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Microbiome Assembly and Function in the Solitary Mason Bee, *Osmia lignaria* (Megachilidae)

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MICROBIOME ASSEMBLY AND FUNCTION IN THE SOLITARY MASON BEE, *OSMIA LIGNARIA* (MEGACHILIDAE)

by

Bailey Crowley

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Approved:

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ABSTRACT

Microbiome assembly and function in the solitary mason bee,

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by

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Utah State University, 2024

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Microbes are increasingly recognized for their role in animal biology, ecology, and evolutionary history. However, the factors that drive microbiome assembly and composition are often host-dependent. Advances in sequencing technologies have permitted the exploration of microbiomes in diverse host taxa, including bees. Early research has revealed that microbial symbionts of social bees prevent spoilage of stored pollen, aid in the digestion of complex macromolecules, and protect the host against pathogens. Moreover, perturbations to an intact microbiome by abiotic stressors, such as increased temperature, may adversely affect the bee host by directly harming the microbial associates or impeding symbiont services. These findings, however, may not extend to ~90% of bee species that do not exhibit sociality. As such, little is known about microbiome assembly across the development of solitary bees as well as how these communities are altered in response to climate warming.

The first study (chapter two) of my thesis characterizes the microbiome of the solitary mason bee, *Osmia lignaria*, throughout its life cycle. Specifically, we employed

16S and ITS amplicon sequencing to describe the bacterial and fungal composition of provisions, larvae, and the gut microbiomes of pre-diapausing, emerged, and dead adults. Bacterial and fungal diversity did not change significantly across larval and adult bee microbiomes. Bacterial and fungal composition, however, was significantly different between larvae and pre-diapausing adults. In addition, many microbial taxa found in stored pollen was also found in immature bees, indicating that larvae acquire their microbiome while consuming the provision. The most prevalent bacterial genus, *Arsenophonus*, was frequently found in provisions and eggs but reached higher read counts in larvae and fully pigmented adults. In this study, the amplicon sequencing variants (ASVs) assigned to *Arsenophonus* had high sequence similarity to the type species that displays the son-killing phenotype. The fungus, *Ascosphaera*, which causes chalkbrood, was also detected in samples from provisions and larvae. Most other microbial taxa detected were plant pathogens or are common in soils, suggesting that they were vectored from environmental sources. These results highlight that solitary bees associate with horizontally and vertically transmitted microbes that have diverse consequences for bee health.

In my second study (chapter three), I conducted an *in vitro* study to examine how temperature-mediated shifts in provision microbiome composition affects the development and health of an early-season solitary bee. Male *O. lignaria* were reared on sterile or microbe-rich provisions within incubators simulating past, current, and projected temperatures for the Great Basin region (USA). As expected, we found a negative relationship between temperature and the duration of larval development. The mean relative abundance of a putative son-killing bacterium, *Arsenophonus*, increased

with warming temperatures. Although sterilization of pollen removed the reproductive parasite, larval survivorship was not significantly different between bees consuming microbe-rich or sterile diets. In contrast to past research, bees reared on sterile provisions weighed more and had higher fat contents than their counterparts reared on microbe-rich pollen, indicating that an intact provision microbiota may not always benefit larval health. Warmer temperatures increased the difference in mean biomass and fat content between larvae feeding on microbe-rich and sterile provisions. This suggests that the provision microbiota may contribute to the decrease in bee body size observed during the past several decades due to warming temperatures.

(131 pages)

PUBLIC ABSTRACT

Microbiome assembly and function in the solitary mason bee,

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Animal-microbe interactions can influence host biology, ecology, and evolution. The assembly and function of microbes found within animal hosts oftentimes depends on which species are involved. Advances in sequencing technologies have permitted the exploration of host-microbe interactions in a variety of animals, including bees. Early research aimed at understanding the microbiomes of social bees, such as honey bees and bumble bees, found that microbes prevent the spoilage of stored pollen, breakdown indigestible nutrients into smaller molecules available for uptake by the host, and also protect the host from pathogens. When environmental stressors, such as increased temperatures, disrupt the microbiome, the host can be negatively affected through direct harm to their symbionts or reductions in symbiont-provided benefits. Only about 10% of bee species worldwide are social however, so the advantages of bee-microbe interactions described above may not apply to most bees species. There is less research describing the microbiomes of solitary bees, including how bacterial and fungal communities change across their bee development and in response to warming temperatures.

