrassella communis_4
Snodgrassella sp024103515
MAG cluster
KEGG module category
Amino acid metabolism
Biosynthesis of terpenoids and polyketides
Carbohydrate metabolism
Energy metabolism
Glycan metabolism
Lipid metabolism
Metabolism of cofactors and vitamins
Nucleotide metabolism
Percent of MAGs with module
0.00
0.25
0.50
0.75
1.00
Enrichment score
60
70
80
90
100

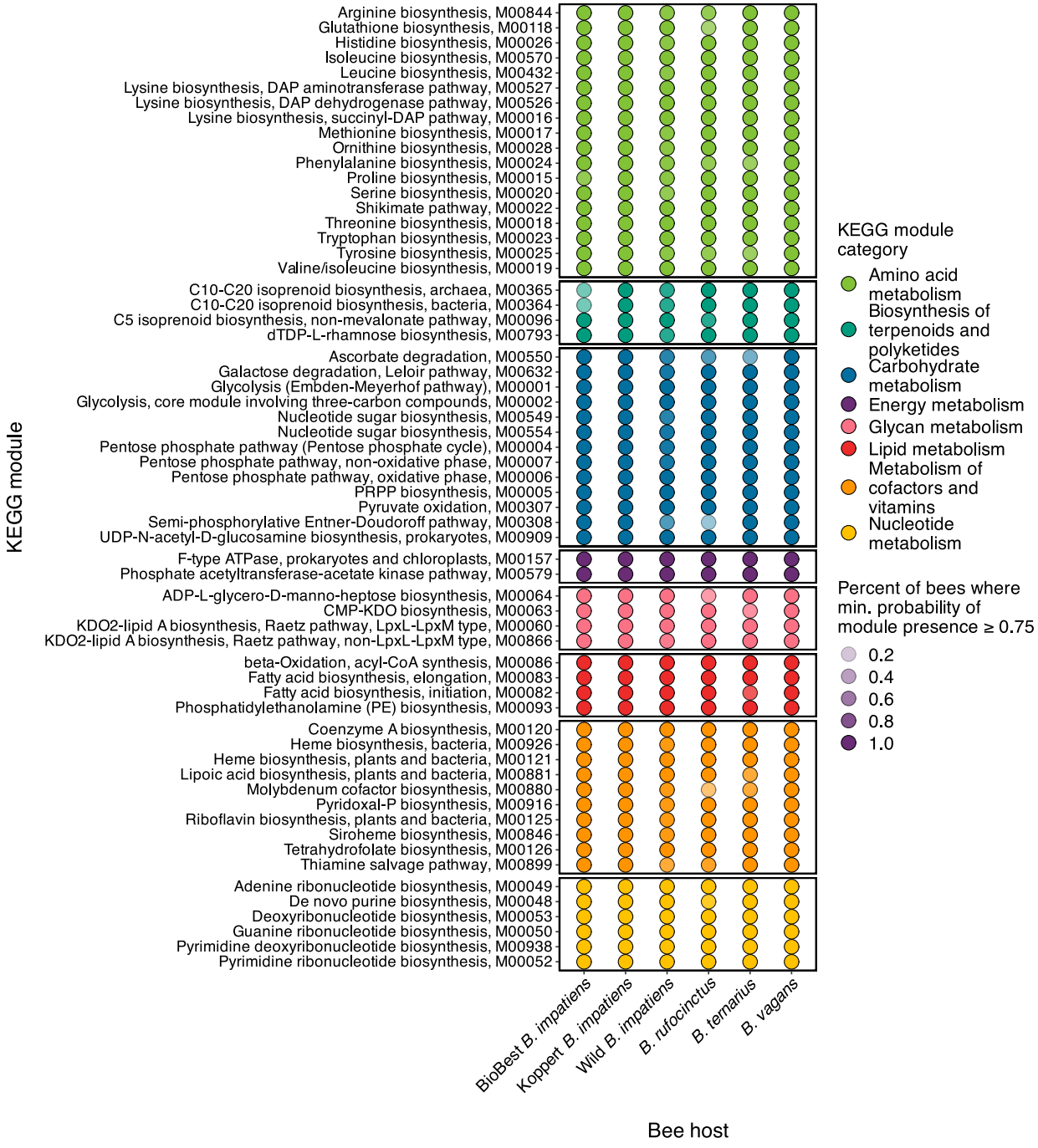
Supplementary Figure 5-11: Plot of differentially enriched KEGG modules in MAG clusters from *Bombus* gut microbiotas.

