The Role of the Environment on Wild Bee Microbiomes

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Abstract

Wild bees are important pollinators that are facing the effects of changing environments due to anthropogenic activities. Particularly sensitive to changes in environmental surroundings are bee microbiomes, or the microorganisms forming symbiotic relationships with their bee host. This thesis examines the role of environmental factors such as land use, microclimates, and pesticide residues on the microbiota within a small carpenter bee, *Ceratina calcarata*. Chapter I provides a review of the literature on bee microbiomes and environmental effects on community composition. In Chapter II, urban land use gradients were examined to characterize the microbiome of wild bees in Toronto. In Chapter III, urban and rural landscapes were studied on a broader scale, finding that bees and their pollen provisions from the city harbour different microbes from their agricultural counterparts. This research provides important implications on how anthropogenic activities may be disrupting bee microbiota and causing dysbiosis potentially harmful to pollinator health.

Dedicated to my parents, Ho and Thuy, who have provided unconditional love and support through all my endeavours.

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Chapter I Introduction: Environmental Effects on Wild Bee Microbiota

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Abstract

Anthropogenic activities and increased land use, which include industrialization, agriculture and urbanization, directly affect pollinators by changing habitats and floral availability, and indirectly by influencing their microbial composition and diversity. Bees form vital symbioses with their microbiota, relying on microorganisms to perform physiological functions and aid in immunity. As altered environments and climate threaten bees and their microbiota, characterizing the microbiome and its complex relationships with its host offers insights into understanding bee health. This review summarizes the role of sociality in microbiota establishment, as well as examines if such factors result in increased susceptibility to altered microbiota due to environmental changes. We characterize the role of geographic distribution, temperature, precipitation, floral resources, agriculture, and urbanization on bee microbiota. Bee microbiota are affected by altered surroundings regardless of sociality. Solitary bees that predominantly acquire their microbiota through the environment are particularly sensitive to such effects. However, the microbiota of obligately eusocial bees are also impacted by environmental changes despite typically well conserved and socially inherited microbiota. We provide an overview of the role of microbiota in plant-pollinator relationships and how bee microbiota play a larger role in urban ecology, offering microbial connections between animals, humans, and the environment. Understanding bee microbiota presents opportunities for sustainable land use restoration and aiding in wildlife conservation.

Keywords: Pollinator health, Bacteria, Fungi, Plant-pollinator networks, Urbanization, Agriculture, Sustainability, One Health

Introduction

The microbiome creates complex relationships between a host and its associated bacteria, fungi, protozoa, as well as viruses leading to networks that contribute to host health and fitness (Rosenberg et al., 2007). Commonly found microbial members across many individuals are characterized as part of the species' core microbiota (Turnbaugh & Gordon, 2009), providing key insights into the members that have co-evolved with the host. Characterizing and analyzing the function of microbiota has been an increasing area of study in bees to understand the factors affecting their microbiota and by proxy, bee health. Another key determinant of host health is the surrounding environment, which directly affects an individual's habitat and indirectly influences microbial composition (Teeling & Glöckner, 2012). As cities expand to accommodate a growing population and anthropogenic activity increases to meet their demand, local environmental changes from land use are inevitable and threaten the landscapes that support the habitats and living conditions of bees and their microbiota. Considering the effects of varying microbial composition alongside the impact of urbanization offers an increasingly relevant perspective as to how bees are responding to changes in land use.

An understanding of how the external surroundings affect bees and microbiota in changing environments will help direct conservation efforts necessary to counteract these human-driven changes and protect pollinators. Bees perform the majority of pollination services and have faced a decrease in population size over the past few decades (Hung et al., 2018;

Kremen et al., 2002; Marshman et al., 2019; Winfree, 2010). Due to their important role in both rural and urban agriculture (Potter & LeBuhn, 2015; Winfree, 2010), pollinator declines have raised the concerns of food insecurity and ecological collapse (Marshman et al., 2019). Worsening the issue, human disturbed landscapes that are common in urban areas and result in habitat loss affect bee abundance and species richness (Ayers & Rehan, 2021; Kelemen & Rehan, 2021; McKinney, 2008; Winfree et al., 2009). There are arrays of local environmental factors, including plant communities, green space availability, microclimate, and types of green space, which filter out certain bee functional traits and alter bee community composition (Ayers & Rehan, 2021; Mathiasson & Rehan, 2020; Theodorou, Radzevičiūtė, et al., 2020). While studies investigating exactly how anthropogenic activities may be responsible for this global pollinator decline over time are ongoing, considerations need to be made to determine how to protect bees from anthropogenic activities.

Here we review the literature on how bee microbiota is established and is altered by their environment, such as in response to urbanization, agriculture, and microclimate change (Figure 1.1). Mainly examining studies using targeted sequencing to characterize bacteria and fungi in bees, we highlight an array of recent and fundamental studies that describe factors influencing beneficial bee microbiota and enable comparisons among bee genera. First, we discuss the role of bee natural history and sociality in determining how microbiota are acquired and maintained. Second, we characterize pollinator susceptibility to changes in their microbiota related to human land use, and further examine which different environmental factors may contribute to altered microbiota. Finally, we examine the role of microbiota in plant-pollinator relationships and how its composition and diversity could illuminate strategies of pollinator conservation. We also highlight the role microbiota play in urban ecology and the sensitivities of symbiotic

relationships to anthropogenic activities. This review offers unique insights into the nature of the bee microbiome to better understand bee declines and the impact of urbanization on wildlife conservation and ecological health.

The Honey Bee Microbiota

Studies of bacteria and fungi in *Apis mellifera* have provided foundational descriptions of the microbial communities associated with bees (Table 1.1, S1.1). There are relatively few bacterial genera found within the honey bee gut, despite high levels of functional diversity within the core microbiota, offering an array of benefits in pathogen defense, immunity, and nutrient utilization (Engel et al., 2012; Martinson et al., 2011). The core honey bee microbiota consists of five species groups comprising as much as 95-99.9% of honey bee bacterial communities (Kwong & Moran, 2016; Moran et al., 2012) (Figure 1.2, Table S1.2). These microbiota are generally considered to belong to a highly conserved core group within corbiculates, including predominantly social honey bees, bumble bees, and stingless bees (Raymann & Moran, 2018). Microbial composition are particularly similar in adult workers, with males and queens displaying more variance in microbial communities due to different social roles (Kwong & Moran, 2016; Tarpy et al., 2015; Vernier et al., 2020; Yun et al., 2018). Recent studies have gone beyond bacteria to explore the fungal communities within the honey bee, finding that commonly occurring and augmented fungi such as *Saccharomyces* and *Aspergillus* can affect microbial communities, immunity, and physiology (Callegari et al., 2021; Tauber et al., 2019; Yun et al., 2018) (Table 1.1). These characterizations of the honey bee microbiota have provided a baseline for microbial diversity and taxonomic composition within bees and have been a vital stepping stone to exploration of other factors that affect the microbiota (Table 1.1, S1.1).

As found mainly in honey bees, microbial communities underscore the role of microbiota in their bee host's physiology and behaviour. Microbiota offer increased immunity by aiding in pathogen defense and protecting against parasites (Engel et al., 2012; Mockler et al., 2018; Rubanov et al., 2019), while also inducing expression of antimicrobial peptides crucial in innate immunity (Kwong, Mancenido, Amanda, et al., 2017; Vásquez et al., 2012). Bee nutrition is benefitted by some microbiota, with functions in carbohydrate breakdown and transport providing detoxification of food components and easily accessible energy sources (Engel et al., 2012; Raymann & Moran, 2018). Bee microbiota alter olfactory learning and memory, as they affect gene expression in the brain (Zhang et al., 2022), and promote long-term memory retention (L. Li et al., 2021). While many microbiota are beneficial to bees, pathogens and parasites can harm these pollinators and dominate beneficial microbiota (Fünfhaus et al., 2018). Some fungal members of the genus *Aspergillus* are opportunistic pathogens that result in stonebrood, or the mummification of honey bee brood (Becchimanzi & Nicoletti, 2022; Foley et al., 2014). However, other *Aspergillus* species do not create toxins and hydrolytic enzymes, rather being mutualistic to bees and competing with other pathogens (Becchimanzi & Nicoletti, 2022). As microbiota affect host health in a variety of avenues, functional characterizations of both bacteria and fungi remain ongoing in efforts to understand how changes to microbial communities go on to affect bee health.

In particular, ubiquitous microbial taxa such as *Apilactobacillus* or *Lactobacillus* have been examined in honey bees. *Lactobaillus kunkeei* acquired within the honey bee nest offer antimicrobial properties due to its ability to outcompete harmful microorganisms and protect against pathogens that lead to honey and bee bread spoilage (Vásquez et al., 2012). Bee bread is fermented pollen and nectar that uses the low pH environment caused by lactic acid bacteria to

allow *Saccharomyces* yeasts to ferment and preserve this dietary source for honey bees (López-Uribe & Lawrence, 2021). Lactic acid bacteria also form biofilms, which create extracellular polymeric substances that help host colonization and cellular recognition, and are predicted to benefit honey bee health (Vásquez et al., 2012). *Lactobacillus* also aid in amino acid digestion by regulating tryptophan metabolism, thereby changing neurological processes within the bee (Zhang et al., 2022). These bacteria even provide resistance against agrochemicals and pathogen adhesion, encouraging their use as probiotics (Pachla et al., 2021; Tlais et al., 2022). As such, loss of critical microbiota can negatively affect both immunity and host fitness, going so far as to delay development in both immature managed and wild bees (Daisley, Chmiel, et al., 2020; Dharampal et al., 2022), leading to weakened immunity and higher mortality (Raymann & Moran, 2018). The documented importance of the microbiota highlights the need for a deeper understanding to provide key insights to better support pollinator health.

Contextual and situational analyses of the honey bee microbiota have uncovered that many different factors affect microbial diversity, composition, and abundance (Table 1.1). Following honey bee queens throughout development has revealed changes in microbial abundance and composition, with queens being more variable in early life and sharing more similarities with the worker microbiota upon queen maturity (Tarpy et al., 2015). Honey bees of different social status possess striking differences in fungal communities. While nurse worker bees are dominated by *Saccharomyces,* foraging workers and queen bees maintain more diversity and reveal an overrepresentation of *Zygosaccharomyces* (Yun et al., 2018). External environments have also been considered, with landscape types such as agriculture shifting the relative abundances of the core microbiota, such that opportunistic bacteria metabolizing insecticides are overrepresented compared to beneficial bacteria (Julia C. Jones et al., 2018;

Muñoz-Colmenero et al., 2020). Even factors such as diet adjustments due to seasonal climate have been shown to change microbial composition, leading to the overrepresentation of beneficial microbiota such as *Bartonella* that aid in metabolism during winters (C. Li et al., 2022). In these contexts, studies in honey bees continue to expand on the sensitivity of microbiota to different factors and explore their role in maintaining bee and hive health.

The Microbiota of Wild Bees

Although honey bees are a popular pollinator of study, the thousands of wild bee species also offer crucial pollination services to both wild and agricultural plants (Demeter et al., 2021; Mallinger & Gratton, 2015). Microbial characterizations of some wild bees have suggested vastly different microbiota from honey bees (Table 1.1, S1.1; Figure 1.2), exacerbating the need for more studies on other bees so that the breadth of microbial diversity can be fully understood (Voulgari-Kokota, McFrederick, et al., 2019). Research questions regarding how wild bee microbiota are shaped, maintained, and affect bee health are ongoing, with emerging studies examining the role of important factors that affect more than just the honey bee. Developmental stages reveal alterations in microbial diversity as bees grow from larvae to pupae and adults in *Nomia melanderi* and *Ceratina calcarata* (Kapheim et al., 2021; Nguyen & Rehan, 2022a). Natural habitat and access to floral resources have been characterized around *Osmia lignaria* to evaluate microbiota in urban bees, finding an association between landscape features and important bacteria (Cohen et al., 2020). Similarly, habitat also influences bumble bee microbiota and the prevalence of pathogens (Bosmans et al., 2018). As more bee genera are studied and technological advancements make it easier to conduct these microbial analyses, gaps in

knowledge surrounding pollinator microbiota are being filled at a rapid pace and confirm that wild bee microbiota are labile and fluctuate over time.

Many questions remain in wild bee microbiota research, and new techniques and integrative methods are leading to deeper insights into the complexities of bee microbiota. Microbiota of bees in the family Apidae and, to a lesser extent the Megachilidae, have been particularly well classified, amidst the wealth of studies examining the corbiculate honey and bumble bees (Table 1.1, S1.1). Detailed characterization of microbiomes from other wild bees, such as those in the understudied bee families Halictidae, Colletidae and Andrenidae, promises a better overall understanding of the role of microbiota in bee health (Table 1.1, S1.1). This also relies on functional descriptions of the key microbial members within the microbiome, such as the non-core microbiota and fungi observed in wild bee species. Most microbiome studies target a locus that classifies reads from the targeted region as bacterial or fungal taxa. These techniques have been and continue to be the standard in microbiome research. While the 16S rRNA region is common for bacteria (Table 1.1), fewer works have implemented targeting of other regions, such as the ITS for fungal reads. Recent studies on bumble bees showcase the importance of characterizing the diversity of fungi and non-core bacteria in different landscapes (Bosmans et al., 2018). Fungi were almost exclusively found in queens from forested habitats and not detected in urban sites. Furthermore, those bumble bees from forested sites harboured more environmental bacteria not included in the bee-specific core, predicting that natural environments may lead to increased microbial diversity (Bosmans et al., 2018). Studies like this reveal that limiting comparisons to the five core bacterial groups over a small group of bees hinders a necessary appreciation of the microbial diversity among wild bees.

Extending analyses towards characterizing the composition of additional microbial taxa and plants is an evolving method of understanding the entire bee microbiota and its interactions with floral resources. Examining the relationship between bees and their associated plants can provide additional ecological insights due to the important role of flowers as pollinator habitat, diet source, and microbial reservoir. For example, the rbcL region has classified an array of pollen types used for brood provisions in small carpenter bee and megachilid provisions, revealing differences in floral usage across landscapes and varying plant associations with bees (Dew et al., 2020; McFrederick et al., 2017; McFrederick & Rehan, 2016, 2019). Future work characterizing the plant composition directly on and within bees will contribute to an understanding of how these plants and associated microbiota become established within the microbiome.

More recent characterizations of all reads unaligned with the host reference genome eliminates the need for targeting regions and allows shotgun metagenomic studies to uncover diverse microbial genes across different taxa such as bacteria, fungi, viruses, plants, arachnids, and protists. Thus, metagenomics opens new avenues to exploring environmental associations between the bee and any organism with which it interacts, without the limitations of deciding upon one or two loci *a priori* (J. Wang & Jia, 2016). Metagenomic analyses in *Apis* bees and their honey have initiated studies of the prevalence of viral, fungal, protozoan, and metazoan species in bees, highlighting the diversity of interactions between bacterial and eukaryotic microbiota and potential applications of environmental DNA (Bovo et al., 2018; Galbraith et al., 2018; Regan et al., 2018). *Ceratina* small carpenter bees have also revealed that bee species associated microbiota are affected by their local environment and can be affected by plant pathogens (Shell & Rehan, 2022). Implementing these strategies and exploring an array of taxa

promises a more complete understanding of bee interactions and its complex role in extensive ecological networks.

Social Maintenance of Microbiota

Solitary bees and eusocial corbiculate bees acquire their microbiota in different ways based on their lifestyle (Figure 1.1). Unlike obligately social bee species, solitary and facultatively social bees often maintain microbiota shaped by their environment because they lack the social interactions that reinforce the consistent core microbiota among nestmates (Dew et al., 2020; Kapheim et al., 2021; McFrederick et al., 2012, 2017; Voulgari-Kokota et al., 2020). Pollen and nectar that nourish bees are also important sources of microbiota (Dew et al., 2020; Figueroa et al., 2021; McFrederick et al., 2017; McFrederick & Rehan, 2016), and can become affected by the pollinators visiting them (Manirajan et al., 2016). Studies on *Ceratina* small carpenter bees and *Osmia* mason bees have highlighted the importance of environmental acquisition of microbiota from flowering plants and transfer to bee pollen provisions (Dew et al., 2020; McFrederick et al., 2017; Voulgari-Kokota, Grimmer, et al., 2019) (Table 1.1). With landscape and local environments likely being directly responsible for changes in microbial communities in bees due to the availability of flowers and green space, looking at wild bee habitats offers new opportunities to understand their microbial communities. The importance of understanding how urbanization, land use change, temperature, and pesticide usage affect plant and microbial communities are crucial due to these sensitivities (Voulgari-Kokota, McFrederick, et al., 2019), raising concerns for how wild bees are forced to re-establish their microbiota as anthropogenic activities increase.

Obligately eusocial corbiculate bees such as *Apis* and *Bombus* maintain a core microbiota through vertical transmission and social interaction between colony members (Kwong & Moran, 2016; Martinson et al., 2012; Powell et al., 2018; Su et al., 2021; Tarpy et al., 2015; Wu et al., 2021). Obligate eusociality is not strictly required in order to maintain core microbiota, as non-corbiculate, facultatively social and gregarious bees that live in shared nesting sites, such as *Xylocopa* species*,* are also dominated by members of the core noted in honey and bumble bees, such as *Bombilactobacillus, Bombiscardovia,* and *Bifidobacterium* (Handy et al., 2022; Holley et al., 2022) (Figure 1.2, Table S1.2). However, these large carpenter bees do interact socially at shared nesting sites and may have experienced a phylogenetic inheritance of common bacteria widespread in the bee family Apidae and also associated with the corbiculate honey and bumble bees (Holley et al., 2022) (Figure 1.2). Interestingly, honey bee genotypes affect which bacterial strains are passed through social transmission, highlighting the importance of ancestry in shaping the microbiota (Wu et al., 2021). Sharing core microbiota is a trait consistent within obligately eusocial bees. Core microbiota such as *Lactobacillus* may then affect social behaviours that potentially reinforces microbiota establishment amongst nest mates through social interactions (Liberti et al., 2022; Vernier et al., 2020). Microbial colonization increases the rate of head-to-head interactions between bees as a result of increased metabolites and amino acids that affect synaptic transmission and encourage social contact, potentially increasing the chances of social interactions (Liberti et al., 2022). Not only are adult honey bee roles affecting bacterial composition, but colony-specific microbiota can also dictate colony membership (Vernier et al., 2020). Shared strain-specific bacteria, particularly the honey bee specific symbiont *Gilliamella apicola*, within colonies can alter individual cuticular hydrocarbon profiles, leading to pheromones and chemical signatures that affect bee nestmate recognition (Vernier et al., 2020).

These social interactions and behaviour have thus created pathways for sharing of microbiota that make up the most commonly established microbiome.

Because eusocial corbiculate bees such as honey bees tend to rely on social transmission to acquire their microbiome, it is relatively easy to describe core microbiota (Raymann & Moran, 2018). However, the idea of a core microbiome has been challenged due to inconsistent definitions and the lack of consensus on the standardized metrics such as exact occurrence and abundance cut-off values used to quantify the core microbiota (Custer et al., 2023; Neu et al., 2021; Risely, 2020). As the focus has traditionally been on these dominating microbiota, determining the roles of relatively less abundant microbiota may reveal neglected but important microbial functions. Life stages and social role impart vulnerability to changes in microbial exposure. Studies comparing the microbial composition across honey bee workers (Anderson & Maes, 2022) and queens (Copeland et al., 2022; Tarpy et al., 2015) have found compositional differences based on behavioural tasks. Conversely, microbiota can define social group membership in colonies, highlighting the correlation between social status and microbial composition (J. C. Jones et al., 2018; Vernier et al., 2020) (Table 1.1). Despite very consistent core microbiota described in adult honey bees, larval microbiota are initially variable and can even lead to differing larval gene expression when there is different bacterial composition (Kowallik & Mikheyev, 2021). Obligately eusocial stingless bee genera *Trigona, Melipona,* and *Partamona* maintain the corbiculate core microbiota, but diet has been shown to add new associations with environmentally acquired acidophilic bacteria that are important in bee nutrition (Figueroa et al., 2021). Thus, these bacteria may supplement and sustain these bees' lifestyle and cyclically allow them to make better use of these environments. Although the idea of a core microbiota remains established within social bees more often than for solitary bees, all

bees seem to be subject to external factors that can contribute to altered microbiota and dysbiosis.

Environmental Influences on Microbial Acquisition

Examining bees from different geographic locations offers a preliminary evaluation of how environment shapes microbiota broadly (Figure 1.1). Given the consistency across the corbiculate core microbiota, microbial changes are less evident in honey bees. Geographic location was not the biggest factor affecting the *Apis* microbiota, even though location may still explain why microbial composition changed over time (Almeida et al., 2022). Geographical distribution was also a poorer predictor of antibiotic resistance genes in honey bee gut bacteria, although there was a geographic pattern of resistance distribution (Sun et al., 2022). This contradictory pattern of local landscape effects has been seen in wild bee microbiota as well, with solitary megachilid bee microbiota associating with host bee species rather than location (Voulgari-Kokota, Grimmer, et al., 2019). Small carpenter bees seemingly maintain different microbiota across geographic distributions and locations (Nguyen & Rehan, 2022b; Shell & Rehan, 2022), although there has not yet been directly comparable and longitudinal studies of a bee genus within a demographic and across different local landscapes. However, metagenomic analysis of small carpenter bees from around the world has revealed associations with host bee species that are strongly affected by local environmental features. For example, population comparisons of *Ceratina australensis* revealed that bacteria favouring saline and marine environments were associated with bees located in beach dunes (Shell & Rehan, 2022). As microbiome research expands across geographic locations, environmental contexts and bee species, these comparisons among regions will become more accessible. Future studies focusing

on specific environmental factors will encourage more detailed comparisons across landscapes and hold great promise for biodiversity conservation.

Climatic variables and environmental conditions such as regional precipitation and temperature have been shown to affect bee microbiomes as well. *Ceratina* from different climatic zones across Australia vary in community composition, with species richness higher in subtropical zones than in temperate zones or grasslands (McFrederick & Rehan, 2019). Microbial diversity and co-occurrences also vary across areas with different mean annual temperature and annual precipitation. Temperature can predict the relative abundances of bacterial and fungal taxa, and vital bacteria such as *Apilactobacillus* are overrepresented in areas with lower annual temperatures (Nguyen & Rehan, 2022b). Precipitation is associated with fungal beta diversity, resulting in areas with higher precipitation having less relatively abundant fungi like *Alternaria* (Nguyen & Rehan, 2022b). Even in honey bees, low temperatures during winters lead to dietary shifts that are associated with the seasonal dominance of the non-core bacteria *Bartonella* (C. Li et al., 2022). These bacteria are capable of metabolizing different energy substrates that may benefit the bee host through producing essential amino acids during restricted diets (C. Li et al., 2022). Honey bees have been shown to display changes in microbial composition in fall and spring time, accommodating the overwintering period in northern temperate climates (Bleau et al., 2020). Thus, environmental factors associated with climate play a role in determining which microbiota are easily accessible and can benefit the host when established.

Access to flowering plants is an important consideration for bee habitats. Floral resources contributing to diet are important factors influencing the bee microbiota because of the availability of microbiota offered in plant-pollinator relationships (Dew et al., 2020; Keller et al.,

2021; Manirajan et al., 2016, 2018; McFrederick et al., 2017; Morris et al., 2020; Sookhan et al., 2021). For example, non-native tropical milkweed in urban landscapes, mainly visited by honey bees and several solitary bees, harboured bacteria attributable to differences in microbial diversity among floral visitors and the environment (Warren et al., 2020). In environments where access to floral resources changes or provisioning requirements becomes more limited, both plant and bee microbiota may experience dysbiosis and a harmful reduction in important microbiota (Christensen et al., 2021; Morris et al., 2020). Visiting an array of plants may also allow bees to become exposed to diverse bacteria and result in improved larval developmental success, as is the case with *Osmia lignaria* pollen provisions with higher bacterial diversity (Westreich et al., 2022). However, this matter is worsened when considering that pollen and nectar in wild flowers can harbour pesticides, which pollinators bring back to their nests, both in agricultural and urban landscapes (Botías et al., 2015, 2017). Therefore, the indirect effects of human activities on bee habitats and flower availability raise additional concerns for pollinator health.

While pollinators, and especially wild bees, are exposed to increased microbial diversity in the form of pollen-associated microbiota, wild bees also play a role in determining microbial composition in the plants they visit. For example, nectar robbing by carpenter bees increases the abundance of Acetobacteraceae in nectar more than pollination by hummingbirds, also resulting in functional enrichment in the bacterial genomes indicating different amino acid and saccharide utilization pathways (Morris et al., 2020). Acetobacteraceae are commonly found in both nectar and bees, playing an important role in pollen germination of nectar and in honey production (Almeida et al., 2022; Christensen et al., 2021). Even yeasts in pollen and nectar have been shown to increase the nutritional value for pollinators (Pozo et al., 2020). Thus, this mutualistic

relationship between plants, bees, and their microbiota suggest that important co-occurrences may be a sign of co-evolution and adaptation and highlights the need to consider plant-pollinator relationships when examining microbial composition and diversity in bees.

Managed bees in agriculture and wild bees in natural habitats house different microbiota, with agrochemicals and pesticides likely acting as a stressor on both bees and their microbiomes (Hotchkiss et al., 2022; Kakumanu et al., 2016; Vidau et al., 2011; Yordanova et al., 2022). As seen in honey bees, beneficial bacteria are more abundant in wild bees from less anthropized environments, while bees from farms harbour more microbiota capable of metabolizing insecticides (Hotchkiss et al., 2022; Muñoz-Colmenero et al., 2020; Tlais et al., 2022). Microbiota of hives sprayed with common pesticides can face altered structural and functional potential, such as how genes for oxidative phosphorylation increased while those responsible for sugar metabolism decreased among bacteria in bees exposed to chlorothalonil (Kakumanu et al., 2016). Glyphosate, a common herbicide for weed control, unexpectedly harms bees due to its ability to change the relative abundances of important bacterial symbionts that protect against opportunistic pathogens (Motta et al., 2018). The effects of pesticides on microbiota are likely specific to hosts. Imidacloprid has been shown to change the digestive physiology and microbiota of *Drosophila melanogaster* larvae and leads to increased mortality and susceptibility to pathogens in honey bees (Raymann et al., 2018). However, this pesticide does not affect gut bacteria in honey bees which cannot metabolize imidacloprid (Raymann et al., 2018). Although it cannot yet be determined that agriculture directly harms bee microbiota, abiotic and chemical factors need to be studied to understand how they interact with the microbiome. While introducing probiotic bacteria can be used to mitigate environmental effects (Daisley et al., 2017;

Motta et al., 2022), careful consideration needs to be given to microbial manipulation and inoculation as a remedy.

Examining the effects of urbanization on bee microbiota has been relatively limited but indicate anthropogenic activities may lead to dysbiosis. Studies on *O. lignaria* have indicated that natural habitat, floral resources, bee community species richness in the area, and garden size are all factors that positively influence the abundance of important bacteria, such as *Lactobacillus* (Cohen et al., 2020, 2022). Likewise, studies on *Bombus terrestris*, comparing forest and urban queens revealed potentially beneficial diversity of environmentally acquired bacteria and fungi exclusive to queens from forests, and a more standard core microbiota in urban bees (Bosmans et al., 2018). Furthermore, studies on small carpenter bees across an urbanization gradient found that important bacteria such as *Acinetobacter* and *Apilactobactillus* were overrepresented in sites with low impervious surface and increased green space availability (Nguyen & Rehan, 2022b). Bees from areas with low land use development also had more plant associated microbiota, whereas microbiota from high land use development areas may be harboured due to their functions in bee development and digestion (Nguyen & Rehan, 2022b). Although urbanization has not been shown to be detrimental to bee health, growing cities do threaten microbial compositions containing known beneficial symbionts. Future studies conducting direct comparisons between urban and rural sites will prove valuable in characterizing the effects of human activities on the established microbial relationships.

The environmental microbiome has changed with anthropogenic activity. Many wild animals such as birds, reptiles, and mammals have begun to develop microbiomes increasingly similar to those of humans, hinting that spillover may be affecting animals in cities (Dillard et al., 2022). Changing microbial composition does not always lead to positive associations and

increases in diversity may potentially lead to dysbiosis. Ants have lost symbiotically beneficial microbiota when transitioning from forested to urban areas, potentially harming host colonies (K. O. Chua et al., 2018). Thus, in considering how anthropogenic activities have come to change the bee microbiome, it may prove useful to account for broader perspectives to determine how microbiomes are changing throughout the surrounding environment and across wildlife. Examining microbial content within the environment, such as in soil and floral resources, will be necessary to characterize bee exposures to microbiota. Specifically, looking at nests and pollen provision microbiota across anthropized landscapes has been unexplored in most species. This would offer insights into the environmental and social transmission of microbiota, potentially opening avenues for discovering how wildlife must adjust to changing environments and microbiota.

Placing bees in a larger ecological context, these pollinators may act as an intermediary carrier of microbiota between plants and other animals due to their role in the ecosystem (Arroyo-Correa et al., 2023; Perfectti et al., 2009). As bees deposit their microbiota on flowers and these plants then offer their collection of bacteria and fungi to other plant visitors (Keller et al., 2021; Manirajan et al., 2016, 2018), the spread of both beneficial and harmful microbiota can occur to other bee species, other pollinators, and other animals. This research ties directly to the One Health concept, which examines the connections between humans, animals, and the environment across disciplines and fields of study to examine the consequences of animal-human-ecosystem networks (Davis et al., 2017; Mackenzie & Jeggo, 2019). This can be applied towards both pathogenic and beneficial microbiota and their impact on bees and their habitats (Trinh et al., 2018). Environmental and wildlife microbiota have been shown to affect human health, reinforcing how changes such as urbanization can lead to cascading effects impacting all

organisms (Trinh et al., 2018). With a broader perspective into the important role bees play within ecosystems and their pollination activities, promoting bee health has implications far beyond this one group of insects. Using bees as a study model, particularly wild bees that may be more sensitive to environmental factors, offers both a unique perspective and a practical avenue to exploring the role of microbiota in One Health.

Future Directions

While changing environments have been shown to affect both solitary and social bee microbiota composition, challenges remain in furthering our understanding of how the environment affects bee microbiota and the degree of harm to bees. In some instances, bees from anthropogenic environments face decreased microbial diversity, although whether this is beneficial or harmful to bees in the long term is still unclear. Wild and captive animal microbiota tend to differ (Chiang et al., 2022; Mays et al., 2021) and the impact of environmental changes differs greatly between bee genera and habitats, leading to difficulties in extrapolating from experimentation in lab and requiring a more robust survey of wild bees. Therefore, establishing a baseline characterization of microbiota across bees to compare and contrast how evolving environmental changes are affecting microbiota will require additional resources and long-term observations. To answer the vast array of questions remaining as to how environmental features are affecting the way microbiota are acquired and maintained, both functional and experimental work needs to be performed across a broader range of bee species. Experiments quantitatively altering the proportion of environmental features, whether through the addition of pesticides, manipulating temperature conditions, or changing access to floral resources, are necessary to characterize changing microbiota effects on their host. Functional research describing the role of

microbiota in symbioses is also much needed, particularly due to the fast pace of characterizing new taxa with no known function. This is also true for pathogens, where it is largely unknown how environmental factors and land use contribute to their presence and abundance. These goals have become more accessible with the recent ease of exploring a wider range of microbiota within the metagenome using shotgun metagenomics and environmental DNA sequencing approaches, including fungi, plants, and viruses. Megabarcoding opens even more avenues for understanding metagenomics, using increasingly affordable high throughput sequencing technologies such as MinION or PacBio for upscaling (P. Y. S. Chua et al., 2023). This will expedite characterization of the bee and its microbiota and will highlight numerous new taxa and functional associations to be studied.

An understanding of the role the microbiota plays in maintaining bee health and how it changes with differing land use offers opportunities for protection efforts by unraveling the cascade of effects that environmental changes have on bees and their microbiota. Using DNA-based techniques, such as metagenomics or megabarcoding, can be extrapolated for use in monitoring pollinators (P. Y. S. Chua et al., 2023). Direct changes through habitat restoration or indirectly through habitat augmentation may be used to supplement floral resources, increase green space availability, and foster better habitats for pollinators (Tonietto & Larkin, 2018; Winfree, 2010). Increasing gardens and green spaces is one way to support plant-pollinator networks and their associated microbiota. Probiotics have also been a consideration for bees, as they can provide crucial bacteria and fungi to bees that may increase immunity. Offering probiotic Lactobacilli in a nutrient patty to honey bee hives has provided colony resistance against American foulbrood (Daisley et al., 2019). However this should be proceeded with caution, as the previously demonstrated probiotic *Parasacharribacter apium* (Corby-Harris et

al., 2014) in practice did not protect bees from European Foul Brood disease caused by the bacteria *Melissococcus plutonius* (Floyd et al., 2020). Thus, supplementing the microbiota and further manipulating bee habitats requires careful implementation in order to prevent dysbiosis within these and other pollinators.

To conclude, environmental features broadly affect microbiota through a variety of avenues, especially solitary bees that largely acquire microbiota from their surroundings and diet. Different regions, environmental temperature, precipitation, pesticide usage, floral resources, and human urbanization are just some factors that change the microbial communities within bees and hosts widely. This generates questions as to how host health is affected in changing environments and inspires studies examining the functional effects of microbiota. As these altered habitats continue to threaten bees, reducing harmful anthropogenic activities and supplementing microbiota with probiotics may be opportunities to protect these important pollinators. Using bees as model organisms will also further provide insights into how a wider range of animals are experiencing changes in their microbiome because of environmental stressors, land use and changes in microclimate. Thus, this field of research has foundational implications for understanding and maintaining the web of interactions that support the overall health of bees, pollinators, wildlife, humans, and the environment.

This thesis considers how these environmental factors interact to establish the microbiome in small carpenter bees. In Chapter II, I characterize how the bacterial and fungal microbiome in *Ceratina calcarata* changes across an urban land use gradient. It was hypothesized that bees in areas with varying percent land use development, percent green space, mean annual temperature, and mean annual precipitation would also harbour a different microbial composition. Increased urbanization was expected to result in lower microbial diversity and the underrepresentation of

beneficial microbes. In Chapter III, a broader study area is examined to compare the difference between urban and rural microbiota and pollen composition across the Greater Toronto Area. This chapter predicted that local land use, climatic changes, and pesticides would contribute to higher microbial diversity and a lack of key symbiotic microbes in urban areas. Finally, this thesis concludes with a discussion of how environmental features impact the wild bee microbiome and are key determinants of maintaining pollinator health.

Tables and Figures for Chapter 1

Table 1.1 Summary of studies characterizing bacteria and fungi within bee genera across different environments and developmental stages. Studies directly comparing bees across developmental stages are noted as a factor affecting the microbiome.

	Bee Family	Bee Genera	Loci	Developmental Stage				Factors Affecting Microbiome					References
				Larvae	Pupae	Callow	Adult	Host Species	Development	Behaviour	Environment	Diet	
Corbiculate	Apidae	*Apis*	16S ITS	X	X	X	X	X	X	X	X	X	Mohr and Tebbe, 2006; Martinson et al., 2012; Moran et al., 2012; Tarpy et al., 2015; Yun et al., 2018; Tauber et al., 2019; Muñoz-Colmenero et al., 2020; Vernier et al., 2020; Callegari et al., 2021; Li et al., 2022
		Bombus	16S ITS	X			X	X	X	X	X		Mohr and Tebbe, 2006; Martinson et al., 2011; Bosmans et al., 2018 Li et al., 2021
		Melipona, Plebeia *Partamona, Scaptotrigona, Scaura,* *Trigona, Tetragonisca*	16S				X	X				X	Figueroa et al., 2021
Non-Corbiculate		*Ceratina*	16S ITS	X	X	X	X	X	X		X	X	Graystock et al., 2017; Nguyen and Rehan, 2022ab Shell and Rehan, 2022
		Xylocopa	16S				X	X					Handy et al., 2022; Holley et al., 2022
	Halictidae	*Augochlora* *Halictus* *Megalopta*	16S	X	X		X	X				X	McFrederick et al., 2012; McFrederick et al., 2014; Graystock et al., 2017
		Nomia	16S	X	X	X	X		X				Kapheim et al., 2021
	Megachilidae	*Heriades* *Lithurgus* *Megachile*	16S ITS	X			X	X				X	McFrederick et al., 2017; Voulgari-Kokota et al., 2019
		Osmia	16S ITS	X			X	X			X	X	Mohr and Tebbe, 2006; McFrederick et al., 2017; Voulgari-Kokota et al., 2019; Cohen et al., 2020

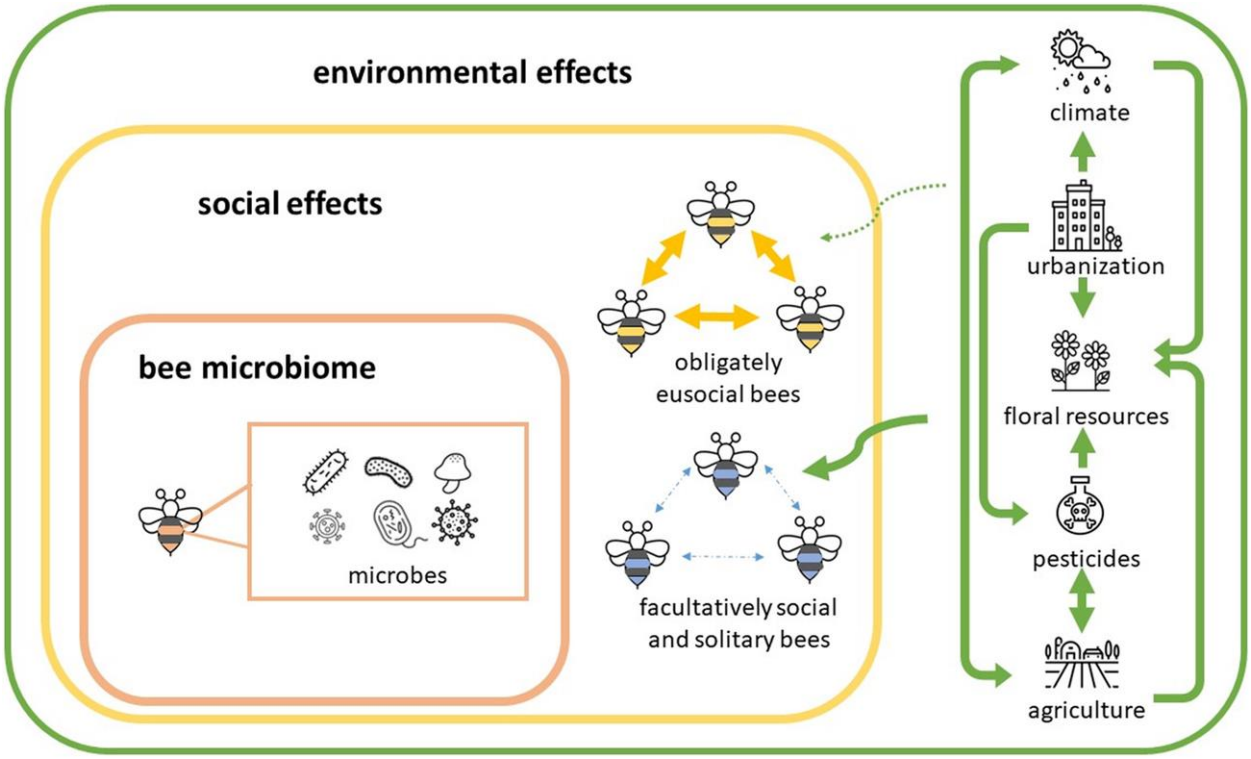

Figure 1.1 Environmental and social effects on the bee microbiome. Thick arrows and lines represent a greater effect and correlation compared to thinner, dashed lines and arrows. Facultatively social and solitary bee microbiomes are more heavily influenced by environmental effects as opposed to social interactions, while obligately eusocial bees tend to share a microbiome socially more distinctly than through environmental acquisition

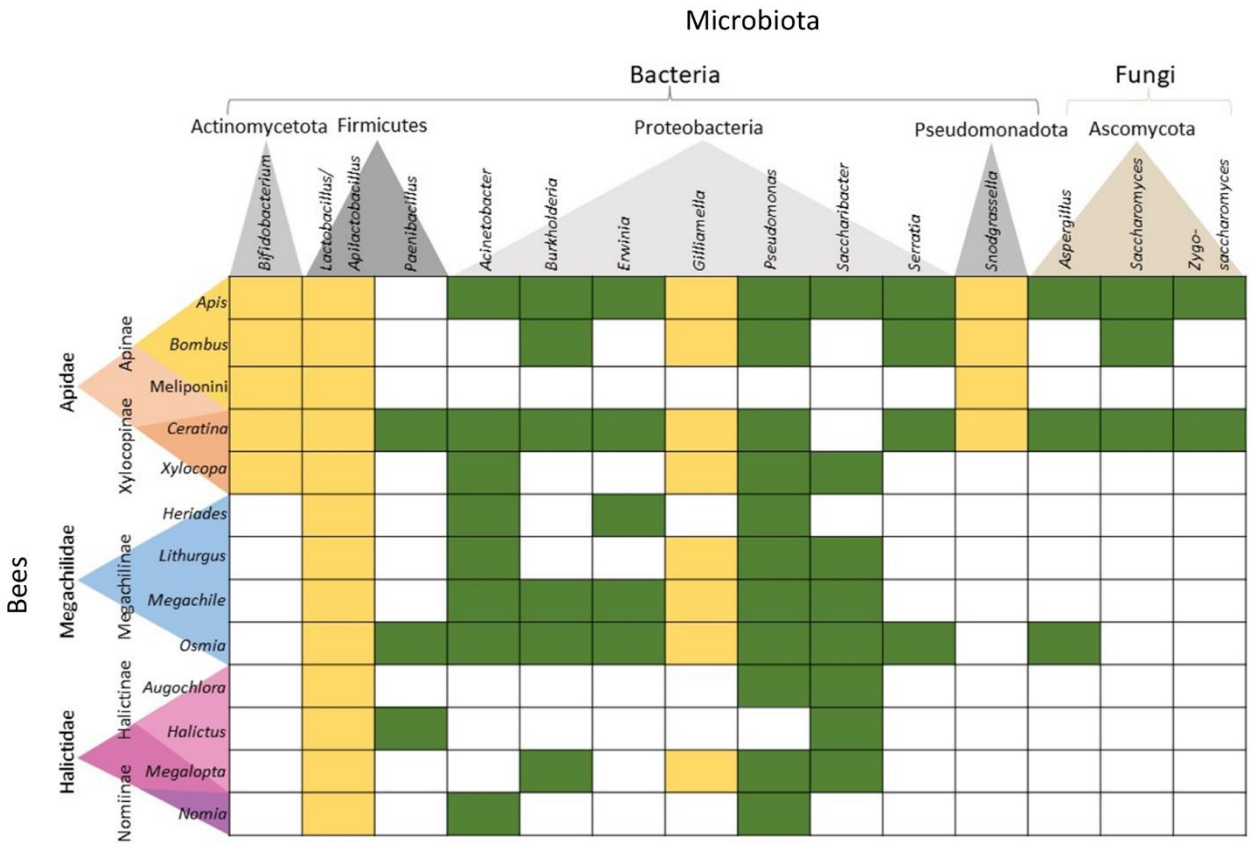

Figure 1.2. Bacterial and fungal genera found in bee genera microbiota. Corbiculate bees and bacteria forming their core are highlighted in yellow. Microbiota not specific to the corbiculate core tend to be environmentally acquired and are shown in green. Bacteria are highlighted in grey, while fungal genera are shown in brown. Additional list and references in Supplementary Table S1.2

Chapter II: The effects of urban land use gradients on wild bee microbiomes

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Abstract

Bees and their microbes interact in complex networks in which bees form symbiotic relationships with their bacteria and fungi. Microbial composition and abundance affect bee health through nutrition, immunity, and fitness. In ever-expanding urban landscapes, land use development changes bee habitats and floral resource availability, thus altering the sources of microbes that wild bees need to establish their microbiome. Here, we implement metabarcoding of the bacterial 16S and fungal ITS regions to characterize the diversity and composition of the microbiome in 58 small carpenter bees, *Ceratina calcarata*, across urban land use gradients (study area 6,425 km^2). By categorizing land use development, green space, precipitation, and temperature variables as indicators of habitat across the city, we found that land use variables can predict microbial diversity. Microbial composition was also found to vary across urban land use gradients, with certain microbes such as *Acinetobacter* and *Apilactobacillus* overrepresented in less urban locations and *Penicillium* more abundant in developed areas. Environmental features may also lead to differences in microbe interactions, as co-occurrences between bacteria and fungi varied across percent land use development, exemplified by the correlation between *Methylobacterium* and *Sphingomonas* being more prevalent in areas of higher urban development. Surrounding landscapes change the microbial landscape in wild bees and alter the relationships they have with their microbiome. As such, urban centres should consider the impact

of growing cities on their pollinators' health and protect wild bees from the effects of anthropogenic activities.