Plot of differentially enriched KEGG modules in MAG clusters from *Bombus* gut microbiotas, faceted by KEGG module category and phylotype. MAG clusters have a point for each module that is enriched in that cluster; for these data, if a MAG cluster does not have a point for a module, the module was absent in all MAGs for that cluster. Point transparency corresponds to the percent of MAGs within each MAG cluster that contain a given module. Point size corresponds to the enrichment score for a given module; the higher the enrichment score, the more strongly associated the module is with the cluster.



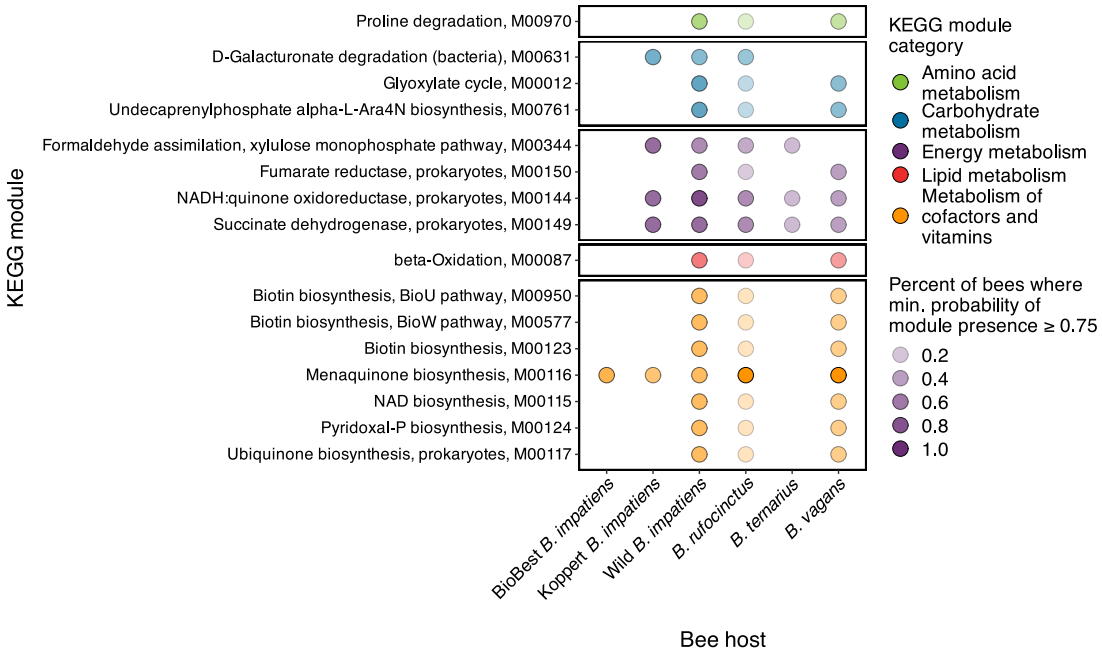
Supplementary Figure 5-12: KEGG module richness and composition of wild and commercial *Bombus* gut microbiotas.

A) Box plot of the number of KEGG modules expected to be present in wild bee gut microbiotas by site (n = 27; n = 3-8 per site). BP = Bruce Pit, CM = Chapman Mills Conservation Area, FG = Fletcher Wildlife Garden, MB = Mer Bleue Bog, ML = Mud Lake, PI = Petrie Island. Boxes represent medians and interquartile ranges; whiskers extend to 1.5 × the interquartile range. n.s. = non-significant. B) Principal coordinates analysis of KEGG module presence/absence in wild bee gut microbiotas using Jaccard distances (n = 27; n = 3-8 per site). C) Principal coordinates analysis of KEGG module presence/absence in wild bee gut microbiotas using Jaccard distances (n = 12; n = 6 per supplier; n = 2 per colony ID).