Keywords: bacteria, fungi, urbanization, microbial diversity, land use, Apidae

Introduction

Research on bee microbiomes uncovered their vital role in many aspects of bee health, including improving immunity (Engel et al., 2012; Mockler et al., 2018; Rubanov et al., 2019), nutrient utilization (Engel et al., 2012; Raymann & Moran, 2018), and reducing metalloid toxicity (Rothman et al., 2019). For example, the presence of non-pathogenic microbial symbionts in honey bees upregulates antimicrobial peptides in the bee and prepares the immune response against pathogenic microbes (Kwong, Mancenido, Amanda, et al., 2017). The importance of microbes can also be implicated more broadly, with the sterilization of larval mason bee microbiomes negatively impacting bee fitness through declining growth rates, biomass, and survivorship (Dharampal et al., 2019). The gut microbiome has even been associated with different behavioural tasks in honey bees and memory retention in bumble bees (J. C. Jones et al., 2018; L. Li et al., 2021). With the common consensus that microbes engage in beneficial interactions with their bee hosts, research continues to examine the factors that influence the establishment and maintenance of the microbiome.

The core microbiome is described as the microbes that are consistently found within many individuals of a species (Danko et al., 2021; Turnbaugh & Gordon, 2009). Social and solitary bees acquire their microbiome in different ways, with honey and bumble bee microbiome composition directly influenced by social interactions with their colony members (Kwong & Moran, 2016; Martinson et al., 2012; Powell et al., 2018; Su et al., 2021; Tarpy et al.,

2015), whereas less social wild bees inherit their microbiome from their surrounding environment and from their diet (Dew et al., 2020; Figueroa et al., 2021; Kapheim et al., 2021; McFrederick & Rehan, 2016; Voulgari-Kokota et al., 2020). Much work has been done on social bees such as *Apis* and *Bombus* to characterize and examine the health effects of an altered core microbiome (Martinson et al., 2011; Raymann & Moran, 2018; Rothman et al., 2019; Su et al., 2021). For solitary wild bees, this research is in its infancy; however, it is known that wild bees do not always maintain the same consistent core microbiome seen in social bees (Graystock et al., 2017; Kwong & Moran, 2016; McFrederick et al., 2012, 2014; McFrederick & Rehan, 2016). An example on how solitary wild bees can have differing core microbiomes can be found in *C. calcarata*, a wild bee displaying different core microbiomes from other bee species and across different regions (Dew et al., 2020; Graystock et al., 2017; Nguyen & Rehan, 2022a; Shell & Rehan, 2022).

A range of variables, including developmental stage (Kapheim et al., 2021; McFrederick et al., 2014; Nguyen & Rehan, 2022a), sociality of host species (Graystock et al., 2017; McFrederick et al., 2014; Mohr & Tebbe, 2006), climate (McFrederick & Rehan, 2019), geographical location (Almeida et al., 2022), and landscape features (Cohen et al., 2020), have been examined to determine their effects on the microbiome. Examining microbiomes across host developmental stages has allowed for closer examinations of the establishment of microbes, showing that diet is the main source of bacteria and fungi for developing solitary bees (Dew et al., 2020; Kapheim et al., 2021; Nguyen & Rehan, 2022a; Pozo et al., 2020). Sociality can influence the bee microbiome by impacting how solitary and social bees interact with food resources, the environment, and other bees to transmit different microbes (Mohr & Tebbe, 2006). Across various bee species, environmental factors such as climate (McFrederick & Rehan,

2019), agriculture (Motta et al., 2018; Muñoz-Colmenero et al., 2020), natural habitat, floral resources, and wild bee diversity in the landscape can all shape microbe composition (Cohen et al., 2020; Shell & Rehan, 2022). In previous studies of *Osmia lignaria,* the presence of some microbes, such as *Apilactobacillus sp.,* was associated with increased green spaces and an increased relative rarified amplicon sequence variant (ASV) abundance in bees from less developed landscapes as opposed to urban and highly developed sites (Cohen et al., 2020). Thus, microbial members within the microbiome are subject to many different factors that can change their abundance, composition, and diversity.

The impact of a changing environment on bees and their microbiome needs to be studied as urbanization and anthropogenic activities continue to alter bee habitats in growing cities (Ayers & Rehan, 2021; Prendergast et al., 2022; Ritchie & Roser, 2018; Wilson & Jamieson, 2019). With more than half of the world currently living in urban areas and projections predicting this number to increase to two thirds of the global population living in a city centre by 2050 (Ritchie & Roser, 2018), the growth of large, developed areas is undeniable. Decreased availability of green space and the urban heat island effect tends to result in increased temperatures and reduced precipitation in these areas (Rinner & Ussain, 2011; Steensen et al., 2022). Urbanization affects the availability of green space, abundance and richness of floral resources, microclimate, and habitat quality for bees, changing the landscape features that can shape bee microbiomes (Ayers & Rehan, 2021; Cohen et al., 2020, 2022; Danko et al., 2021; Goulson et al., 2015). Floral abundances and garden sizes have a direct, positive effect on parasite and pathogen richness that is harmful to bumble bees, attributable to increased transmission from more resource provisioning (Cohen et al., 2022). Wild bumble bees have also been shown to harbour pesticides in both agricultural and urban landscapes (Botías et al., 2017),

potentially jeopardizing microbial composition (Kakumanu et al., 2016; Rothman et al., 2020). Characterizing the microbiome of urban bees and how its composition and diversity varies across different landscapes offers an essential step towards understanding contributing factors to changes in bee health.

This study examines the small carpenter bee *C. calcarata* Robertson (Hymenoptera: Apidae). These subsocial bees nest within pithy stems, laying eggs on mass provisions that will provide brood the total nutrition required until they are fully grown (Michener, 2007; Rehan & Richards, 2010). Numerous studies have characterized diversity and composition of the microbiome and pollen provisions in *C. calcarata* (Dew et al., 2020; Graystock et al., 2017; McFrederick & Rehan, 2016; Nguyen & Rehan, 2022a). In adult bees this core microbiome consists of 13 bacterial phylotypes, including *Lactobacillus, Acinetobacter, Methylobacterium, Pseudomonas,* and *Gilliamella* (Graystock et al., 2017; Shell & Rehan, 2022), several of which are common in other bee microbiomes as well (Kapheim et al., 2021; Vásquez et al., 2012; Voulgari-Kokota, Grimmer, et al., 2019). The *C. calcarata* fungal microbiome includes members such as *Alternaria, Ascosphaera* and *Penicillium* (Nguyen & Rehan, 2022a). However, despite various characterizations of this small carpenter bee bacterial and fungal microbiome, closer investigations into the specific factors driving differences in microbial composition and diversity, as well as the functional role of different microbial taxa on maintaining bee health, are fundamental.

The aim of this study is to determine whether the microbiome of adult *C. calcarata* differs across an urbanization gradient including local environmental features: percent land use development, percent green space, temperature, and precipitation. Using 16S and ITS metabarcoding, we examined the respective bacterial and fungal composition and diversity

within 58 female small carpenter bees collected across a densely urban landscape, with different levels of urbanization. Here, we hypothesize that bees living under different environmental conditions across an urban land use gradient will result in varying microbial composition. We predict lower microbial diversity and the underrepresentation of beneficial microbes in more urban and developed areas with less available green space, increased temperatures, and reduced precipitation. This research aims to understand the differences in the microbiomes of wild bees living under different levels of urbanization.

Methods

In June-July 2019 and 2020, 58 female individuals of *C. calcarata* Robertson (Hymenoptera: Apidae) were collected across 29 sites within Toronto, Canada (43.6532° N, 79.3832° W) (Figure 2.1). Between one and three bees were selected from each site and sites were chosen to cover a widespread area across the city. *Ceratina calcarata* is a native small carpenter bee commonly found in urban and rural contexts across eastern North America, including within the city of Toronto (City of Toronto, 2016; Dew et al., 2020; Kelemen & Rehan, 2021; Packer et al., 2007; Shell & Rehan, 2016). Nests established in the pithy stems of sumac, *Rhus typhina,* were opened with the lone adult female being removed from each collected nest, flash frozen in liquid nitrogen, and stored at -80°C until DNA extractions.

The collection map was created using the Sentinel-2 land use/land cover timeseries from 2017-2021 by Impact Observatory, Microsoft and Esri at a resolution of 10m (Karra et al., 2021) (Figure 2.1). Collection sites were characterized into five different levels of urban intensity using measurements of the developmental percent, percent green space, mean annual temperature, and

annual precipitation (Table S2.1). These categories were assigned by evenly dividing the range of values for each environmental variable into five categories ranging from 1 (very low) to 5 (very high). Landscape features of developmental percent and percent green space were calculated at each collection site using the Ontario Land Cover Compilation (OLCC) v.2.0 in ArcGIS as a percentage of landscape cover within a 500m radius from the collection point (Land Information Ontario, 2019). Climate data, including mean annual temperature and annual precipitation, were calculated using the same process with WorldClim v.2.0 data at a resolution of 30 seconds (Fick & Hijmans, 2017). These features provide an overall characterization of urban land use gradients in the study region and were divided into the five categorical levels for later analyses (Table S2.1).

DNA extractions were performed using the Omega-Biotek E.Z.N.A. Soil DNA kit, following the manufacturer's protocol for 100-250 mg samples, with some modifications as described in Nguyen & Rehan (2022). This included the addition of 100µg of 1xPBS, 30µL of proteinase K, 5µL of RNAse and manual crushing of the bees using a sterile pestle. DNA concentrations were checked using a QuBit HS DNA assay (Invitrogen) prior to submission to the Génome Québec Centre D'Expertise et de Services (Montreal, Canada), who conducted library preparation and sequencing. Illumina MiSeq amplicon sequencing with 300bp paired-end reads was conducted using the 16S rRNA region for bacteria with the V5-V6 fragment (forward primer 799F-mod3 CMGGATTAGATACCCKGG and reverse primer modified 1115R AGGGTTGCGCTCGTTG) as in McFrederick and Rehan (2020) and the ITS region for fungi with the ITS1 fragment (forward primer ITS1F CTTGGTCATTTAGAGGAAGTAA and reverse primer ITS2 GCTGCGTTCTTCATCGATGC).

Qiime2 was then used to process reads for microbiome analysis (Bolyen et al., 2019). Demultiplexed sequences underwent sequence quality control using the DADA2 pipeline, which filters phiX reads, chimeric sequences, and joins paired ends (Callahan et al., 2016). Sequences were omitted when quality scores dropped below 30 and read lengths fell below 283 bases for forward reads and 260 bases for reverse reads. Qiime2 was also used to generate feature tables, representative sequences, and taxonomy tables (Bolyen et al., 2019; Katoh & Standley, 2013; McDonald et al., 2012; Price et al., 2010; Weiss et al., 2017a). ASVs were tested against the SILVA 128 99% OTUs full length sequences classifier for 16S bacterial sequences and the UNITE 99% OTUs classifier for ITS sequences using the q2-feature-classifier and classify-sklearn pipeline (Abarenkov et al., 2021; Bokulich et al., 2018; Pedregosa et al., 2011; Yilmaz et al., 2014). The SILVA database with 99% sequence identity was used for its refinement and removal of duplicate sequences (Glöckner et al., 2017). Taxonomic classifications were then cross referenced against the NCBI nt database using BLAST, where classifications from the NCBI database were used to clarify and prioritized when there were any discrepancies within the two classifiers (Johnson et al., 2008; Johnson et al., 2021).

Resultant amplicon sequence variants (ASVs) read counts and taxonomic classification tables for each ASV were imported into R (version 3.6.1) for further statistical analysis (R Core Team, 2019). ASVs of the genera *Wolbachia* and *Sodalis* were removed as they are common intracellular endosymbionts present due to mite contamination (Graystock et al., 2017). While one blank did not contain any reads, ASVs identified in the other two of blanks were reagent or human-sourced contaminants and either absent in all samples or had low read counts of less than 50 reads. Using the “phyloseq” package, reads from three blanks were proportionally removed (McMurdie & Holmes, 2013).

Alpha and beta diversity analyses, measured using the Shannon diversity index and Bray-Curtis dissimilarity respectively, were conducted using the "phyloseq" package (McMurdie & Holmes, 2013). The adonis function was used to conduct permutational multivariate analyses (PERMANOVA) that test whether microbial composition varies significantly in the different levels of urbanization (Oksanen et al., 2020). Assumptions required for the PERMANOVA test were validated using the betadisper function and significant results followed up with Tukey's HSD test (Oksanen et al., 2020).

Bipartite networks were created using the "bipartite" package in R, examining associations between the top 18 bacterial and fungal taxa and land use development gradients (Dormann, 2011; Dormann et al., 2008, 2009). Statistics were calculated at the species level, examining the degree of connectance, effective number of interacting partners, Shannon diversity of interactions, and closeness centrality in a weighted network across five categories of land use development (Dormann et al., 2008). Redundancy analyses (RDA) were conducted using the rda function from the "vegan" package (Oksanen et al., 2020). Using the decostand function in "vegan", the Hellinger transformation was applied to taxa abundances and the environmental variables were standardized prior to RDA analyses (Legendre & Gallagher, 2001). An ANOVA like permutation test was performed with the anova.cca function to determine the significance of which environmental features could model microbe abundance.

Similarity percentage (SIMPER) values were calculated within the PAST (version 4.07) program to identify taxa predominantly leading to differences in diversity (Hammer et al., 2001). Furthermore, correlation analyses using CoNet and SparCC were conducted to find co-occurring bacterial and fungal taxa amongst all the bees. CoNet was performed using the package "CoNetinR" and edge scores calculated with Spearman, Bray, Pearson, and Kullback-Leibler

(Faust & Raes, 2016). The package "SpiecEasi" was used to conduct SparCC analyses with 100 bootstrap replicates (Friedman & Alm, 2012).

Results

Metabarcoding of the 58 adult *C. calcarata* resulted in an average of 31,394 reads, ranging from 19,860 to 43,593 paired-end reads per sample. The average quality of these reads was 34.5. A total of 192 bacterial and 367 fungal amplicon sequence variants (ASVs) with a mean sequence length of 317 bp were found and compared across 58 bee samples.

Diversity

Microbial community composition did not reveal differences across urban land use gradients through alpha diversity or due to sample collection date over the range of two years (Figure S2.1). Sample collection year did not associate with any differences in the alpha diversity, beta diversity, or relative abundance of bacterial and fungal taxa. Shannon diversity index comparisons across each environmental variable revealed no overall significant differences in microbial alpha diversity among the five categorical levels of developmental percent, green space percent, temperature, or precipitation (Figure S2.1).

Bray-Curtis dissimilarities revealed bacterial and fungal differences in beta diversity across three environmental variables including land use development, precipitation and temperature (Figures 2.2, S2.2). Land use development percent was associated with both bacterial and fungal beta diversity (PERMANOVA; bacteria, $R^2 = 0.10$, df = 4, $p = 0.017$; fungi, $R^2 = 0.10$, df = 4, $p = 0.023$; Figure 2.2). Figure 2.2A indicates that individuals from moderate to very high levels of development were similar in microbial composition and dissimilar to

individuals from sites with very low to low levels of development. Samples from sites with very high development had more bacterial genera richness than sites with low development (Table S2.2), corroborating that development positively associates with bacterial richness. However, green space percent was not a significant factor in determining differences in Bray-Curtis diversity (PERMANOVA; bacteria, $R^2 = 0.07$, df = 4, $p = 0.18$; fungi, $R^2 = 0.09$, df = 4, $p = 0.056$; Figure S2.2AB).

Temperature explained variations in fungal beta diversity (PERMANOVA; fungi, $R^2 = 0.09$, df = 4, $p = 0.021$; Figure S2.2D), while bacterial beta diversity did not pass the test for homogeneity of multivariate dispersions with temperature (betadisper; bacteria, F = 3.13, df = 4, $p = 0.018$; fungi, F= 0.33, df = 4, $p = 0.855$; Figure S2.2C). As for precipitation gradients, fungal beta diversity differences were detected, while bacterial beta diversity differences were insignificant (PERMANOVA; bacteria, $R^2 = 0.08$, df = 4, $p = 0.181$; fungi, $R^2 = 0.12$, df = 4, $p = 0.002$; Figure S2.2E-F). Clear clustering was less evident for the fungal PCoA, suggesting increased dissimilarity between individuals (Figure 2.2B). One group of fungal samples that were clustered, indicating similar beta diversity, tended to have moderate to high annual temperatures, and low to moderate annual precipitation (Figure S2.2 D&F). This was also consistent comparing the average number of genera across the environmental variables, which saw that the low to moderate development, high temperature, and low precipitation categories had the highest fungal genera richness (Table S2.3). However, the individuals grouped closely on the PCoA were spread across different levels of land use development and green space, suggesting the environmental variables are not always correlated with each other. As temperature, green space, and precipitation were not significant environmental variables for

bacteria, this comparative analyses between the environmental variables could not be performed for the bacterial PCoA.

An RDA was conducted and analyzed using an ANOVA with 999 permutations on all four environmental variables to determine which variables were associated with bacterial and fungal taxa (Figure 2.3). Development was significant (ANOVA; bacteria, F= 1.86, df = 1, p = 0.032; Figure 2.3A*)* in associations between urbanization level and bacterial taxa. Green space was significantly associated with both bacterial and fungal taxa (ANOVA; bacteria, F = 1.86, df = 1, p = 0.037; fungi, F = 2.45, df = 1, p = 0.002; Figure 2.3AB). Precipitation was also key in the RDA analyses for fungal taxa (ANOVA; fungi, F = 1.86, df = 1, p = 0.012; Figure 2.3B) with variation in precipitation explaining variation in fungal taxa. In addition to the RDA with all environmental variables, forward selection modelling was performed to select the driving environment variables that could predict diversity. Bacterial taxa revealed a significant model (ANOVA; bacteria, F = 1.80, df = 1, p = 0.016, adjusted R-squared = 0.017) associated with development (ANOVA; bacteria, F = 1.86, df = 1, p = 0.036) and temperature (ANOVA; bacteria, F = 1.74, df = 1, p = 0.046). The fungal model resulted in a different significant model (ANOVA; F = 1.97, df = 1, p = 0.007, adjusted R-squared value of 0.17) involving only temperature (ANOVA, fungi, F = 1.97, df = 1, p = 0.005) predicting fungal taxa.

Taxonomy

Across the 58 samples, the bacterial genera *Acinetobacter, Apilactobacillus*, *Nocardia,* and *Saccharibacter* had the greatest summed relative abundances amongst all the bacterial genera (Table S2.2). Particularly notable, *Apilactobacillus* had a relative abundance of over 50%

of the total reads in 25 samples (Figure 2.4A). A low amount of bacterial diversity is noticed amongst the adults, as 26 samples contained reads from only one genus and 12 samples had two bacterial genera (Figure 2.4A, Table S2.2). Overall, there was an average of 3.6 bacterial genera associated with each bee. In terms of fungi, *Alternaria, Ascosphaera*, and *Penicillium* had the greatest summed relative abundances and were common genera in the bee microbiome (Table S2.3). Fungal genera richness was higher than bacterial, with an average of 6.5 genera per individual (Figure 2.4B, Table S2.3).

Similarity percentage (SIMPER) analyses corroborated bacterial and fungal relative abundances were driven by environmental features (Table S2.4). Some bacteria and fungi are typically overrepresented at either high levels of development or green space, suggesting patterns along an urbanization gradient (Table S2.4). For example, *Acinetobacter* and *Saccharibacter* had high abundances in very low levels of development (Table S2.4). On the contrary, *Lactobacillus* bacteria were found mostly in areas with moderate to high levels of development (Table S2.4). *Apilactobacillus* was simultaneously overrepresented in areas with the highest amount of green space, least amount of development, and high levels of development (Table S2.4). In terms of fungi, *Ascosphaera* was similarly abundant at both ends of the spectrum at low, high, and very high levels of development (Table S2.4). *Taphrina* fungi were overrepresented in areas with very low levels of development, whereas the opposite was true for *Zygosaccharomyces* being overrepresented in areas of high development (Table S2.4). Differences in taxa abundances were also apparent across varying precipitation and temperature gradients, with *Alternaria* being underrepresented with increased levels of precipitation and *Apilactobacillus* most abundant in environments with low annual temperature (Table S2.4). In examining the clustered individuals on the fungal PCoA from sites with low precipitation and high temperatures (Figure S2.2 D&F),

Alternaria was present in all these samples, with other common fungi including *Mortierella* and *Ascosphaera*. Thus, urbanization markedly characterizes disparate overrepresentations in bacteria and fungi.

To examine the uniqueness and connectedness of the microbiome across an urbanization gradient, bipartite networks were used to examine associations between different levels of development and microbial genera (Table 2.1, S2.5, Figure S2.3). The bacterial network resulted in overall lower levels of connectance (bacteria, 0.54; fungi, 0.82), links per species (bacteria, 2.13; fungi, 3.26), and Shannon diversity (bacteria, 1.56; fungi, 2.53) when compared to the fungal network. Bees from sites with the greatest percent development were found to have a higher degree of bacterial connectance, Shannon diversity of interactions, and effective partners, while also inversely showing low weighted closeness, when compared to areas with the least percent development (Table 2.1). Sites with low to moderate and very high percent development showed more abundant but less specialized relationships between bacteria and bees. This pattern held for fungi at a moderate level of land use development (Table 2.1). Thus, fungal networks maintained more consistency across the urban land use gradients. Networks revealed certain microbes like *Apilactobacillus, Alternaria, Ascosphaera*, and *Penicillium* had high degrees of connectance across all five development levels, whereas others had low degrees of connectance and were associated with an urbanization level. For example, *Clostridium* and *Saccharibacillus* were only found in very high levels of development and *Enterobacter* and *Samsoniella* were associated with very low levels of development.

Bacterial and Fungal Co-Occurrences

CoNet and SparCC analyses were used to determine if any bacterial and fungal associations were found among the top 10 bacterial and fungal taxa across all 58 samples (Table 2.2, Tables S2.6-S2.7). Using all individuals, CoNet revealed 17 associations (Table S2.6), while SparCC presented 28 total co-occurrences of positive and negative correlations (Table S2.7). Interestingly, a positive association between bacteria-bacteria was found to be significant across both statistical analyses in *Sphingomonas* and *Saccharibacillus* (CoNet, correlation = 0.31, p = 0.019*;* SparCC, correlation = 0.53, $p < 0.01$; Tables S2.6-S2.7). Additionally, separate analyses for each land use development level revealed patterns in co-occurrences. Through CoNet analyses, *Metholybacterium* was only found not correlated to *Sphingomonas* in the very low and moderate development levels, while *Acinetobacter* and *Sphingomonas* were associated only in the moderate to high development levels (Table 2.2). Associations between fungi-bacteria and fungi-fungi were absent in densely urban areas and only seen in sites with the lowest land use development, with the rest of the associations only existing between bacteria (Table 2.2).

Discussion

This study examines the bacterial and fungal microbiome in 58 *C. calcarata* individuals across an urban land use gradient. Percent development, percent green space, annual temperature, and annual precipitation were examined to determine how environmental factors may drive differences in microbial diversity and composition. While alpha diversity did not differ across the city, beta diversity and redundancy analysis modelling could be predicted by percent development and temperature. Taxonomic comparisons also revealed some bacterial and fungal taxa were more commonly found in either very low or highly developed areas of the city, indicating differences in the microbiome between urban land use gradients. Different levels of

land use development also result in varying degrees of connectance in networks and different co-occurrences between microbes.

Microbial diversity

Shannon's diversity indices, a measure of alpha diversity, did not vary with environmental variables of development, green space, precipitation, or temperature and yielded low values of bacterial diversity, where more than half of the samples only contained one or two genera (Figure S2.1). This aligns with a previous study of *C. calcarata* in Toronto, which found little change and overall low microbial alpha diversity as bees matured from brood to adults (Nguyen & Rehan, 2022a). Similarly, a study comparing the stingless bee *Tetragonula carbonaria* microbiome between two different sites also showed alpha diversity remaining consistent, despite climatic and floral resource differences (Hall et al., 2021). Although studies with stingless bees have revealed the presence of environmental bacteria in the microbiome (Cerqueira et al., 2021; Kwong, Medina, et al., 2017), social bees often have a core microbiota and low diversity (Kwong & Moran, 2016). However, this is not representative of solitary wild bees, such as in a study with *Osmia lignaria* across different environmental contexts, which found that environmental factors drove differences in relative ASV abundances and alpha diversity (Cohen et al., 2020). Yet, as many factors affect microbiome alpha diversity it remains difficult to segregate how factors are affecting overall diversity in isolation and how a combination of environmental or situational variables co-occurring can affect alpha diversity.

Beta diversity, represented by Bray-Curtis dissimilarity matrices (Figure 2.2), was able to capture more of the microbial differences driven by urban land use gradients. Percent

development was the most significant factor, with development associating with both bacterial and fungal Bray-Curtis dissimilarities, high development showing increased bacterial genera richness, and with development able to predict bacterial diversity through the redundancy modelling analyses (Figures 2.2 & 2.3, Table S2.2). Annual temperature was another considerable variable, yielding a significant ability to model differences in both bacterial and fungal microbiomes and associate with fungal beta diversity (Figure S2.2F). Collectively, the interplay between these environmental characteristics may be dynamically changing microbial diversity, as features such as high temperature and low precipitation can act together to foster higher fungal genera richness and clustered Bray-Curtis dissimilarities, as seen in the circled individuals on the PCoA plots (Table S2.3, Figures S2.2 D & F). However, these samples did not cluster with either land use development or green space, suggesting that the correlation between environmental variables is unclear (Figures 2.2 & S2.2). Environmental features were expected to be a factor determining differences in beta diversity, as was initially seen in a study comparing two colonies of stingless bees at different locations (Hall et al., 2021), and in *O. lignaria* when dissimilarity matrices could be predicted by percent natural cover, number of trees and shrubs, bee species richness, and bare soil (Cohen et al., 2020). Similarly, McFrederick and Rehan (2019) found different species richness of fungi and bacteria when comparing subtropical, temperate and grassland zones across Australia, suggesting that climate shapes the *C. australensis* microbiome. Thus, environmental characteristics describing both land use and climate affect the microbial diversity of individual *C. calcarata* microbiomes.

Microbial composition

Apilactobacillus, *Alternaria*, *Penicillium* and *Ascosphaera* were the most prevalent and abundant bacterial and fungal genera found across the city (Table S2.2, S2.3). *Apilactobacillus* are common beneficial bee symbionts (Tlais et al., 2022) and were established as part of the core microbiome in *C. calcarata* in New Hampshire, a more rural landscape (McFrederick and Rehan, 2016; Graystock et al., 2017). In urban cities such as Toronto, *Apilactobacillus* was previously largely absent in adult *C. calcarata* (Nguyen & Rehan, 2022a) and was found to be underrepresented at sites with moderate levels of land use development, overrepresented in sites with the most green space, and overrepresented at sites with lower annual temperatures in this study (Table S2.4). Thus, urban bees reveal a different microbiome from those in rural contexts and of particular concern is the varying abundance of *Apilactobacillus*.

The fungal genus *Ascosphaera* contains both pathogenic and apathogenic fungi (Klinger et al., 2013), and the species *A. major* was common in *C. calcarata* (Table S2.3). This species has caused chalkbrood-like diseases in *Megachile centuncularis* and *Apis mellifera*, but can also live relatively harmlessly as a facultative parasite within bee nests on pollen provisions and larval feces, including other wild bees such as *Osmia bicornis* (Bissett, 1988; Holm & Skou, 1972; Anja A. Wynns et al., 2013). Therefore, the abundance of *Ascosphaera* may indicate a commensalism between *C. calcarata* and these bee specialist fungi. Future studies are needed to determine the fitness effects of *Ascosphaera* on this species.

Overrepresentations of certain bacterial and fungal taxa at sites of varying land use development may indicate such factors are affecting microbial composition. Areas with a low percentage of development were found to have a greater abundance of *Acinetobacter, Ascosphaera, Saccharibacter,* and *Taphrina* (Table S2.4). *Acinetobacter* is a flower-associated species of bacteria also commonly associated with yeasts in nectar which can induce germination

and pollen bursting that then benefits pollinators by way of improved nutrition from nectar (Christensen et al., 2021; Rering et al., 2021). Another flower-associated bacteria, *Saccharibacter*, is closely related to the bacteria *Bombella apis* which is known to protect developing honey bees from fungal pathogens and contains genetic loci involved with nutrition, microbial and host interactions, and immunity (Smith & Newton, 2020). Thus, the overrepresentation of beneficial microbes in areas with low land use development is promising for these pollinators. On the contrary, the two fungal genera found in high abundance in more rural areas, *Ascosphaera* and *Taphrina,* are facultative bee and plant pathogens respectively (Cissé et al., 2013; Anja A. Wynns et al., 2013). However, these genera were also previously seen in immature *C. calcarata* and it is unclear if they pose any threat to this species (Nguyen & Rehan, 2022a). *Ascosphaera* was also overrepresented at high and very high development levels (Table S2.4, Figures 2.4B), suggesting this fungi may not be limited to rural areas.

The overrepresented genera present in sites with a high percentage of development, such as *Lactobacillus*, *Penicillium,* and *Zygosaccharomyces* (Table S2.4)*,* were not microbes that are known to be harmful to bee health. *Lactobacillus spp.,* such as *L. crispatus* and *L. intestinalis*, have been seen in *A. mellifera, Bombus terrestris,* and *O. bicornis* (Mohr & Tebbe, 2006)*,* and many studies have uncovered the important and beneficial relationship between *Lactobacillus* and bees (L. Li et al., 2021; Rothman et al., 2019). *Penicillium* molds are commonly found in *Melipona scutellaris* (Barbosa et al., 2018) and *A. mellifera* bee bread and is of importance as it produces enzymes involved in lipid, protein, and carbohydrate metabolism that can even protect bees against fungicides (Gilliam et al., 1989; Yoder et al., 2013). *Zygosaccharomyces sp.* are fungi that have been shown to provide steroid precursors crucial for the pupation of the stingless bee *Scaptotrigona depilis* (Paludo et al., 2018). Hence, while differing from areas of low land use

development, bacteria and fungi found in urban bees may be supported by their own beneficial properties to their bee hosts. Regardless of urbanization, different overrepresentations of bacteria and fungi may serve varying, but equally beneficial, purposes. Areas with low development seem to harbour plant associated microbes that may be associated with natural plant availability, whereas high development sites contain microbes more associated with bee development and digestion.

Co-occurrences between microbes have been studied in bees and pollen to examine how microbial members interact and establish the microbiome (Dew et al., 2020; Graystock et al., 2017; Manirajan et al., 2018; Russell et al., 2011). This study found a strong positive relationship between *Sphingomonas* and *Saccharibacillus* when examining adults (Table S2.6-S2.7). While *Saccharibacillus* has been found in commercial bee pollen from Europe, little is known about its interactions and presence in bee microbiomes (Andrade et al., 2019). Bacteria of the genus *Sphingomonas* have been shown to be negatively correlated with *Fusarium* species that cause Fusarium Head Blight in maize crops (Cobo-Díaz et al., 2019). In *C. calcarata*, *Sphingomonas* co-occurred positively with the fungal genera *Pantoea* (Nguyen & Rehan, 2022a), a genus prevalent in *C. australensis* (Shell & Rehan, 2022), *A. mellifera* (Wright et al., 2001), and stingless bees (Leonhardt & Kaltenpoth, 2014). Additionally, *Sphingomonas* is a dominant bacteria found in the nests of stingless bees *Frieseomelitta varia, Melipona quadrifasciata,* and *Tetragonisca angustula* (de Sousa, 2021) and is also found in *A. mellifera* (Anjum et al., 2018; Muñoz-Colmenero et al., 2020) and *O. bicornis* microbiomes (Mohr & Tebbe, 2006), thus this bacteria co-occurs naturally with wild bees. In regards to urban and agricultural bees, this bacteria may be particularly beneficial as it contains enzymes that degrade organochlorides in

insecticides (Russell et al., 2011). Therefore, commonly occurring bacteria, such as *Sphingomonas*, may be playing an underappreciated role in the wild bee microbiome.

Microbe correlations can be examined considering land use development to determine if environmental factors may be affecting the stability of these associations. *Methylobacterium* was correlated with *Sphingomonas* in all but the low and moderate percent development sites (Table 2.2). *Methylobacterium* have shown beneficial relationships with plants and bacteria, sometimes even relying on growth factors produced by other microbes (Iguchi et al., 2015; Manirajan et al., 2018). The number of correlations present also increased with percent developed area, suggesting that urbanized areas may be associated with more positive co-occurrences between bacteria. This has been seen in urban soils, where environmental features altered microbial networks (H. Wang et al., 2018). Therefore, landscape features may be changing the way bacteria and fungi are supported, which can in turn affect the presence and abundance of these and other microbes. Further examination into the functional role of specific microbes as well as how they exist in symbioses is needed to explain how these networks are maintained.

Effects of a changing environment

Changes in microbial diversity and composition are of potential concern because bee-microbe symbioses play a key role maintaining bee health (Dharampal et al., 2019, 2020; Engel et al., 2012; Mockler et al., 2018; Rothman et al., 2019). While this study described several abundant bacterial and fungal genera dominating the microbiome, this is a large contrast to previous studies of *C. calcarata* that revealed 13 core bacterial phylotypes in bees and much more diversity (Graystock et al., 2017). The reason for bacteria showing decreased diversity

compared to fungi, as seen in lower Shannon diversity measures, lower degrees of network connectance, and fewer effective partners (Table 2.2), remains unclear. This low bacterial diversity in adults was also found in a previous study of *C. calcarata* in Toronto, particularly when compared to developing brood, and suggests a persistent and concerning decrease in microbial diversity in urban landscapes (Nguyen & Rehan, 2022a). Ongoing long-term and additional studies are needed to examine whether the few bacterial genera currently making up the microbiome are excluding other bacteria and/or whether bees in cities have generally less diverse microbiomes in this and other wild bee species.

As the urban land use gradients such as percent development and temperature reveal their effects on the bee microbiome, rapid urbanization becomes increasingly alarming. Urbanization and anthropogenic activities are a worsening problem driving declines in bee populations, altering bee community compositions, and negatively affecting certain bee species (Ayers & Rehan, 2021; Prendergast et al., 2022; Ritchie & Roser, 2018; Wilson & Jamieson, 2019). In addition, the presence of pesticides in urban areas may be driving declines in bee health and in bee microbiome structure, composition, and diversity (Botías et al., 2017; Hotchkiss et al., 2022; Kakumanu et al., 2016). Future work comparing a wider range of rural and agricultural landscapes across multiple regions will help determine how bee microbiomes change with land use. Additional studies examining pesticides present in bee habitats in urban and rural areas and how these accumulate in bees and their pollen provisions will also be important (Botías et al., 2017; Hotchkiss et al., 2022; Kakumanu et al., 2016; Pisa et al., 2014). As research continues to untangle the variables that work together to establish and maintain the wild bee microbiome, the ever-changing landscape in cities adds new considerations for possible environmental stressors.

In conclusion, this study examines the bacterial and fungal composition and diversity in adult *C. calcarata* across an urban land use gradient, revealing differences explained by percent land use development, green space, precipitation, and temperature. Individuals from low to moderate development levels tended to share similar bacterial composition within one cluster, while those from moderate to high development levels grouped separately. In examining fungal taxa, individuals showed greater dissimilarity in beta diversity, with climatic variables as possible drivers. The interplay of environmental factors across urbanization gradients act on microbial community composition, with overlapping characteristics such as annual temperature and annual precipitation coinciding with fungal beta diversity. Microbial composition in rural areas were dominated by genera such as *Acinetobacter* and *Apilactobacillus,* beneficial microbes that support bee health and may affect bee survival. Specific taxa varied across different levels of urbanization, which may be explained by co-occurrences between bacteria and fungi that varied amongst different land use development, and suggests that microbial relationships are dependent on changes in environment. These complex networks reveal that urban areas may exhibit a stronger degree of connectance in bacteria, while lower levels of urbanization foster greater connectance within the fungal microbiome. Overall, increased urbanization has led to a significant impact on microbial composition and diversity. As cities continue to expand and urbanization rises globally, it is increasingly important to understand how landscapes affect bee health through their microbiome.

Tables and Figures from Chapter II

Table 2.1. Summary of bipartite network level statistics comparing the association between the top 18 bacterial and fungal taxa across five levels of development.

	Network level statistics	Dev 1	Dev 2	Dev 3	Dev 4	Dev 5
Bacteria	Degree	8	6	10	10	15
	Effective partners	1.45	2.15	2.11	1.06	2.33
	Shannon diversity	0.34	0.77	0.75	0.06	0.85
	Weighted closeness	0.55	0.03	0.06	0.51	0.12
Fungi	Degree	14	14	17	14	16
	Effective partners	1.97	3.43	4.50	2.07	3.80
	Shannon diversity	0.68	1.23	1.50	0.73	1.33
	Weighted closeness	0.35	0.08	0.19	0.34	0.28

The five categories range from Dev 1 (very low development) through Dev 5 (very high development).

Table 2.2 CoNet correlations between bacteria-bacteria, fungi-fungi, and fungi-bacteria within the top 10 bacterial and fungal genera found in 58 Ceratina calcarata across five levels of land use development.

Development level	Correlated taxa	Correlation	*p*-value
DEV-1 ($n = 16$)	*Alternaria – Penicillium*	0.81	0.013
	Sarocladium – Saccharibacter	0.75	0.015
DEV-2 ($n = 6$)	*Carnimonas – Erwinia*	1.00	<0.01
	Methylobacterium – Sphingomonas	1.00	<0.01
DEV-3 ($n = 11$)	*Acinetobacter – Sphingomonas*	1.00	<0.01
DEV-4 ($n = 13$)	*Acinetobacter – Methylobacterium*	1.00	<0.01
	Acinetobacter – Sphingomonas	1.00	<0.01
	Methylobacterium – Sphingomonas	1.00	<0.01
DEV-5 ($n = 12$)	*Acinetobacter – Saccharibacillus*	1.00	<0.01
	Acinetobacter – Erwinia	1.00	<0.01
	Erwinia – Saccharibacillus	1.00	<0.01
	Methylobacterium – Sphingomonas	0.98	<0.01

The five categories range from Dev 1 (very low development) through Dev 5 (very high development). Sample sizes at each land use development level are provided in brackets. A full representation of all correlations is available in the supplement (Supplementary Table S6).

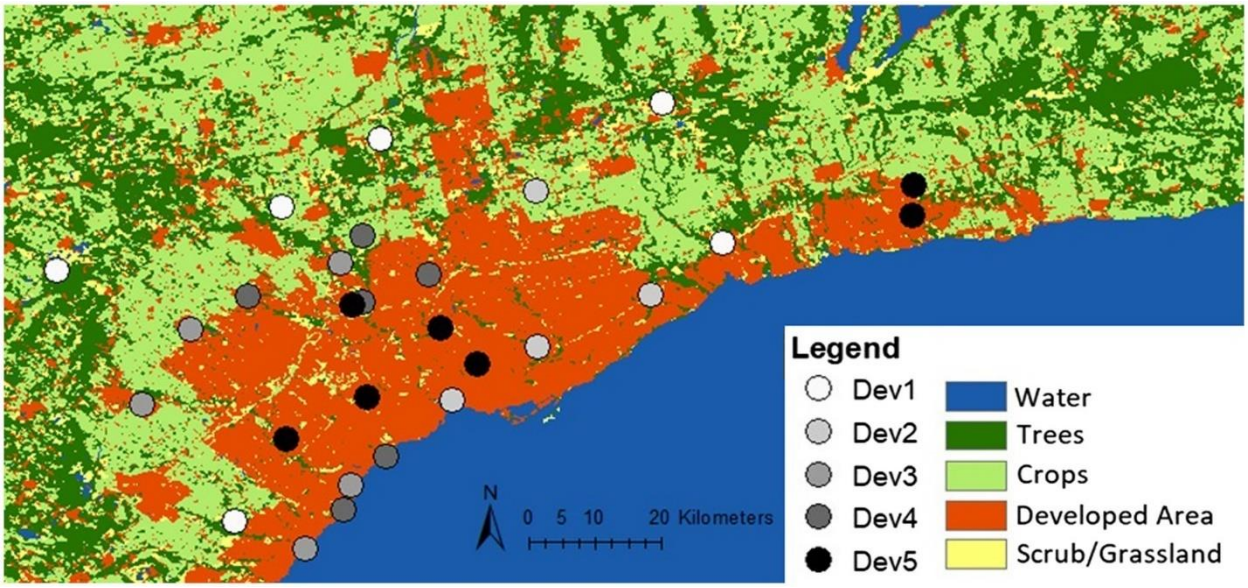

Figure 2.1. Development distribution and collection locations in and around Toronto, Canada using the Sentinel-2, 10 m land use time series from 2017 to 2021 by ESRI. Circles represent 29 collection sites. The trees class includes trees and flooded vegetation. Crops include human planted grass and crops below tree height. Scrub/grassland consists of bare ground and rangeland, or open areas with homogenous grasses. Developed areas are built areas with human made structures, roads, and impervious surfaces.