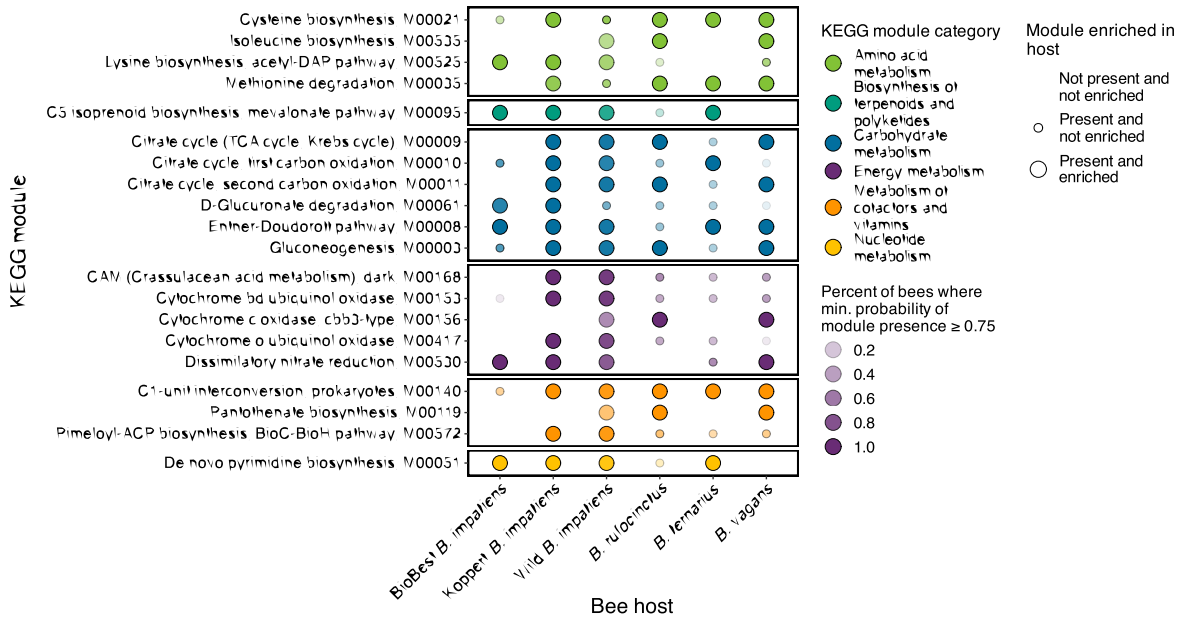
Supplementary Figure 5-13: Plot of KEGG modules that have consistently high prevalence across all bee hosts.

Points are coloured by KEGG module category. Point shading corresponds to the percent of bee gut microbiotas in each host where the minimum probability of module presence is ≥0.75.



Supplementary Figure 5-14: Plot of KEGG modules that have consistently low prevalence across all bee hosts. Points are coloured by KEGG module category.

Point shading corresponds to the percent of bee gut microbiotas in each host where the minimum probability of module presence is ≥0.75.



Supplementary Figure 5-15: Plot of differentially enriched KEGG modules by bee host.

Points are coloured by KEGG module category. Point shading corresponds to the percent of bee gut microbiotas of each host where the minimum probability of module presence is ≥0.75. Point size corresponds to whether the module is present and enriched in a given bee host.

Chapter 6

Discussion

The gut microbiota is an important facet of host health and performance in social bees. Consequently, research on social bee gut microbiotas has greatly accelerated in the past decade (Hammer et al., 2021; Kwong & Moran, 2016; Motta & Moran, 2023b). However, the gut microbiotas of bumble bees remain understudied when compared to those of honey bees, despite bumble bees' status as key, native pollinators across a variety of ecosystems and climates. My thesis makes significant contributions to our understanding of bumble bee gut microbiotas, particularly how they do—and do not—vary in response to natural and anthropogenic factors, including pesticide exposure, diapause, and host species and origin.

6.1 Pesticide-induced variation in social bee gut microbiota composition

Bumble bees are important pollinators in agro-ecosystems (e.g., Button & Elle, 2014; Eeraerts et al., 2020; Garibaldi et al., 2013; Lyu et al., 2023; Nayak et al., 2019; Roldán Serrano & Guerra-Sanz, 2006; Willmer et al., 1994), but by pollinating plants in these environments, they are exposed to a variety of pesticides (Gradish et al., 2019; Hladik et al., 2016; Nicholson et al., 2023; Rondeau et al., 2022). Understanding how pesticides impact bumble bees, including their gut microbiotas, is important to ensure thriving bumble bee populations in agricultural areas.

This thesis makes several contributions to our understanding of how pesticides can alter bee gut microbiotas. In Chapter 2, I synthesized literature on pesticide-induced alterations in bee gut microbiotas. While reviews had been written on the effects of pesticide exposure on animal and human gut microbiotas (Syromyatnikov et al., 2020; Tsiaoussis et al., 2019; Zhou & Zhao, 2021), mine was the first to focus solely on bees. Key findings from this review included that core microbial phylotypes differ in their sensitivity to pesticide exposure (Figures 2-4, 2-5) and that a variety of factors, such as pesticide concentration, exposure duration, season, and concurrent stressors, contribute to the occurrence and severity of pesticide-induced alterations in gut microbiota community structure. I also identified a critical scarcity of studies on the consequences of pesticide-disturbed gut microbiotas for host health and performance, in addition to a shortage of studies using non-honey bee hosts (Figure 2-1A), examining fungicides and herbicides (Figure 2-3), or investigating post-disturbance recovery.

In Chapter 3, I addressed some of these research gaps by testing whether exposure to the fungicide chlorothalonil altered bumble bee fecal microbiotas. Chlorothalonil is commonly used on field

and greenhouse crops which bumble bees pollinate (e.g., Kurz et al., 2008; Putnam et al., 2002), making it a relevant fungicide to test for effects on bumble bee gut microbiotas. I found that exposure to chlorothalonil for a short, field-realistic duration did not alter bumble bee fecal microbiotas, regardless of concentration (Figure 3-2). Furthermore, microcolony production did not suffer from exposure (Figure 3-1), providing evidence that at least when exposure durations are brief and unaccompanied by other stressors, bumble bee hosts and microbiotas are resilient to chlorothalonil exposure. Another goal of this chapter was to investigate post-disturbance recovery in bumble bee microbiotas, but as fecal microbiotas were not disturbed by chlorothalonil, this was not accomplished. The capacity of social bee gut microbiotas to recover from pesticide-induced disturbances remains largely unstudied but is highly relevant to the health of agricultural social bee populations; thus, future research should be dedicated to this topic.

In Chapter 4, I explored post-disturbance recovery in a different context: I investigated whether pesticide exposure could impede post-diapause recovery in bumble bee gut microbiotas. Bumble bee queen gut microbiotas are disturbed during diapause, as seen in my study and others (Chapter 4; Bosmans et al., 2018), but these microbiotas are a key source of gut microbes for colony workers (Hammer et al., 2021; Su et al., 2021). Therefore, post-diapause gut microbiota recovery is important not just for queen health and performance but the health and performance of the entire colony. I did not see an effect of glyphosate exposure on bumble bee gut microbiota recovery post-diapause, likely due to severe declines in and/or losses of *Snodgrassella* during diapause (Figure 4-2), the core phylotype that glyphosate consistently affects (Blot et al., 2019; Motta et al., 2018; Motta & Moran, 2020, 2023a). I also only examined gut microbiotas over a one-week period post-diapause, which was not sufficient for recovery (Figures 4-1, 4-2). Post-diapause recovery is a crucial stage in the bumble bee life cycle wherein queen health and performance determine the success of her future colony and fluctuating gut microbiotas may be particularly vulnerable to disturbance. Future studies should continue to investigate whether exposure to pesticides or toxicants impedes post-diapause gut microbiota recovery using longer-duration longitudinal designs.

Another goal for future research in pesticide-bumble bee gut microbiota interactions should be to expand beyond commercial populations and traditional host species. To date, all studies on the effects of pesticides on bumble bee gut microbiotas have been conducted using commercial colonies of either *B. impatiens* or *B. terrestris* (Cullen et al., 2023; Helander et al., 2023; Motta & Moran, 2023a; Rothman et al., 2020; Straw et al., 2023; Wintermantel et al., 2018). In Chapter 5, I found that community composition and structure of bumble bee gut microbiotas can vary with population origin (i.e., commercial vs. wild) and between commercial suppliers (Figures 5-2, 5-3, 5-4), with some commercial *B.*

impatiens colonies gaining or losing entire phylotypes relative to wild populations. I also observed substantial differences between *B. impatiens* gut microbiotas and those of other wild bumble bee species (Figures 5-2, 5-3, 5-4). As some pesticides appear to affect specific phylotypes (Chapter 2), my findings imply that research conducted using commercial colonies from one supplier may not translate well to other bumble bee populations, within or beyond the same species; thus, a greater diversity of wild populations and species should be studied directly. One potential study would be to sample bumble bee gut microbiotas from multiple species at field sites that differ in pesticide use. Comparing gut microbiotas between site types would provide valuable information on the consistency of pesticide-induced alterations across host species in wild populations and environments.