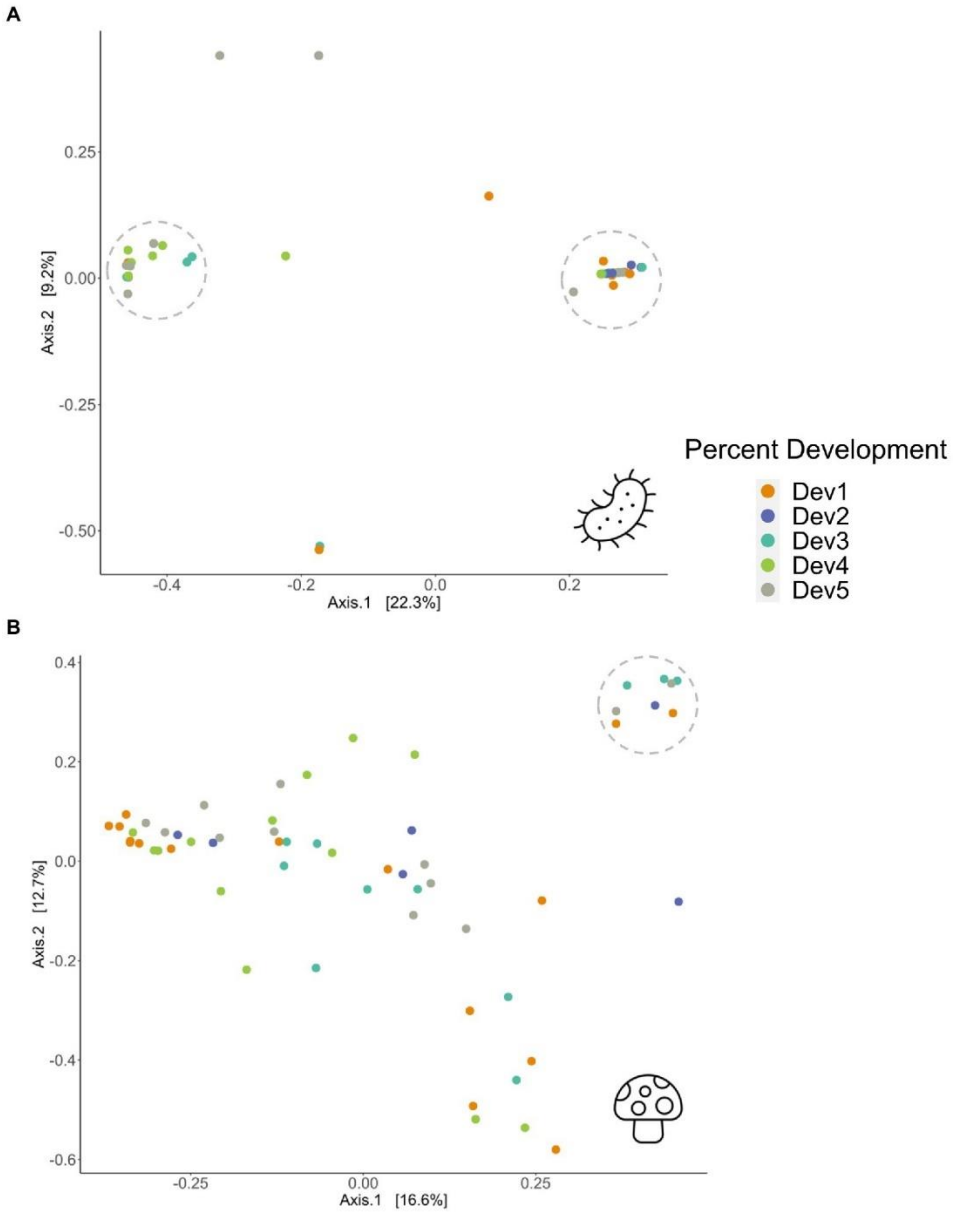

Figure 2.2 PCoA plots of Bray–Curtis dissimilarity matrices showing (A) bacterial ($p = 0.017$) and (B) fungal ($p = 0.023$) beta diversity in 58 adult Ceratina calcarata across five levels of percent development, ranging from Dev 1 (very low development) through Dev 5 (very high development). For exact development percentages, see Supplementary Table S2.1. Dotted circles represent clusters of individuals with similar beta diversity.

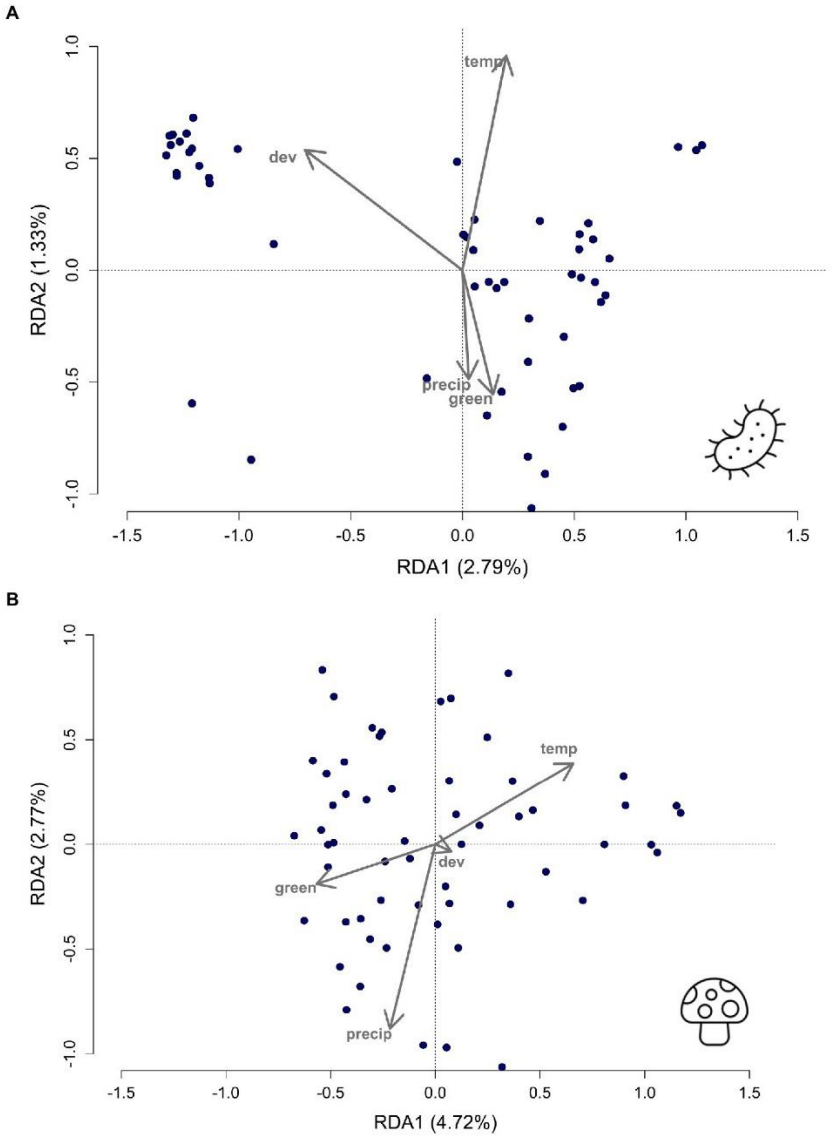

Figure 2.3. Redundancy analyses (RDA) plot showing whether (A) bacterial ($p = 0.030$) and (B) fungal ($p = 0.001$) taxa are associated with the environmental variables of dev = development, green = green space, temp = temperature, and precip = precipitation. Bacterial taxa are influenced by the development ($p = 0.032$) and green space ($p = 0.037$) variables. Fungal taxa are driven by green space ($p = 0.002$) and precipitation ($p = 0.012$).

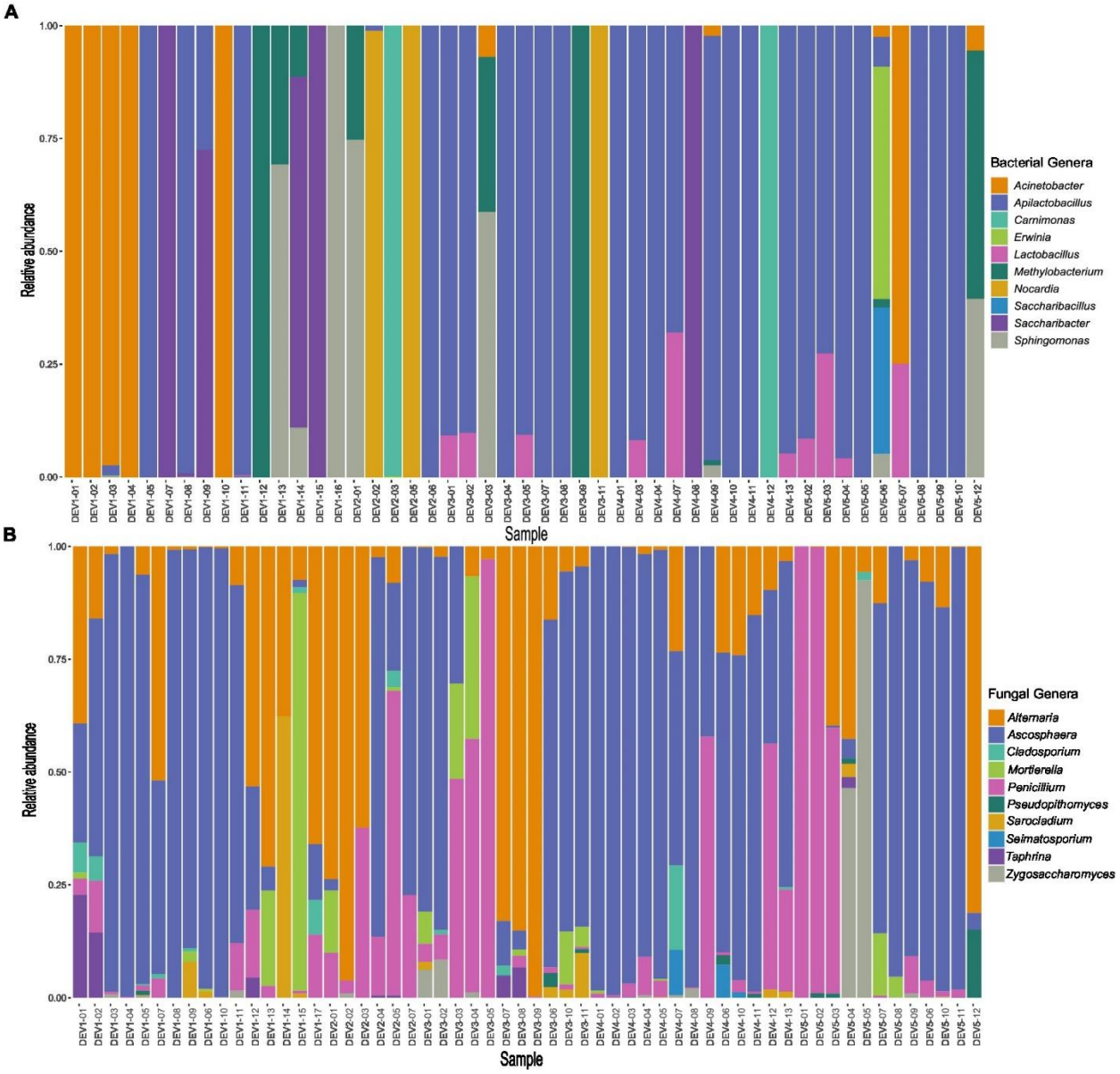

Figure 2.4. Top 10 (A) bacterial and (B) fungal genera found in 58 Ceratina calcarata from different levels of development across Toronto. The five categories range from Dev 1 (very low development) through Dev 5 (very high development).

Chapter III: Wild bee microbiomes across an urban rural divide

Phuong N. Nguyen

Abstract

Wild pollinators and their microbiota are sensitive to land use changes from anthropogenic activities that disrupt landscape and environmental features. As urbanization and agriculture affect the availability of floral resources, green space, microclimates, and pesticide exposures, human led disturbances are driving changes in bee microbiomes, potentially leading to dysbiosis detrimental to bee fitness. This study examines the bacterial, fungal, and plant compositions of the small carpenter bee, *Ceratina calcarata,* and its pollen provisions across an urban rural divide. We performed metabarcoding of *C. calcarata* and provisions in Toronto by targeting the 16S, ITS, and rbcL regions. This showed similar plant composition and diversity despite a greater microbial diversity in pollen provisions than in bees. By characterizing the differences in land use, climate, and pesticide residues that differentiate urban and rural landscapes, we find that urban areas support elevated levels of microbial diversity and more complex networks between microbes and plants than rural areas. However, urban areas may lead to lower relative abundances of known beneficial symbionts and increased levels of pathogens, such as *Ascosphaera* and *Alternaria* fungi. Further, rural pollen provisions indicate elevated pesticide residues that may negatively affect bee health and dysregulate symbiosis. As anthropogenic activities continue to alter land use, ever changing environments threaten microbiota crucial in maintaining bee health.

Introduction

Changes in land use resulting from anthropogenic activities have widescale environmental and ecological implications. The surgency of urbanization has increased demand for expanding cities to support growing populations (Ritchie & Roser, 2018), thereby encouraging the implementation of impervious surfaces and a decline in greenspace. To meet the rising demands for food supply, agricultural expansion is well documented, resulting in increased managed and rural areas used for crops (Laurance et al., 2014). As land use continues to fragment natural environments, the distinction between urban and rural dichotomies are being blurred (van Vliet et al., 2020). By examining and characterizing the environmental features in urban and rural landscapes, a more comprehensive understanding of how human disturbances to landscapes have impacted these ecosystems.

Bees are vital pollinators that are facing climate change and altered habitats, creating threats to global food security (Hung et al., 2018; Marshman et al., 2019). Dependent on species' functional traits, some bees are better able to cope in urban or rural landscapes (Ayers & Rehan, 2023; Escobedo-Kenefic et al., 2022; Hall et al., 2019; Kelemen & Rehan, 2021; Wilson & Jamieson, 2019). However, these pollinators are largely dependent on environmental features such as the floral resources or the types of green space available, meaning some are particularly vulnerable to land use changes that filter bee functional traits and ultimately changes bee community composition (Ayers & Rehan, 2021; Mathiasson & Rehan, 2020). In addition, the unintended side effects of pesticide contamination in both agriculture and urban locations can be detrimental to bee health (Botías et al., 2017; Goulson et al., 2015; Pisa et al., 2014; Raymann et al., 2018; Willis Chan & Raine, 2021). The combination and interaction of all these factors continuously creates challenges for pollinator conservation.

In addition to directly harming certain bees, changes in the environment may also disturb bee microbiota (Nguyen & Rehan, 2023). This disrupts the microbiome, the complex network of bacteria, fungi, and other microorganisms that create important symbioses within bees (Engel & Moran, 2013; Rosenberg et al., 2007). The microbiome plays an important role in maintaining bee health. For example, beneficial bacterial symbionts can offer increased immunity, protection against parasites, and detoxification (Daisley et al., 2020a; 2020b; Rothman et al., 2019; Tauber et al., 2019). The effects can also be physiological, with bee microbes affecting gene expression that improves memory retention (Li et al., 2021; Zhang et al., 2022). An imbalance in these beneficial relationships may lead to dysbiosis or a disruption to the microbiome (Belizário & Faintuch, 2018), further inhibiting bees' abilities to survive stressors and negatively affecting fitness (Daisley et al., 2020; Dharampal et al., 2019; Li et al., 2019).

The small carpenter bee *Ceratina calcarata* is a generalist native to eastern North America and a subsocial bee often nesting in pithy stems such as raspberry and sumac (Michener, 2007; Packer et al., 2007; Rehan & Richards, 2010). *C. calcarata* will lay eggs on pollen provisions within brood cells, which then provides sustenance and a source for microbes that the brood will be exposed to while it is developing (McFrederick et al., 2014, 2017; Michener, 2007; Nguyen & Rehan, 2022a). The core microbiome of *C. calcarata* and its pollen provisions have been previously described to include 13 and 19 bacterial phylotypes, respectively (Graystock et al., 2017; Shell & Rehan, 2022). Metagenomics of *Ceratina* have also identified strong associations between microbiota and host bees which are sensitive to local environmental features (Shell & Rehan, 2022). A characterization of the *C. calcarata* microbiome in Toronto has been examined across developmental stages and an urban land use gradient (Nguyen & Rehan, 2022a, 2022b).

This study seeks to characterize the bacterial and fungal microbiome as well as the plant composition found within small carpenter bee adults and their pollen provisions in Toronto. This is the first characterization of plant compositions and their involvement in microbial networks within these bees and pollen provisions in this study region. Second, this study aims to classify an urban rural divide using land use development, green space availability, annual temperature, precipitation, and pesticide concentration to holistically examine environmental impacts on bee microbiota. It is hypothesized that local land use (Cohen et al., 2022; Dew et al., 2020; McFrederick et al., 2012; McFrederick & Rehan, 2016), climatic changes (C. Li et al., 2022; Nguyen & Rehan, 2022b), and pesticides (Botías et al., 2015, 2017; Julia C. Jones et al., 2018) will be key factors in contributing to a higher microbial diversity and a lack of key symbiotic microbes in urban areas (Bosmans et al., 2018; Cohen et al., 2020; Nguyen & Rehan, 2022b).

Methods

From May to July of 2021 and 2022, adult female *Ceratina calcarata* Robertson (Hymenoptera: Apidae) and an associated pollen provision from the same raspberry stem were collected from five urban (n = 72) (43.7°N-79.5°W) and two agricultural sites (n = 46) (43.4°N, -79.9°W) in the Greater Toronto Area (Figure 3.1). Rural samples were taken from farms at least 40km away from the nearest urban location. All adult bees were flash frozen in liquid nitrogen and stored at -80°C, whereas pollen provisions were kept at -20°C until DNA extraction.

Creation of the collection map was performed in ArcGIS using the Sentinel-2 land use/land cover timeseries from 2017-2021 by Impact Observatory, Microsoft and Esri at 10m resolution (Karra, 2021). The percentage of land use development and green space were

calculated using the Southern Ontario Land Resource Information System (SOLRIS) v3.0 as a percentage of landscape cover within a 500m radius of the collection point (Ministry of Natural Resources and Forestry, 2019). Mean annual precipitation and mean annual temperature were calculated using WorldClim v.2.0 data at a resolution of 30s (Fick & Hijmans, 2017). These environmental variables validated differences between the urban and rural study areas when compared using Kruskal-Wallis rank sum tests in R (R Core Team, 2019), due to non-normality in these variables. Toxicological analyses of pollen balls and adult moms were conducted in two rounds at the Agriculture and Food Laboratory at the University of Guelph Laboratory Services using a TOPS 142 LC-MS/MS multi-residue pesticide screen. This screen utilized pooled 1g samples, with one replicate of pooled bees and two replicates of pooled pollen for each urban and rural area.

DNA extraction was performed using the Qiagen DNeasy Blood and Tissue kit, including modifications previously described (Nguyen & Rehan, 2022a, 2022b). QuBit HS DNA assays (Invitrogen) and a tape station were used to check for quality and concentration prior to sequencing. Urban samples were submitted to the Génome Québec Centre D'Expertise et de Services (Montreal, Canada) and rural samples to the Canadian Centre for DNA Barcoding (Guelph, Canada) where library preparation, sequencing, and demultiplexing were performed. Paired-end Illumina MiSeq amplicon sequencing at 300bp targeted the 16S rRNA region for bacteria (V5-V6 fragment), the ITS region for fungi (ITS1 fragment), and the rbcL region for plants (RBCL7 fragment) as in McFrederick and Rehan (2019).

Raw reads were processed through Qiime2 using DADA2 (Bolyen et al., 2019; Callahan et al., 2016). Amplicon sequence variants (ASVs) were truncated at a forward read length of 150bp for rbcL and 250bp for 16S and ITS reads prior to the creation of feature tables and

representative sequences (Bolyen et al., 2019; Callahan et al., 2016; Katoh & Standley, 2013; McDonald et al., 2012; Weiss et al., 2017b). Taxonomy was preferentially classified using the NCBI nt database using BLAST (Johnson et al., 2008, 2021). Bacterial taxa were additionally tested against the SILVA 128 99% OTUs classifier and fungal taxa tested against the UNITE 99% OTUs classifier for ITS sequences (Abarenkov et al., 2021; Bokulich et al., 2018; Glöckner et al., 2017; Pedregosa et al., 2011; Yilmaz et al., 2014). Plant taxa were manually checked against the USDA Plants Database (USDA, 2023) for native status and further review.

Amplicon sequence variants (ASVs) read counts and taxonomic classification tables were then imported into R, where contaminants of the genera *Wolbachia* and *Sodalis* were removed, along with any ASVs making up less than 1% of the reads within any sample (version 3.6.1) (R Core Team, 2019). Contaminant reads found in blanks were minimal and proportionally removed at counts of less than 100 within an individual using the *phyloseq* package (McMurdie & Holmes, 2013). At this stage, urban and rural samples were combined into one dataset and reads were converted to relative abundance for further analyses.

The *phyloseq* package was also used to measure the alpha and beta diversity metrics of Shannon diversity index and Bray-Curtis dissimilarity (McMurdie & Holmes, 2013). Shannon diversity indices were compared using a GLM and estimated marginal means with the lmer function. The adonis function in the *vegan* package allowed for a PERMANOVA that tested whether diversity differed between rural and urban samples, with assumptions checked using the betadisper function and pairwise comparisons conducted with Tukey tests (Oksanen et al., 2020). Redundancy analyses (RDA) used the rda function from the *vegan* package, following a Hellinger transformation for standardization of taxa and environmental variables (Legendre &

Gallagher, 2001; Oksanen et al., 2020). Pesticide concentrations were included in the environmental variables using the average concentration (ppm) from LC-MS screening.

DESEQ2 analyses were conducted in R to select for taxa most significantly different in relative abundance between urban and rural individuals (Love et al., 2014). Similarity percentage (SIMPER) statistics were calculated in the PAST (version 4.0.7) software to determine taxa overrepresented in urban or rural areas (Hammer et al., 2001). Bipartite networks were created using the *bipartite* package in R, examining interactions between the top 10 relatively abundant bacterial, fungal, and plant taxa (Dormann, 2011; Dormann et al., 2008, 2009). Similarly, the *mixOmics* package was used for DIABLO analyses that integrated bacterial, fungal, and plant genera into networks (Singh et al., 2019). A keepX value of 10 was used for each loci and the plotDiablo function used to calculate correlations values between bacteria, fungi, and plants.

Results

Microbial Characterization of Pollen and Bees

Metabarcoding of adult female *Ceratina calcarata* resulted in an average read count of 2,627 bacterial, 5,845 fungal, and 20,480 plant reads; while pollen provisions averaged 8,222 bacterial, 10,967 fungal, and 24,803 plant reads per sample (Table S3.1-S3.3). The most abundant bacteria overall in both adult bees and their pollen provisions are *Acinetobacter* and *Pantoea* (Table S3.1, Figure 3.2A). *Apilactobacillus* was the eighth most relatively abundant bacteria in both bees and in provisions, following the bacteria *Carnimona*s, *Nocardia,* and *Sphingomona*s (Table S3.1). The top three relatively abundant fungal genera were common between bees and pollen, consisting of *Alternaria, Ascosphaera*, and *Aureobasidium* (Table S3.2,

Figure 3.2B). Top plant genera with high relative abundance across both bees and pollen were *Trifolium* (clover), *Hydrophyllum* (waterleaf), *Rubu*s (raspberry), and *Lonicera* (honeysuckle) (Table S3.3, Figure 3.2C).