6.2 Variation in bumble bee gut microbiotas through diapause

In addition to exploring changes in bumble bee gut microbiotas due to pesticides, I also investigated natural drivers of variation in bumble bee gut microbiota community structure and function, like diapause. Diapause is a key, solitary life stage in the temperate bumble bee colony life cycle that acts as a bottleneck for the transmission of gut microbes between generations (Hammer et al., 2021). In Chapter 4, I conducted the first study that quantified changes in bumble bee gut microbiota community structure during diapause and early diapause recovery. Despite limitations imposed by my methodology, I found that gut microbiotas undergo large reductions in microbial abundance and dramatic restructuring during diapause (Figures 4-1, 4-2). In fact, multiple core phylotype populations declined below my detection limit and did not recover by one week after the end of diapause. My research thus implies that diapause may constrain the transmission not only of microbial strains to the next generation (Powell et al., 2016), but entire microbial phylotypes.

Despite the importance of this life stage, gut microbial community dynamics during diapause have received little research attention. My study is one of only three that have examined bumble bee queen gut microbiotas before and after diapause (Bosmans, Pozo, Verreth, Crauwels, Wäckers, et al., 2018; Wang et al., 2019), all of which were conducted in lab environments. More fundamental research is required to fully characterize shifts in queen gut microbiota community structure and function before, during, and after diapause, and to determine the consequences of these shifts for host and colony health. While difficult to implement, studies on wild queens in natural environments, ideally encompassing multiple species, will be critical to this effort.

6.3 Variation in bumble bee gut microbiotas across populations and species

In Chapter 5 of this thesis, I present the first study to compare genome-level taxonomic composition and metabolic potential of bumble bee gut microbiotas between commercial bumble bee colonies from different suppliers and between commercial and wild bumble bee populations of the same species. In Chapters 3 and 4, I conducted lab work using commercial *B. impatiens* colonies. However, after purchased colonies repeatedly lacked certain core phylotypes, I began to wonder to what extent the gut microbiotas of these colonies taxonomically and functionally reflected those of wild *B. impatiens* populations in eastern Canada (the geographic region in which these bees are being purchased to pollinate). In Chapter 5, I compared gut microbiota community structure and function between wild *B. impatiens* populations and two commercial *B. impatiens* suppliers. I found that community structure differed between wild and commercial gut microbiotas, and between the commercial suppliers themselves, with the most notable difference being a complete lack of *Snodgrassella* in Biobest colonies (Figure 5-2). However, metabolic potential was largely consistent across populations (Figure 5-5). Understanding that commercial and wild bumble bee gut microbiotas can differ in community structure but remain metabolically consistent will help researchers better assess how studies in commercial colonies translate to wild *B. impatiens* populations. Studies with more extensive sampling of commercial *B. impatiens* colonies, and that conduct commercial vs. wild gut microbiota comparisons in *B. terrestris*, another common commercial species, will help verify and complement my results.

Beyond *B. impatiens*, I also characterized *B. rufocinctus*, *B. ternarius*, and *B. vagans* gut microbiotas for the first time in Chapter 5. Despite gut microbiotas having been taxonomically profiled across multiple bumble bee species (Bosmans, Pozo, Verreth, Crauwels, Wilberts, et al., 2018; Cariveau et al., 2014; Dong et al., 2024; Kwong et al., 2017; Li et al., 2015; Lim et al., 2015; Parmentier et al., 2018; Powell et al., 2016; Praet et al., 2018; Villabona et al., 2023), little work has been done comparing gut microbiota functional profiles (though see Dong et al., 2024). Thus, Chapter 5 also represents the most extensive effort to date to compare gut microbiota function across different bumble bee species. The gut microbiotas of *B. rufocinctus*, *B. ternarius*, and *B. vagans* differed from each other and from those of *B. impatiens*, with many individuals lacking certain core phylotypes and gaining others (Figures 5-2, 5-4). These differences highlight the need to expand bumble bee gut microbiota research to include more host species. Yet, despite taxonomic variation, gut microbiota metabolic potential was largely consistent across all surveyed species (Figure 5-5), as it was across *B. impatiens* populations. This observation supports the existence of a pool of core phylotypes from which multiple combinations of phylotypes can support host health and performance. Still, the gut microbiotas of a great number of bumble bee species

native to disparate biomes (e.g., Arctic tundra, neotropics, forests, fields, bogs) remain to be characterized to further support this hypothesis. Additionally, studies employing functional 'omics techniques will be essential to determine if consistency in metabolic potential translates to consistency in metabolic function.

In Chapter 5, I observed that multiple populations and species of bumble bees had lost specific core phylotypes, such as *Snodgrassella*, *Bombilactobacillus*, and *Lactobacillus* (Figure 5-2). Based on past studies (Bosmans, Pozo, Verreth, Crauwels, Wäckers, et al., 2018; Bosmans, Pozo, Verreth, Crauwels, Wilberts, et al., 2018) and my work in Chapter 4 (Figure 4-2), it is possible that diapause is a driver of these losses and thereby variation in phylotype composition both within and between bumble bee species. An interesting avenue for future studies would be to investigate the role that diapause plays in driving intra- and inter-specific variation in gut microbiota composition. One could leverage the existence of bumble bee species with "atypical" diapauses for this purpose (i.e., diapauses that differ from those of temperate bumble bee species). For example, some neotropical bumble bee populations never undergo diapause (Cameron & Jost, 1998; Sakagami & Sakagami, 1976), while in some Arctic species diapause can last more than 10 months (Heinrich, 2004). Characterizing gut microbiota composition and diversity in bumble bee species living at either end of the diapause spectrum will provide key insight into the role that diapause plays in structuring gut microbiotas.

6.4 How to study bumble bee gut microbiotas

In addition to examining variation in bumble bee gut microbiotas, my thesis also explores and validates techniques for bumble bee gut microbiota research. In Chapter 3, I tested the validity of using fecal microbiotas as a proxy for gut microbiotas. Fecal microbiotas are often used as proxies for gut microbiotas in vertebrate research and are convenient in that they do not require destructive sampling of specimens. Thus, they are an attractive option for studying bumble bee gut microbiotas, particularly those of at-risk species and queens. However, I found that the composition and structure of bumble bee fecal microbiotas does not reflect the structure of gut microbiotas (Figures 3-4, 3-5). While large disturbances in gut microbiota structure likely alter fecal gut microbiota profiles, it would not be possible to determine how well the alterations in fecal microbiota structure reflected those in the gut. Thus, I recommend that gut microbiotas be sampled for bumble bee gut microbiota research whenever possible.

I also demonstrated the necessity of microbial enrichment when examining diapause microbial communities in Chapter 4. While core bumble bee gut phylotypes are all bacteria, bumble bee guts can also harbour fungi (Brysch-Herzberg, 2004; Pozo et al., 2018) and likely viral communities as well, as in honey bees (Bonilla-Rosso et al., 2020; Deboutte et al., 2020). Furthermore, diapausing queen guts are thought to play an important role in ferrying yeasts from one growing season to the next (Brysch-

Herzberg, 2004; Pozo et al., 2018). Metagenomic surveys are an efficient method to obtain taxonomic and functional information on all organisms within a sampled community, and thus are useful to conduct cross-domain profiling of bumble bee gut microbiotas. However, I found that bumble bee gut microbial abundance can decline by an order of magnitude during diapause (Figure 4-1), greatly shifting the ratio of host to microbe DNA in favour of the host, leading to biased sequencing (Supplementary Figure 4-1A). Therefore, it is important to enrich for microbial cells before sequencing, even if it is at the expense of eukaryotic microbes (Ellegaard & Engel, 2019). Future studies should incorporate appropriate enrichment methods such as physical filtering (Ellegaard & Engel, 2019) or cell sorting (Dungan et al., 2023).

Finally, as discussed previously, my work in Chapter 5 characterizing the community structure and metabolic potential of commercial and wild bumble bee gut microbiotas will help researchers determine how well bumble bee gut microbiota research translates beyond the populations in which it was conducted.

6.5 Conclusion

My thesis makes valuable contributions to our knowledge on variation in bumble bee gut microbiotas. I synthesized and investigated pesticide-induced alterations in bumble bee gut microbiota community structure, a relevant topic given the importance of bumble bees for agricultural pollination. Furthermore, I characterized natural variation in bumble bee gut microbiotas across diapause, a key life stage that may play important roles in host-microbial symbioses, and across host populations and species, of which there are still many more with disparate ecologies to investigate. Finally, I strengthened our understanding of how to study bumble bee gut microbiotas and how studies in commercial colonies may—or may not—translate to wild bumble bee populations.

Overall, the results of my thesis enrich our knowledge of bumble bee gut microbiotas, a key facet of bumble bee health and performance. My research will provide a strong foundation for future studies as we continue to explore the fascinating symbiosis between this important group of pollinators and the microbes that call them home.

6.6 References

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