Pollen provisions harboured a more diverse bacterial microbiome than adult bees by alpha diversity (Tukey test bees-pollen, estimated pairwise difference = -0.593, df = 209, $p < 0.0001$) (Figure 3.3). Bipartite networks comparing the top ten bacteria, fungi, and plants showed that microbial and plant nestedness and diversity were higher in pollen than in bees, despite similar network connectance and links per genus (Table S3.4). DIABLO analyses examined the interactions between the top 10 genera for each of the three loci, revealing positive Pearson correlation coefficients of above 80% between fungi-plants within adult bees, as well as between bacteria-fungi and bacteria-plant taxa within pollen provisions (Figure S3.1AB). Not limiting to the top 10 taxa for each locus, all the pairwise relationships between bacteria, fungi, and plant taxa were positively correlated above 84% in bees (Figure S3.1C). In pollen provisions, the positive correlation between bacteria-fungi was 87%, indicating strong microbial correlations within the pollen balls (Figure S3.1D).

Environmental Characterization

Environmental features of the urban collection sites differed from those in rural areas (Table S3.5). Percentage land use development and mean annual temperature were higher in urban sites, while the amount of available green space and mean annual precipitation were lower in the city. Land use development was below 6% for all the agricultural sites, with urban areas ranging from 20-90% impervious surface (Kruskal-Wallis; $X^2 = 9.86$, df = 1, $p = 0.001$) (Table

S3.5). Urban annual mean precipitation ranged from 804-848mm while rural sites saw 879-897mm (Kruskal-Wallis, $X^2 = 6.04$, df = 1, p = 0.014), and urban annual mean temperatures were 7.68-8.64°C in the city and 6.99-7.54°C in agricultural areas (Kruskal-Wallis; $X^2 = 9.90$, df = 1, p = 0.002) (Table S3.5). The differences in land use and climate between these areas support the classification of urban and rural sites for this study.

LC-MS screening indicated the presence of pesticides in pollen samples, but a complete absence in any adult bees across both urban and rural sites (Table S3.6). Notably, the fungicide imazalil was found in four out of five urban pollen provision samples. Urban pollen provisions harboured less than 0.005ppm of pesticides, while pollen from the northern three collection sites away from the downtown core had at least 0.009ppm (Figure 3.1, Table S3.6). From both agricultural sites, pollen provisions contained herbicides and insecticides (Table S3.6). Dipropetryn and imidacloprid, a neonicotinoid, was found in one farm at concentrations less than the detectable and quantifiable limit, respectively (Table S3.6). The other agricultural site presented the herbicide napropamide at less than quantifiable limits and the insecticide spirotetramat at concentrations between 0.0013 and 0.0016ppm.

Microbial Diversity Differences Between Urban and Rural Areas

Urban bees and their pollen provisions had significantly elevated levels of alpha microbial diversity compared to rural sites (emmeans and Tukey test rural-urban, bacterial pairwise difference = -0.751, df = 209, $p < 0.0001$; fungal pairwise difference = -0.131, df = 233, p = 0.0155) (Figure 3.3). Through beta diversity comparisons using Bray Curtis dissimilarities, urban and rural samples resulted in two distinct clusters in both bacterial and fungal diversity

PCoAs, highlighting an urban rural divide (Tukey test urban-rural; bacteria, $p = 5.3x10^{-6}$, fungi, $p < 13x10^{-6}$) (Figure 3.4AB). This was confirmed with bipartite networks having a higher Fisher alpha diversity and more effective partners in urban compared to rural sites (Table S3.4).

Similarity percentages (SIMPER) from adult bee samples and pollen provisions indicated bacterial and fungal taxonomic differences in urban and rural sites (Table S3.7). For example, the bacteria *Schmidhempelia* were found exclusively in rural sites and the bacteria *Nocardia* had over twice the mean relative abundance in rural sites (Table S3.7). DESEQ2 analyses corroborated this overrepresentation of *Schmidhempelia* in adult bees from rural sites and also found that *Sphingomonas* and *Carnimonas* were overrepresented in urban adult bees (Figure S3.2, Table S3.8). Fungal reads in rural bees were low, leading to the significant overrepresentation of *Alternaria* and *Ascosphaera* in urban bees (Table S3.7). In pollen provisions, *Pantoea, Yersinia*, and *Clostridium* bacteria and *Alternaria, Ascosphaera,* and *Mycosphaera* fungi were overrepresented in urban areas (Table S3.7). DESEQ2 comparisons uncovered high variation between bacterial genera in pollen and slightly less variation in fungal genera, confirming SIMPER results as to key taxa driving differences in urban and rural pollen (Figure S3.2, Table S3.8).

Plant diversity largely remained consistent between urban and rural bees and pollen provisions (Tukey test rural-urban, estimated pairwise difference = -0.0132, df = 232, $p < 0.78$; bees-pollen, estimated pairwise difference = 0.09, df = 234, $p = 0.054$) (Figure 3.3). Bray-Curtis clusters were less evident when comparing plant diversity but still statistically different (Tukey test urban-rural, plant, $p = 0.004$). This suggests more dissimilarity and more diversity in plant composition within urban and within rural areas (Figure 3.4C). *Hydrophyllum, Trifolium, Plantago*, and *Rubu*s were greatly overrepresented in rural bees (Table S3.7). This aligned with

DESEQ2 analyses, which also further indicated that *Fragaria* and an Asteraceae sp. were more relatively abundant in bees from rural sites and *Syringa* and *Sambucus* plant genera relatively abundant in urban sites (Figure S3.2B, Table S3.8). As for pollen provisions, aside from the plant genus *Lonicera* which was overrepresented in urban pollen, *Hydrophyllum, Trifolium*, and *Syringa* were more relatively abundant in rural pollen provisions (Table S3.7). Plant taxa in pollen provisions in rural areas mirrored those in agricultural bees, however plants including *Rhus* and *Gleditsia* were overrepresented in urban areas (Figure S3.2E).

Redundancy analyses (RDA) revealed that microbial and plant differences are explained by the interaction of the studied environmental variables and relative pesticide concentrations located in each area across the three loci. Environmental features explain 20% of the variation in bee microbial and plant diversity and 31% of the diversity in pollen provisions (ANOVA bees, adj $R^2 = 0.20$, df = 7, p = 0.001; pollen, adj $R^2 = 0.31$, df = 7, p = 0.001) (Figure 3.5AB). Particularly, forward selection modelling on adult bee samples indicated that longitude, latitude, precipitation, green space availability, and pesticide concentration predict microbial and plant diversity (Table S3.9). RDA for pollen provisions revealed that temperature also predicts bacterial, fungal, and plant composition of the pollen provisions, suggesting that urban sites with higher mean annual temperatures harbour different microbiomes and plant communities than rural sites with lower temperatures (Tables S3.5, S3.9).

Discussion

Differing land use and associated abiotic factors present an array of variables that affect the microbial and plant diversity within adult bees and their pollen provisions. This study

characterized the small carpenter bee and pollen provision microbial and plant diversity and composition across an urban rural divide for the first time. We predicted that due to the availability of different plants (Cohen et al., 2022; Dew et al., 2020; McFrederick et al., 2012, 2017; McFrederick & Rehan, 2016), microclimates (Li et al., 2022; Nguyen & Rehan, 2022b), and an array of pesticides (Botías et al., 2015, 2017; Daisley et al., 2020; Jones et al., 2018), rural adult bees and pollen provisions will experience decreased microbial diversity while urban sites may lack important microbial taxa recognized to be beneficial to bee health (Bosmans et al., 2018; Cohen et al., 2020; Nguyen & Rehan, 2022b).

Microbiome and Plant Composition Characterization

The bacterial and fungal taxa most relatively abundant in the urban and rural bees and pollen provisions have been documented in previous studies of *Ceratina calcarata* in Toronto, including *Acinetobacter, Pantoea, Nocardia, Sphingomonas, Alternaria, Ascosphaera,* and *Penicillium* (Table S3.1-S3.2, Figure 3.2) (Nguyen & Rehan, 2022a, 2022b). In particular, *Acinetobacter* and *Sphingomonas* have been characterized as part of the core microbiome in *Ceratina* and their pollen provisions (Graystock et al., 2017), although earlier studies have also examined the presence of the other bee symbionts in *Ceratina* (Nguyen & Rehan, 2022a, 2022b; Shell & Rehan, 2022). The relative abundance of the bacterial genus *Acinetobacter* is of particular interest as they are important symbionts usually present in provisions and that stimulate pollen germination, bursting, and protein release that is beneficial to pollinators (Christensen et al., 2021; Crowley & Russell, 2021; Dew et al., 2020).While the functions of these microbes are largely still unknown in wild bees (Nguyen & Rehan, 2023), the reoccurring

presence of certain microbes suggests that small carpenter bees from this region are consistently forming relationships with bacterial symbionts year over year.

Plant taxa identified within bees and pollen provisions with the most relative abundance were native and known to be common in the study areas, including *Trifolium* (clover), *Rubus* (raspberry), *Hydrophyllum* (waterleaf), and *Lonicera* (honeysuckle) (Figure 3.2C, Table S3.3) (USDA, 2023). These genera have largely also been previously examined in *Ceratina calcarata* provisions, including the nutrient rich clover (Lawson et al., 2020). While the presence of *Rubus* and *Rhus* are largely explained by the raspberry and sumac stems that small carpenter bees nest in, not all provisions contained these plant genera (Figure 3.2C, Table S3.3), confirming no dependence on nesting material for pollen provisions (Dew et al., 2020). Although these bees may be visiting an array of different flowers for each provision (Figure 3.2C), the plants identified on bees and in their pollen balls exhibit lower floral diversity than microbial diversity and tend to be limited to 2-3 different floral genera per sample (Figure 3.3). This may suggest that foraging flower constancy may be occurring and reflecting the symmetry between bee and pollen plant composition (Kobayashi-Kidokoro & Higashi, 2010). On the contrary, the presence of more than one plant taxa in each pollen provision also indicates that multiple floral resources are visited within each foraging day, thus offering an array of different microbes associated with those flowers with each provisioning trip (Graystock et al., 2017).

Microbial and plant diversity were more elevated in pollen provisions than in adult bees (Figures 3.3, S3.1; Table S3.4). Likewise, the strength of correlations between bacterial, fungal, and plant taxa were higher in pollen than in bees (Figure S3.2), suggesting co-occurrences are more frequent between microbes and plants. This may be a characteristic trait of adult wild bees, which have been previously shown to have low levels of microbial diversity when compared to

pollen provisions or juvenile bees (Graystock et al., 2017; Nguyen & Rehan, 2022a). This indicates the microbes transferred and maintained in the bee microbiome until adulthood are limited, although a greater microbial diversity in pollen provisions can be beneficial for bees by aiding with metabolism and detoxification (Ghosh et al., 2022; Gilliam et al., 1989; Yoder et al., 2013). The importance of microbes in pollen is highlighted in experimental studies on honey bees that face increased virus replication and reduced survivorship and head mass when exposed to antibiotics, yet can be rescued with the introduction of untreated pollen (Li et al., 2019). Thus, although the role of the microbiome remains important for a myriad of functions in immunity (Tauber et al., 2019), metabolism (Zhang et al., 2022), and fitness (Dharampal et al., 2019, 2022), bees may be less reliant on a diverse microbiome and rather more dependent on an abundance of certain functional microbial taxa. Further experimental studies on the function of microbes and quantifying changes in microbial diversity are needed to better understand the effects of low overall diversity on bee fitness.

Urban and Rural Land Use Effects on the Microbiome and Plant Composition

Urban and rural bees and pollen provisions harboured different microbial and plant diversities (Figure 3.3), with urban study areas correlated with a higher diversity than rural locations (Figure 3.4, Table S3.4). Previously, urban land use gradients were not associated with differences in microbial alpha diversity of small carpenter bees in the City of Toronto (Nguyen & Rehan, 2022b). In this study, with a larger land use differential comparing urban and rural locations, urban bees were found to be more diverse and harbored previously documented microbes such as *Sphingomonas, Carnimonas, Alternaria*, and *Ascosphaera*, whereas rural bees were overrepresented in *Schmidhempelia,* a bacterial genus not previously noted in any bees

aside from *Bombus impatiens* (Langridge, 2014; Martinson et al., 2014; Nguyen & Rehan, 2022a, 2022b) (Table S3.7, S3.8). Candidatus *Schmidhempelia bombi* is a close relative of *Gillia apicola,* a prominent bacteria in honey bees, bumble bees, and other bees including carpenter and leafcutter bees (Nguyen & Rehan, 2023). This may be indicative of certain bee symbionts being shared between bee species in rural areas via complex pollinator-microbe-plant interactions (Francis et al., 2021; Keller et al., 2021; McFrederick et al., 2017). With the unexpected overrepresentation of *Schmidhempelia* in rural *Ceratina,* cross-species transfer of microbes may be causing rural areas to foster a more limited microbial diversity that is dominated by microbes previously unseen.

Accompanying increased urban microbial diversity are concerns as which microbial taxa are most relatively abundant. *Ascosphaera* commonly causes chalkbrood disease in honey and leafcutter bees (Aronstein & Murray, 2010; James, 2005; Anja Amtoft Wynns et al., 2012), but have also been found in other wild bees including small carpenter and mason bees (LeCroy et al., 2023; Nguyen & Rehan, 2022b). *Alternaria,* another fungal genera overrepresented in urban sites (Table S3.7), are plant pathogens with antifungal activity that can produce phytotoxic metabolites (Dalinova et al., 2020), although this may paradoxically be beneficial to honey bees in protecting them against other pathogens, such as those causing chalkbrood disease (Ye et al., 2021). *Apilactobacillus* were slightly underrepresented in urban bees, despite being overrepresented in urban pollen provisions when compared to rural counterparts (Table S3.7). *Apilactobacillus* is a common symbiont in bees with a plethora of functions in immunity (Daisley et al., 2020; Parichehreh et al., 2018; Rothman et al., 2019), biofilm formation (Pachla et al., 2021; Vásquez et al., 2012), and metabolism that aids in memory and behaviour (Zhang et al., 2022). This reoccurring pattern of less relatively abundant *Apilactobacillus* in small carpenter

bees may be associated with this city, as higher annual temperatures within the City of Toronto have previously associated with the underrepresentation of this bacteria (Nguyen & Rehan, 2022b). In addition to the overrepresentation of pathogens in urban areas, high microbial diversity in urban areas may not necessarily indicate a healthier microbiome.

Attributing to the difference in urban and rural plant composition is access to different floral resources in agricultural sites compared to areas within the city (Figures 3.2-3.3). Particularly at rural sites, the presence of *Fragaria* and *Rubus* were explained by proximity to berry crops in bloom near the time of provisioning and collection. However, the flowers found in urban areas such as the introduced *Syringa* (lilacs), native *Gleditsia* (locusts), and both native and introduced *Sambucus* (elderberries) can be expected in gardens and parks throughout the city (USDA, 2023). Floral resource availability can vary greatly within land use types and even within cities. Increased floral diversity and hot spots for bees can be found more in urban areas than agricultural areas, yet habitat loss and fragmentation from urbanization can also threaten flowering plant species richness (Ayers & Rehan, 2021; Lynch et al., 2021; Theodorou et al., 2020a; 2020b). This increased floral diversity may lead to more diverse floral hubs of microbes for pollinators, again further contributing to increased microbial diversity in urban settings (Keller et al., 2021; McFrederick et al., 2017). Thus, while largely dependent on the local landscape and surrounding floral resources, urban areas can still provide a variety of floral resources for bees to forage from and hence offer higher microbial diversity as well.

The interaction between the environmental variables of longitude, latitude, mean annual precipitation, green space availability, and pesticide concentration predicted microbial and plant relative abundances (Figure 3.5, Table S3.9). The interactions between different local and landscape features dictate microbial composition in wild bees (Cohen et al., 2020; Nguyen &

Rehan, 2022b, 2023). In *Ceratina calcarata,* land use development and mean annual temperature have been able to predict bacterial diversity and temperature could predict fungal diversity in adult bees (Nguyen & Rehan, 2022b). In *Osmia lignaria*, features such as the natural cover and floral abundance predicted the average abundance of *Wolbachia* and *Lactobacillus* respectively (Cohen et al., 2020). Urban heat island effects, typified by increased temperatures and decreased precipitation in urban areas, may also affected microbial composition (Rinner & Ussain, 2011; Steensen et al., 2022). In honey bees, microbial composition changes in advance of overwintering in the fall and spring time in northern temperate climates, with low temperatures in the winter leading to the seasonal dominance of bacteria aiding in metabolism (Bleau et al., 2020; Li et al., 2022). With all these environmental variables acting in concert, it remains difficult to disentangle or quantify the effects of a single factor, sparking the need for further experimental studies.

The impact of pesticides on microbial composition on pollen provisions raises concerns in both urban and rural bees (Botías et al., 2017; Hotchkiss et al., 2022; Kakumanu et al., 2016; Meftaul et al., 2020; Yordanova et al., 2022). Detected in urban pollen at concentrations higher than other pesticides in rural pollen (Table S3.6), imazalil (also known as enilconazole) is a systemic fungicide and antifungal agent used to treat ringworm and fungal infections in cats and dogs in the form of a wash (Moriello, 2016; Moriello et al., 2017; United States Environmental Protection Agency, 2005). Thus, these residues being found in pollen provisions may indicate that veterinary usage of imazalil in shampoos may be contaminating the environment and reducing fungal diversity. The only quantifiable pesticide present in rural provisions was spirotetramat, ranging between 0.0013-0.0016 ppm (Table S3.6). This insecticide only caused high mortality of *B. impatiens* by chronic ingestion at field rates of 176ppm (Ramanaidu &

Cutler, 2013). Other pesticides in rural provisions include the neonicotinoid imidacloprid and the herbicide napropamide, which present low acute risks to honey bees but still require further assessments for the effects of its metabolites in pollen and larvae (European Food Safety Authority et al., 2018). Imidacloprid can increase mortality and reduce immunity in honey bees and in squash bees, although its harm may be unrelated to negatively affected bacterial microbiomes (Almasri et al., 2022; Raymann et al., 2018; Willis Chan & Raine, 2021). Rather, it may be the established core microbiota that aids in detoxifying pesticides, suggesting that dysbiosis between bees and their bacteria threatens the bees' abilities to defend against these pesticides (Almasri et al., 2022; Muñoz-Colmenero et al., 2020). This is especially of concern in agricultural areas with reduced microbial diversity and the greatest risks of pesticide exposure.

The bacterial, fungal, and plant composition of urban and rural wild bees in the Greater Toronto Area mimics previous findings for this bee and region. Microbial diversity and network nestedness were greater in pollen provisions than in bees, as well as in urban areas as opposed to rural sites. This lack of diversity may be caused by the environmental differences in the urban-rural divide, including available heterogeneity of green space and floral resources, the microclimate differences, and the effects of pesticides contaminating both rural and urban landscapes. Further studies testing these land use and landscape variables are required to decipher their functional effects on the microbiome and subsequently, on bee health. Although urban bees and their provisions exhibit greater levels of microbial diversity, they are harbouring low relative abundances of known beneficial symbionts and higher abundances of pathogens. On the contrary, rural bees may be more precarious in the face of environmental stressors due to their decreased microbial diversity and chronic exposure to pesticides. Thus, while this short-term study cannot determine which land use region is more stable for bee microbiomes and

health, long term studies are needed to track anthropogenic activities and human land use impacts on the microbes and plant resources that wild bees rely on. With consequences associated with both urban and rural locations, human disturbances continue to pose threats to these important pollinators both directly and indirectly.

Tables and Figures from Chapter III

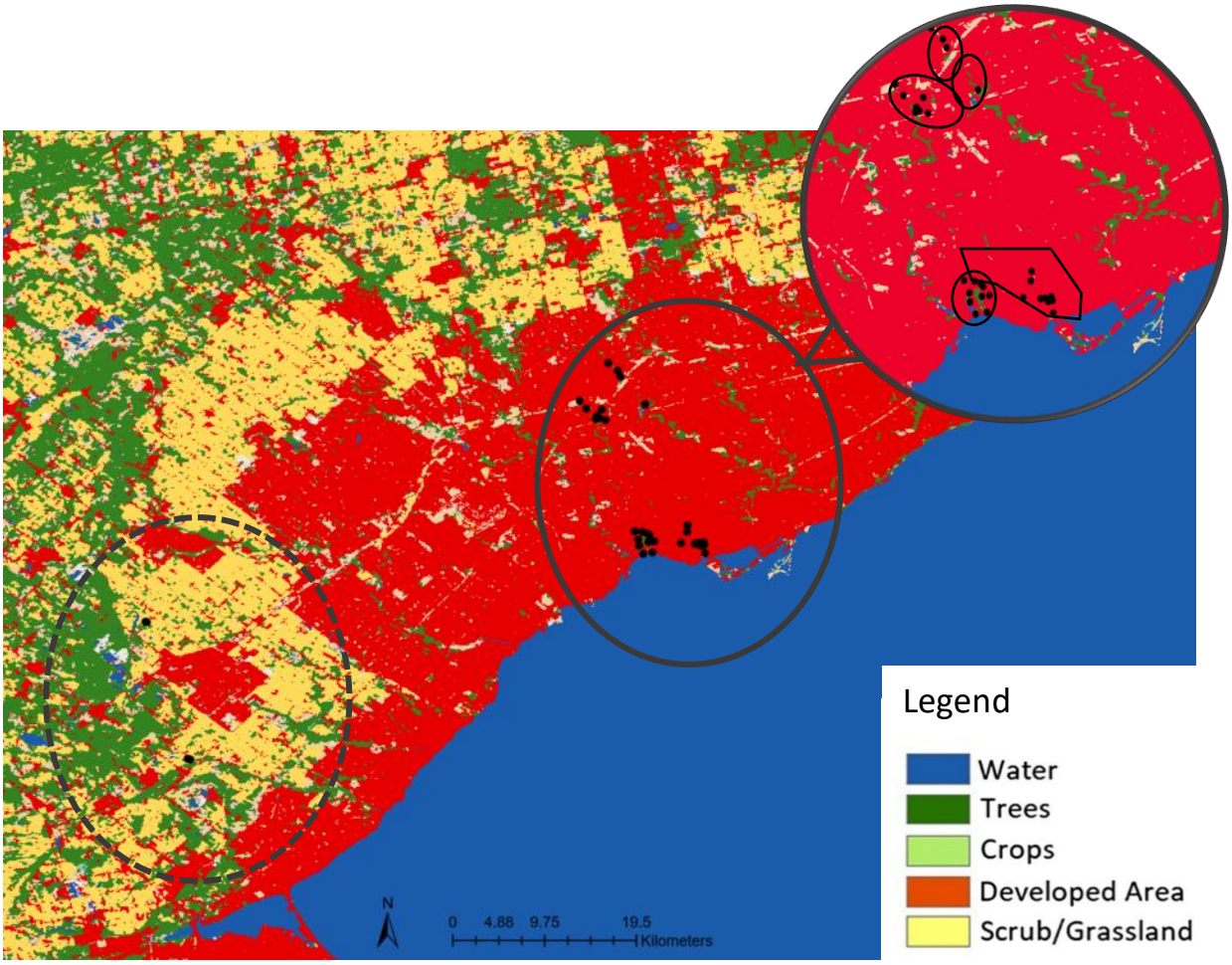

Figure 3.1. Collection map with sampling locations around Toronto, Ontario using the land use series from 2017-2021 Sentinel-2 map by ESRI at a resolution of 10m. Sites within the dashed circle represent rural samples from agricultural areas and solid circles indicate urban areas in the city. Urban sites were further divided into five locations throughout the city.

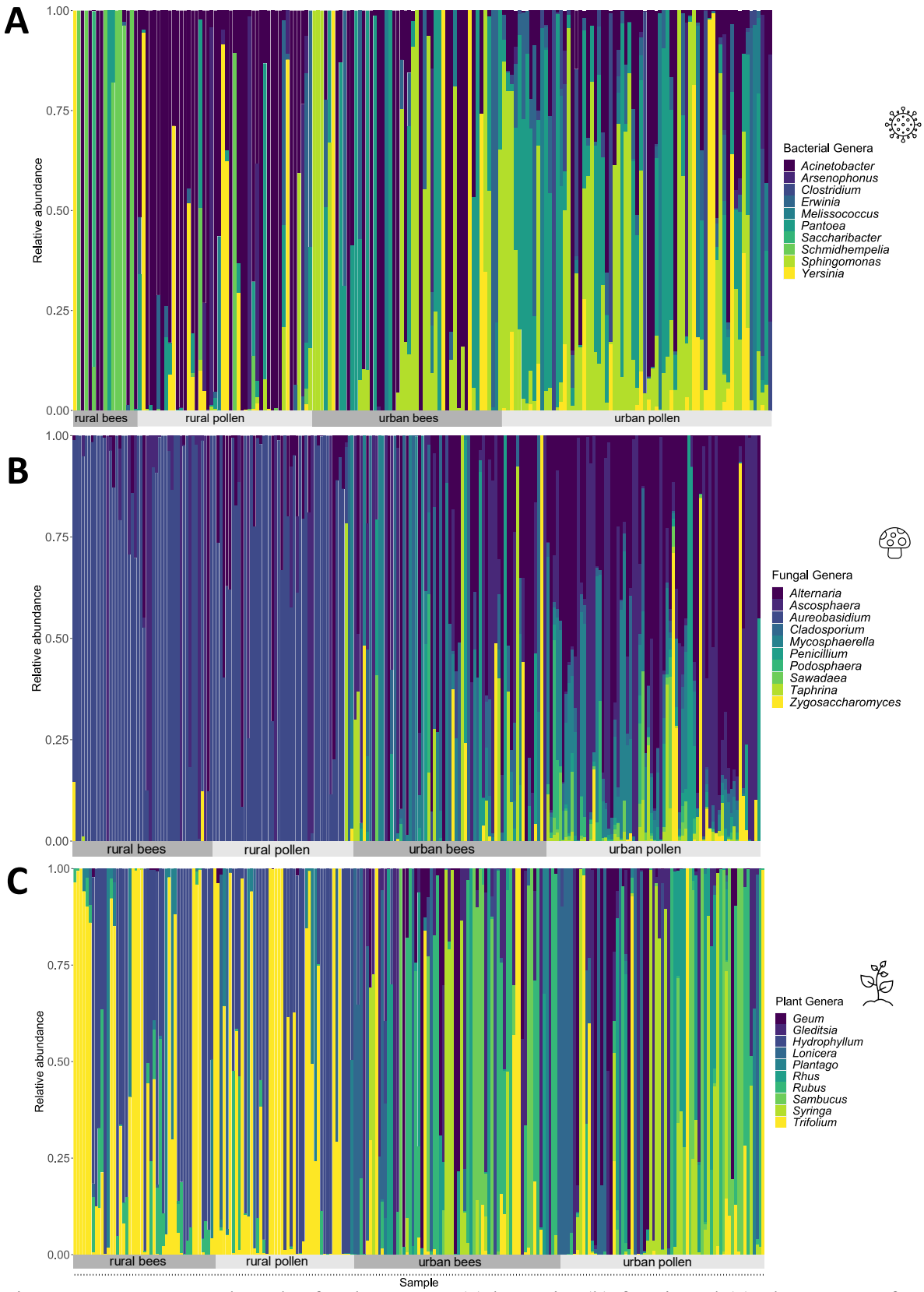

Figure 3.2. Taxonomy bar plot for the top ten (a) bacteria, (b) fungi, and (c) plant genera found within adult bees and pollen provisions in urban and rural areas.

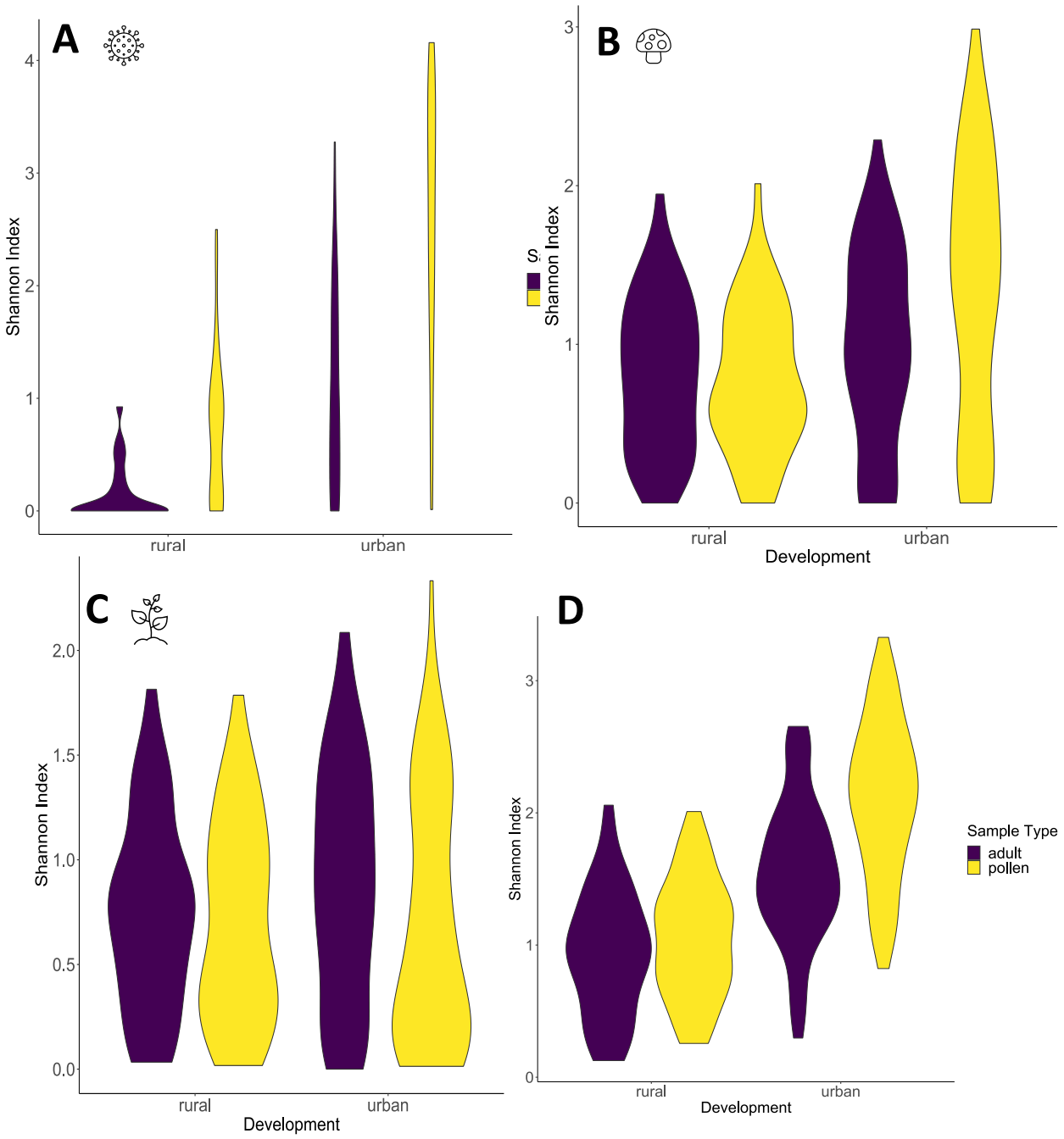

Figure 3.3 Alpha diversity measured using Shannon diversity for (a) bacteria, (b) fungi, (c) plant composition, and (d) all three loci within adult bees and pollen in rural and urban contexts. Urban bees and pollen harbour a greater bacterial and fungal diversity than rural communities. Pollen provisions have a higher bacterial diversity than adult bees.

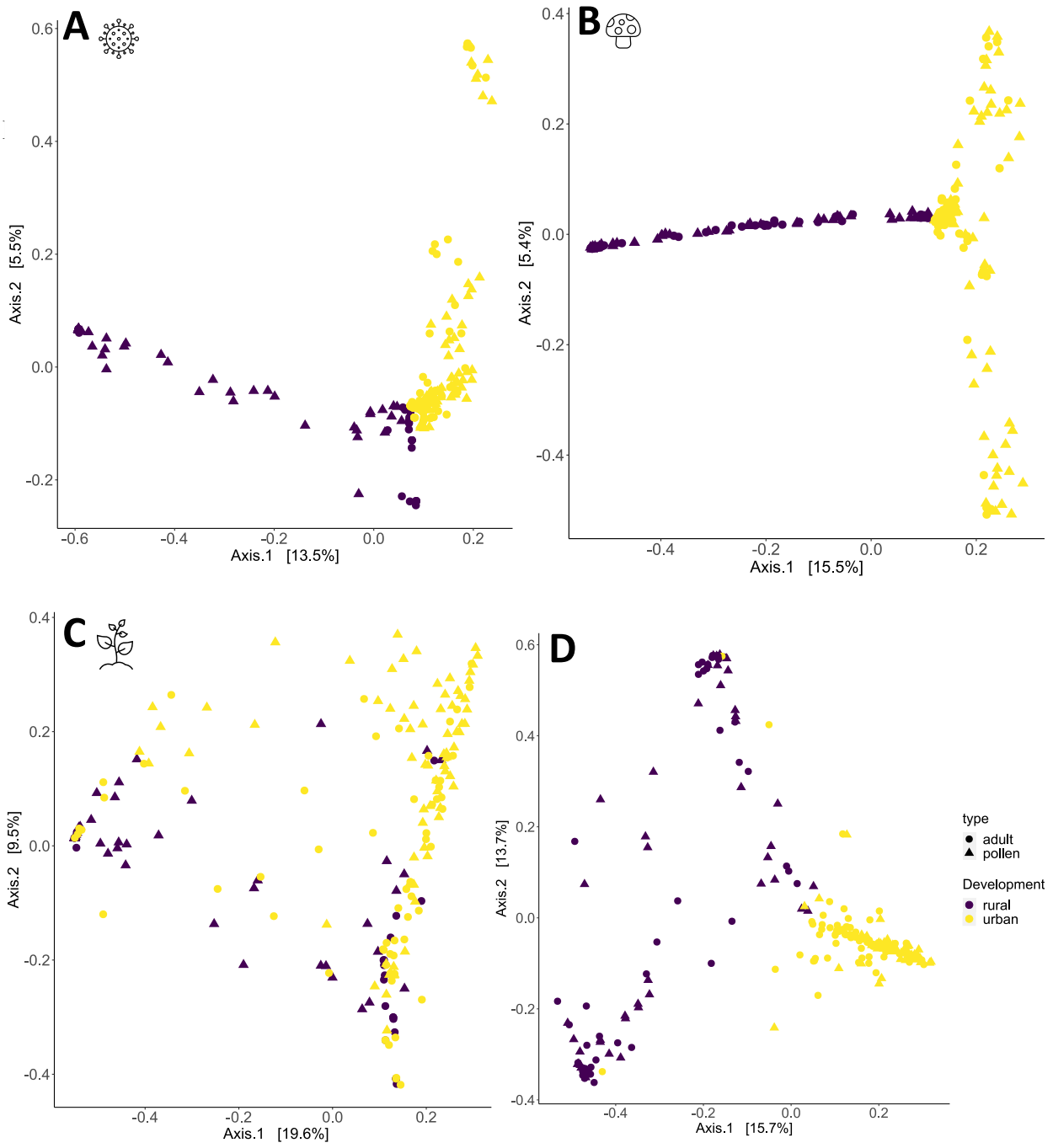

Figure 3.4. Beta diversity measured with Bray Curtis dissimilarities for (a) bacteria, (b) fungi, (c) plant compositions, and (d) all loci within adult bees and pollen in urban and rural areas. Tukey tests indicate urban and rural differences in bacterial ($p = 5.3x10^{-6}$), fungal ($p < 13x10^{-6}$), and plant ($p = 0.004$) diversity.

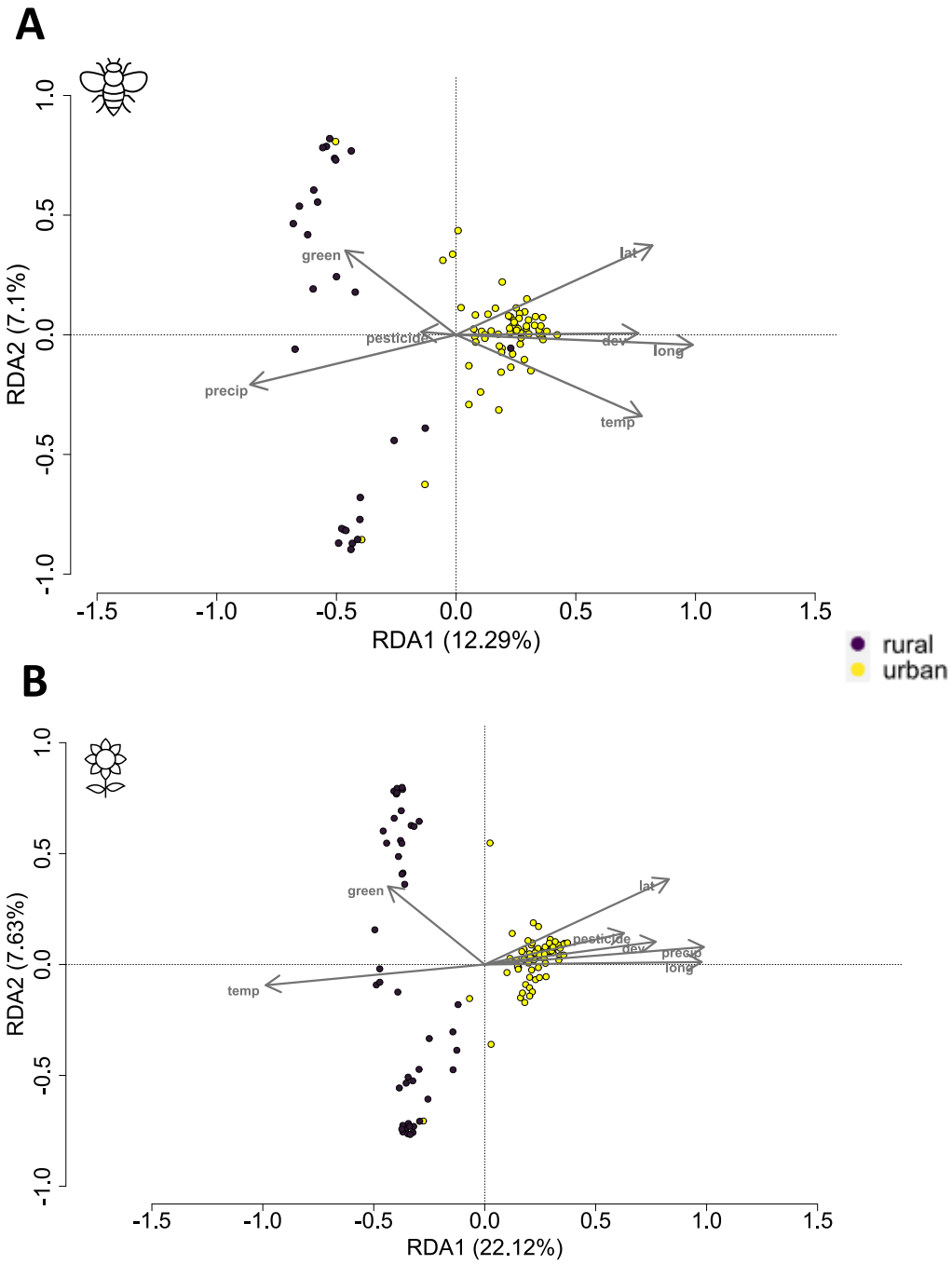

Figure 3.5. Redundancy analyses depicting the interaction of environmental variables on the microbial and plant diversity of (a) adult bees (adj $R^2 = 0.20$, $p = 0.001$) and (b) pollen provisions (adj $R^2 = 0.31$, $p = 0.001$). Purple circles indicate rural urban while yellow circles are from urban areas. Environmental variables include land use development (dev), availability of green space(green), mean annual precipitation (precip), mean annual temperature (temp), longitude (long), latitude (lat), and average pesticide concentration (pesticide).

General Discussion

As human disturbances change the environmental features that shape pollinator habitats, it is increasingly important to characterize how anthropogenic activities are disrupting bees and the microbes that support them. Microbes offer a wide array of benefits to bees and could be protecting them amidst these altered environments by increasing immunity (Daisley, Chmiel, et al., 2020; Tauber et al., 2019), protecting against pathogens (Daisley et al., 2019; Voulgari-Kokota et al., 2020), and aiding in detoxification (Rothman et al., 2019). This thesis aims to understand how environmental variables, such as types of land use and climate, are contributing to the diversity and composition of bacteria and fungi that are established within wild bees. In studying a small carpenter bee, *Ceratina calcarata*, across Toronto, this thesis seeks to identify benefits and disturbances that can be extrapolated to other cities, land uses, and other wild bee species, many of which are currently understudied despite their importance in providing pollination services. My research provides insights into the effects of urban and rural landscapes on the bee microbiome, offering a better understanding of ways in which pollinators can be potentially protected and supported.

In Chapter I, I provide an overview of the literature on the role of geographic distribution, temperature, precipitation, floral resources, agriculture, and urbanization on bee microbiota (Nguyen & Rehan, 2023). While obligately eusocial bees may not predominantly obtain their microbes from the environment like solitary bees (Martinson et al., 2011; Voulgari-Kokota, McFrederick, et al., 2019), all studied bee microbiomes have revealed sensitivities to changes in the environment. This is of particular importance when the role of microbes and bees are intertwined into plant-pollinator relationships and large-scale interactions between animals,

human, and the environment (Ayers & Rehan, 2023; Christensen et al., 2021; Daisley et al., 2022). Thus, this research encourages further studies that will help expand knowledge surrounding microbes and their role in urban ecology.

In Chapter II, I examine the microbiome of *Ceratina calcarata* across an urban land use gradient, categorizing land use using levels of impervious surface, green space, precipitation, and temperature (Nguyen & Rehan, 2022b). While it was hypothesized that microbial diversity would be lower in more developed areas, it was found that environmental features characteristic of urbanization associate with differences in microbial diversity and higher fungal genera richness. Increased levels of development also resulted in lower relative abundances of certain beneficial bacteria known to offer an array of benefits to bee immunity and metabolism (Christensen et al., 2021; Pachla et al., 2021; Vásquez et al., 2012). Furthermore, the co-occurrences of bacteria and fungi differed across a land use gradient, suggesting interactions between microbes may also be altered across different environments. Overall, this study raises concerns as to how expanding cities and further urbanization disrupts the healthy symbiotic relationships established within bees and jeopardizes wild bee health.

In Chapter III, I analyze the bacterial, fungal, and plant compositions of both *Ceratina calcarata* and their pollen provisions in agricultural and urbanized areas. Expanding the study area allowed for characterization of the environmental effects on the microbiome across a distinct dichotomy. It was hypothesized that urban land use would foster greater levels of microbial diversity, as found in Chapter II (Nguyen & Rehan, 2022b). Through characterizing differences using land use, climate, and pesticide residues, these environmental features confirmed a distinct urban and rural divide occurring within the Greater Toronto Area. This urban rural divide was able to capture more distinct differences between microbial diversity and

composition than the urban land use gradients from Chapter II. While confirming much of the taxonomic composition previously found within this bee and its pollen provisions (Dew et al., 2020; Graystock et al., 2017; Nguyen & Rehan, 2022a, 2022b; Shell & Rehan, 2022), this study found increased microbial diversity in urban areas that were composed of elevated levels of pathogens and decreased relative abundances of beneficial symbionts. In addition, the presence of pesticides within pollen provisions were found in both urban and rural landscapes, although higher concentrations were associated with agricultural applications. Pesticide residues are to be expected in urban and rural settings (Brain & Anderson, 2020; Meftaul et al., 2020), and their negative impact on bee microbiota and bee health reinforces the role of human led disturbances in disrupting pollinator habitats (Botías et al., 2015; Hotchkiss et al., 2022; Ramanaidu & Cutler, 2013). Therefore, these results further contribute to our understanding the impacts of land use in cities and in agriculture on wild bees and their microbiomes.

Having unveiled environmental features that contribute to differences in bacterial and fungal microbiota within bees, this thesis confirms the indirect human impact on the environmental microbiome and a unique avenue in which anthropogenic activities are interrupting pollinator health. In both studies, sensitivities to differences in land use and climate imply these effects may worsen with increased urbanization and agricultural expansion (Laurance et al., 2014; Ritchie & Roser, 2018). As the benefits of microbial diversity on maintaining bee health are largely still unknown, it may be the taxonomic composition of the microbiome that is particularly of concern in altered environments. The increased presence of pathogens and reduction in beneficial bacteria such as *Lactobacillus* in urban bees may provide insights into actionable ways of fostering healthier environments for bees. This might include probiotics that can help with immunity, pathogen defense, and detoxification (Daisley, Chmiel, et

al., 2020; Motta et al., 2022; Pachla et al., 2021). Other possibilities include offering green spaces and floral resources that can support beneficial plant-pollinator-microbe interactions (Christensen et al., 2021; Francis et al., 2021; Trinh et al., 2018). Overall, an understanding of the environmental features that shape the wild bee microbiome illuminates the impact humans have on these pollinators.

To conclude, this thesis presents the wild bee microbiome within an urban land use gradient and at a broader scale comparing urban and rural landscapes. While further research examining the functions of most of these microbes and their role in bee health are necessary, these studies offer a baseline characterization of the microbiota across landscapes and find that bee microbiomes are susceptible to environmental impacts. These results offer a preliminary understanding of how wild bees interact with plants, other animals, and the environment, tying into the One Health concept (Mackenzie & Jeggo, 2019; Trinh et al., 2018). Using this broader perspective, the vast network of microorganisms within bees can provide wide-scale implications on ecological systems.

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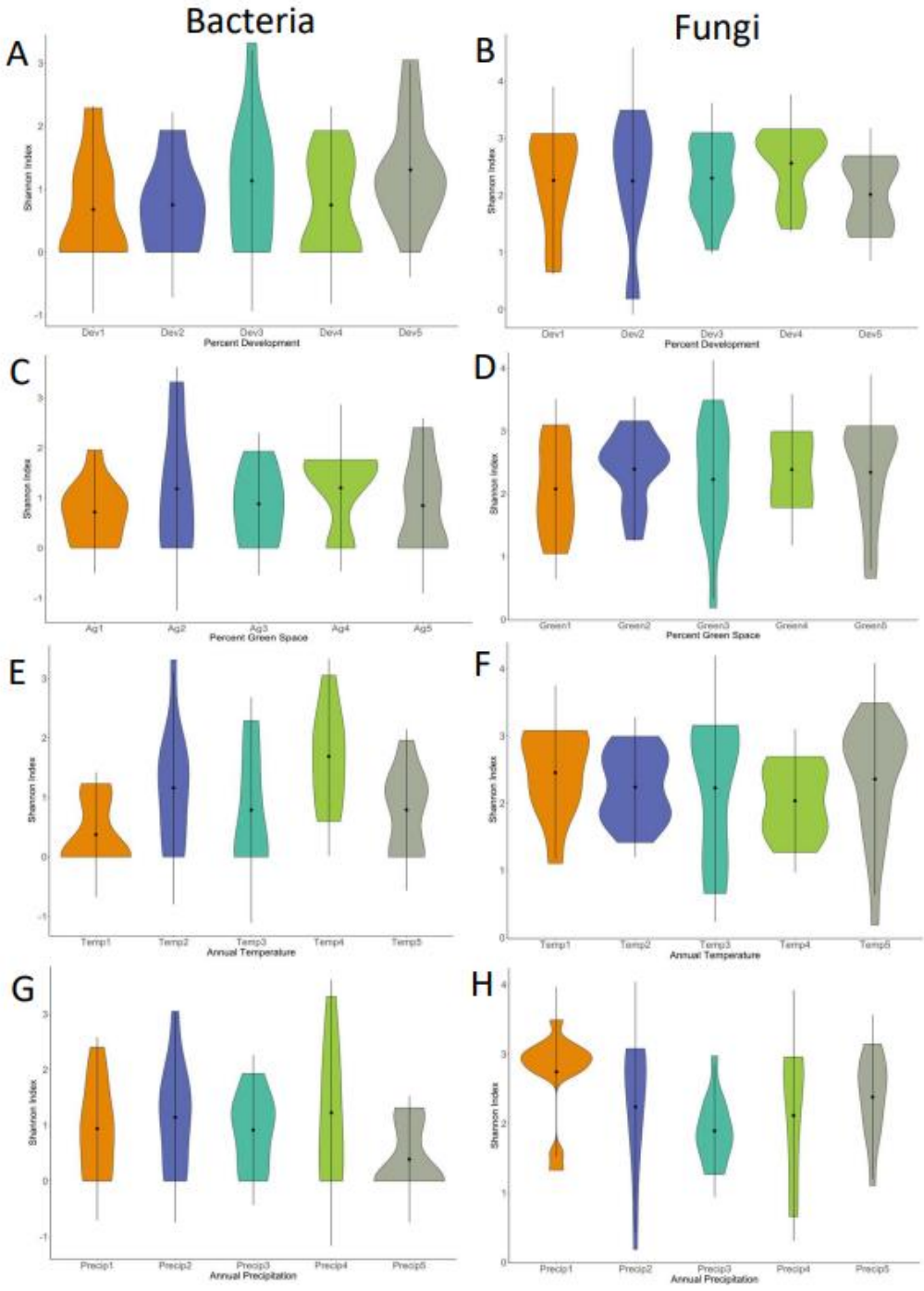

Figure S2.1. Violin plots depicting Shannon diversity of bacteria (left) and fungal (right) taxa across four environmental variables: (a-b) development, (c-d) green space, (e-f) temperature, and (g-h) precipitation. Exact categorical divisions can be found in Table S2.1. Vertical lines indicate the range of Shannon indices and the shape widths show distribution of samples.

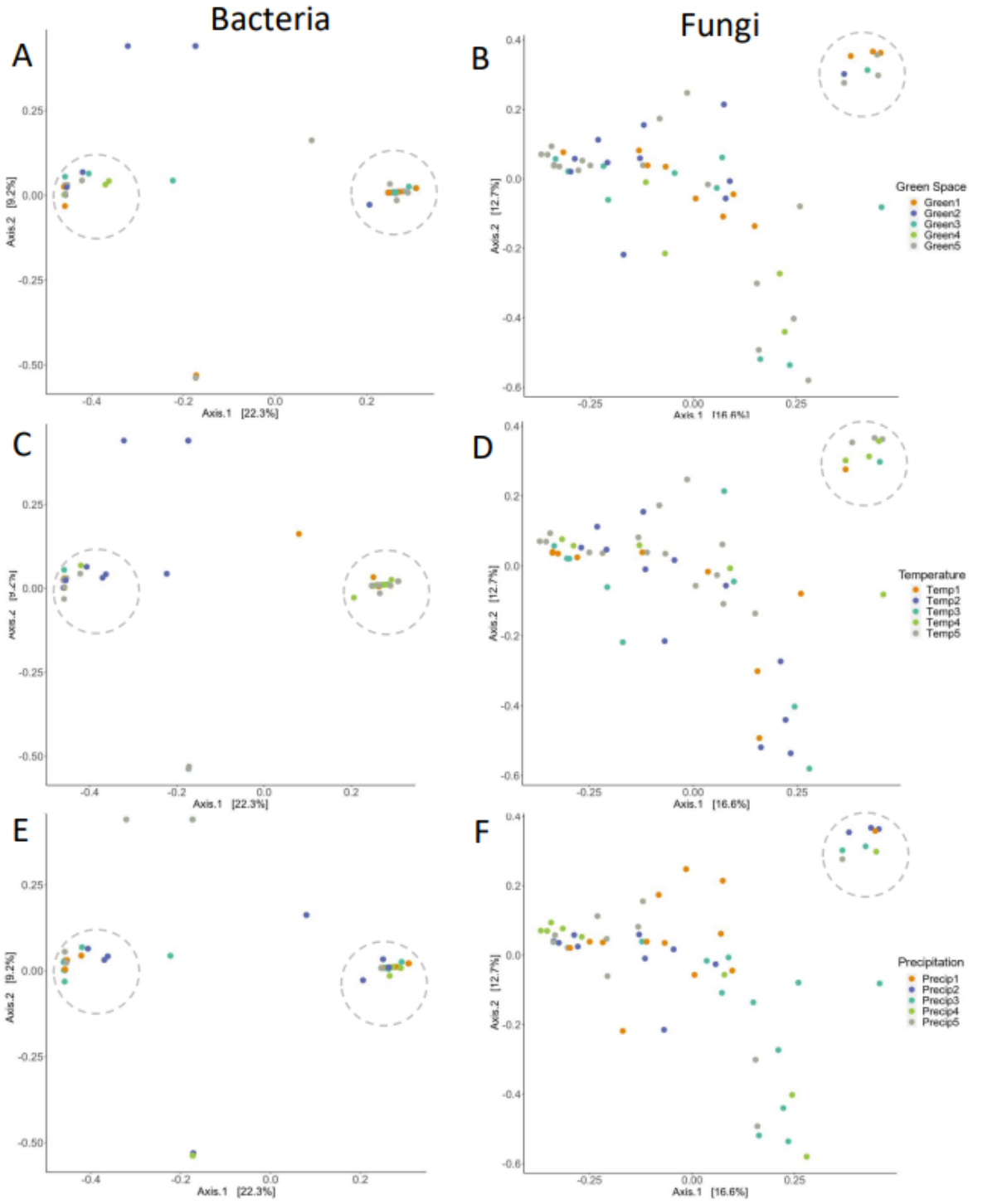

Figure S2.2. PCoA plots of Bray-Curtis dissimilarity matrices showing bacterial (left) and fungal (right) beta diversity when considering the variables of (a-b) green space (bacteria, p = 0.18; fungi, p = 0.056), (c-d) temperature (bacteria, p = 0.013; fungi, p = 0.021), and (e-f) precipitation (bacteria, p = 0.181; fungi, p = 0.002). Each category ranges from 1 (very low) to 5 (very high). Exact categorical divisions can be found in Table S2.1. Dotted circles represent clustered individuals with similar beta diversity.

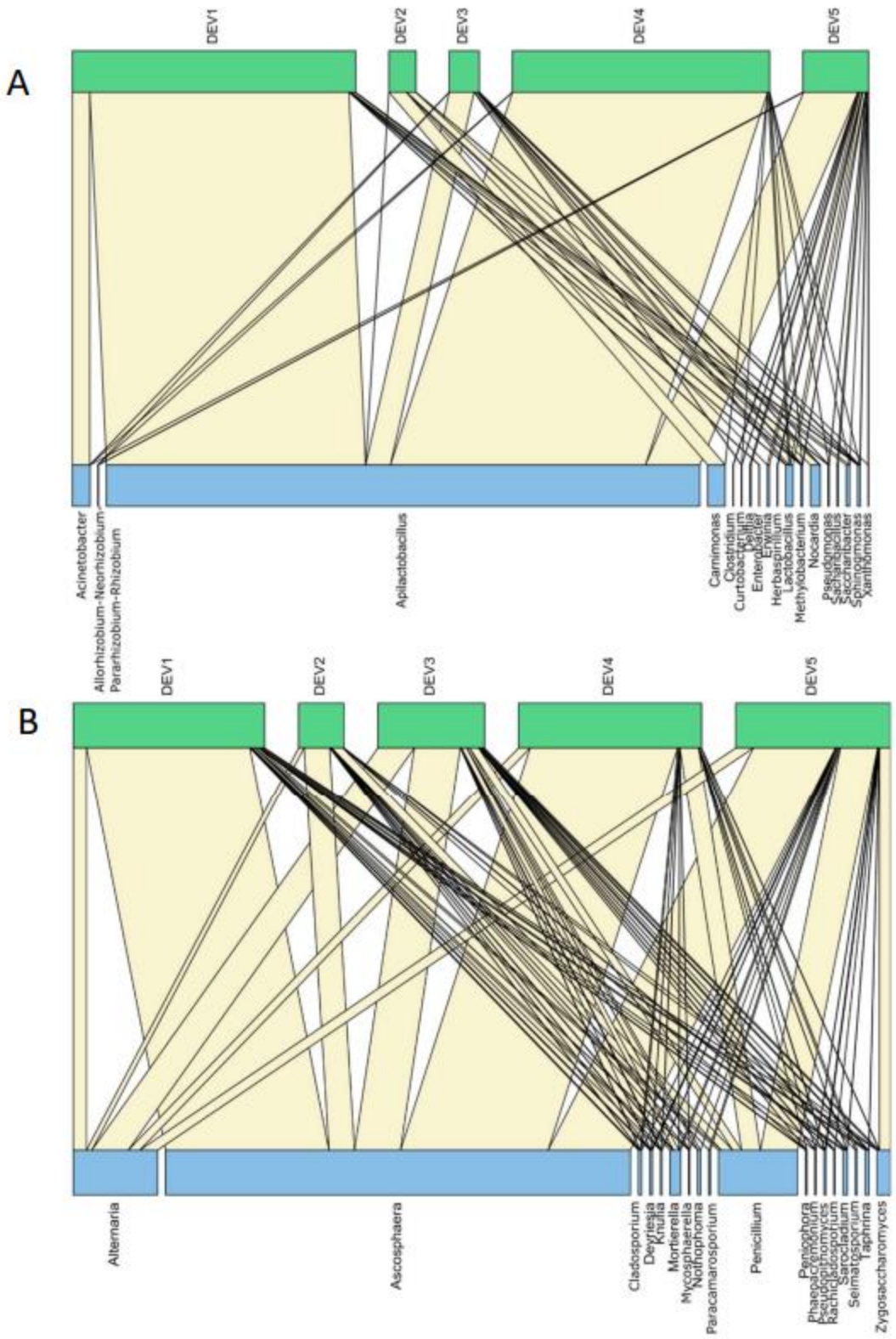

Figure S2.3. Bipartite networks comparing the association between the top 20 (a) bacterial and (b) fungal taxa and five levels of development. The five categories range from Dev 1 (very low development) through Dev 5 (very high development).

Appendix C: Supplementary Materials from Chapter III

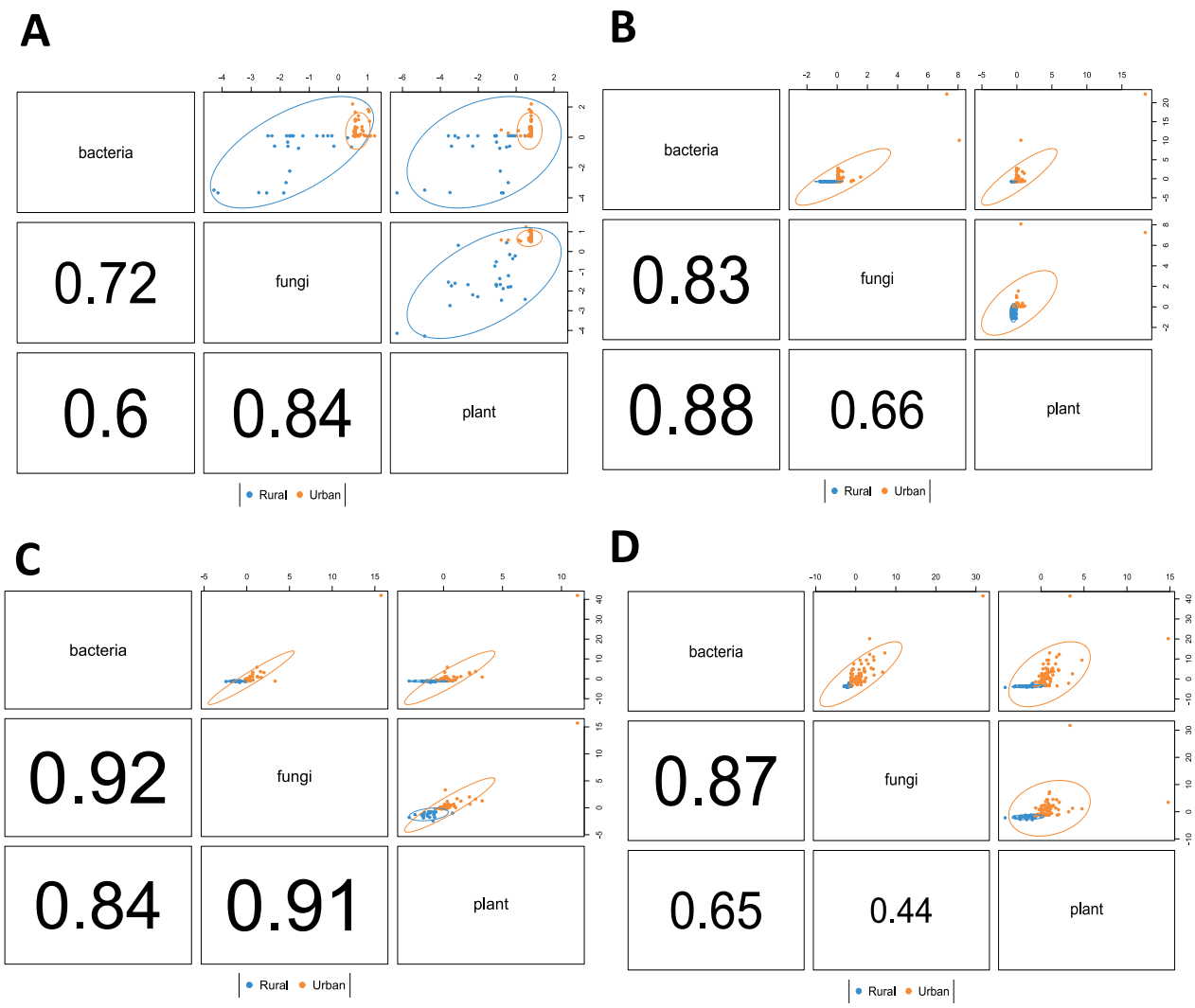

Figure S3.1. DIABLO correlations between the top 10 most significant bacterial, fungal, and plant genera in (a) adult bees and (b) pollen provisions and all the bacterial, fungal, and plant genera in (c) adult bees and (d) pollen provisions.

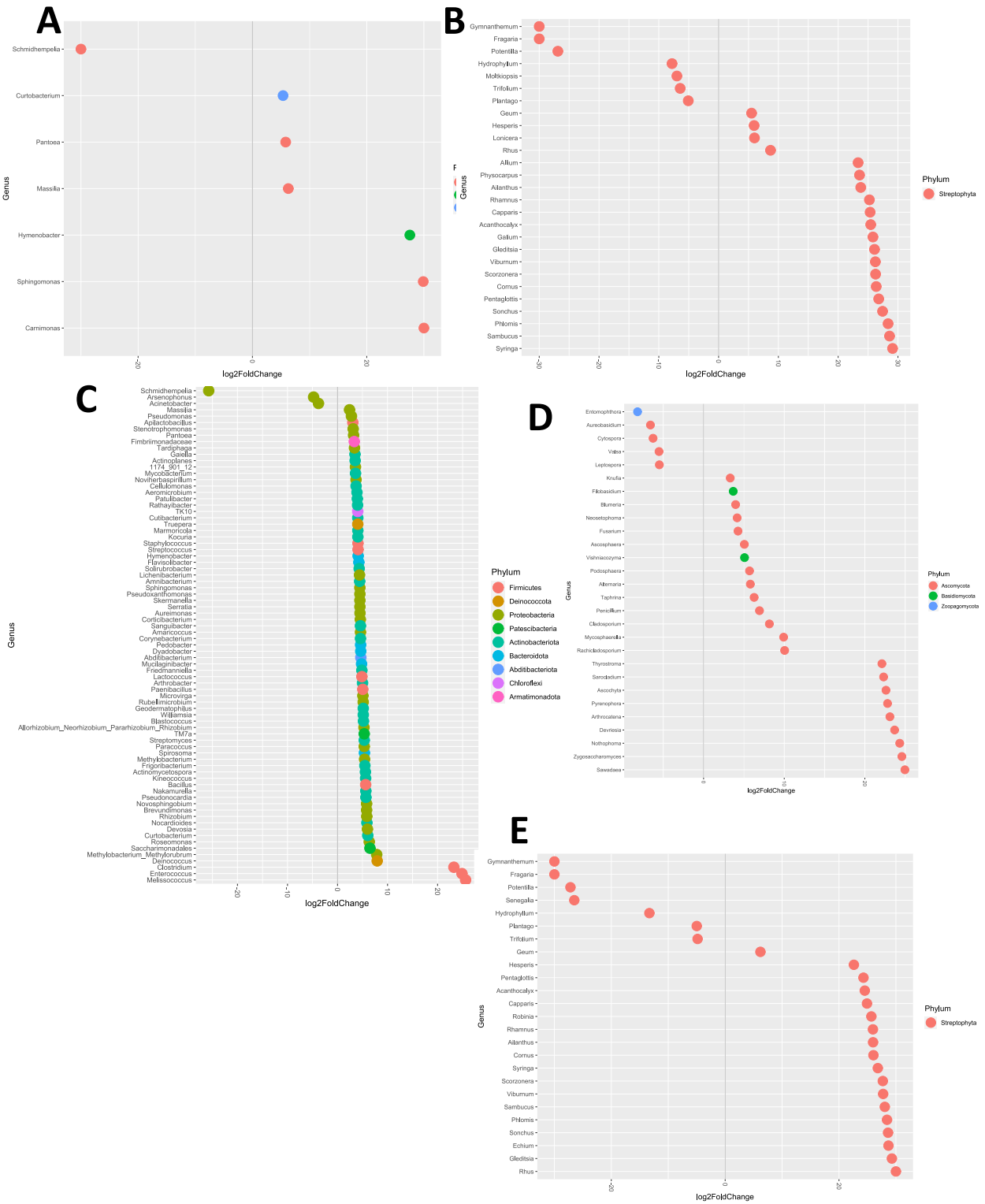

Figure S3.2. DESEQ2 analyses comparing (a) bacterial, and (b) plant genera in bees and (c) bacterial, (d) fungal, and (e) plant genera in pollen provisions across an urban (negative log2FoldChange) and rural (positive log2FoldChange) divide. Fungal genera in bees were not differentially abundant in rural and urban areas. Further details are available in Table S3.7.