In the first study of my thesis, I characterized the microbiome of the solitary mason bee, *Osmia lignaria*, across its development. Specifically, I used amplicon sequencing to determine which bacterial and fungal species are present in provisions (larval food), larvae, and the guts of adult bees before and after overwintering. I found

bacterial and fungal diversity did not change across bee samples. However, the composition of bacterial and fungal communities was significantly different between larvae and adult bees before the onset of winter. Larvae seem to acquire their microbiome from provisions, as many of the bacterial and fungi found in pollen was also detected in larvae. Notably, *Arsenophonus* was the most abundant bacterial genus, and had high sequence similarity to a vertically transmitted species that results in the death of male offspring. The causative agent of chalkbrood, *Ascosphaera*, was also found in provisions and larvae. Most other bacteria and fungi present were plant pathogens and those commonly found in soil. This study suggests that solitary bees harbor microbes with diverse functions that are acquired from the environment or are maternally transmitted.

For my second project, I conducted an experimental study to determine how temperature affects the microbiome composition of provisions and the resulting effects on solitary bee development and health. Specifically, we measured the body weight and fat content of male *O. lignaria* reared on sterile or microbe-rich provisions within incubators emulating past, current, and projected temperatures for the Great Basin region (USA). As anticipated, the time it took larvae to develop shortened with increasing temperatures. We detected a positive relationship between temperature and the mean relative abundance of *Arsenophonus*, a putative male-killing symbiont. While our sterilization method removed the reproductive parasite from pollen in the sterile treatment, there was no difference in larval survivorship between bees reared on sterile and microbe-rich provisions. Contrary to past research, larvae reared on microbe-rich provisions had a lower biomass and total fat content than those reared on sterile provisions, indicating that an intact provision microbiota may not always be beneficial to

larval health. Temperature increased the difference in the mean weight and fat content between larvae consuming sterile and microbe-rich provisions, with the warmest microclimate having the greatest effect. We conclude that microbes found in the provision of solitary bees may have played a role in decreasing bee body size over the past several decades of warming.

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Introduction

Investigating the establishment, maintenance, and function of microbial symbionts associated with animals is important for a complete understanding of host biology. However, the nature of these interspecific interactions is often host-dependent. Advances in sequencing technologies have permitted the exploration of host-microbe interactions in non-model species, such as bees. Early research into bee microbiomes found that social species harbor a core hindgut bacterial community that facilitates the digestion of complex macromolecules and aids in disease prevention by directly outcompeting pathogens and priming the host immune system (Zheng *et al.* 2019, Steele *et al.* 2021, Kwong *et al.* 2017). Yet only approximately 9.4% of bee species worldwide are social (Ascher & Pickering 2018), inviting the characterization of non-social bee microbiomes. In the introduction of this thesis, I describe the ecological processes shaping microbial community assembly in non-social bees, using comparisons to social bees, when applicable, to illuminate differences between host systems.

The assembly of communities, including the microbiomes of bees, can be organized into several processes: selection, dispersal, diversification, and drift (Vellend 2010, Nemergut *et al.* 2013, Zhou & Ning 2017). Underlying these ecological processes are determinism and stochasticity, which vary in their relative importance to govern community structure. Deterministic processes are niche-based mechanisms that result in differences in the number of species and their relative abundances. Meanwhile, stochastic processes can only generate variation in the abundance of species due to random births, deaths, and colonization. The first ecological process, selection, is a purely deterministic and causes fitness differences between species due to the outcome of biotic interactions

(e.g., competition, mutualism, host filtering) and variation in species' ability to withstand abiotic conditions (e.g., sugar and oxygen concentration). Dispersal is the movement or colonization of species across space. Both deterministic and stochastic processes affect dispersal, as the rate of dispersal can depend on population size (stochastic) and species traits (deterministic). Likewise, diversification, or new genetic variation, is also affected by both deterministic and stochastic processes. Mutations randomly produce genetic variation (stochastic) but this can also affect species' functional traits or identity (deterministic), depending on the region of DNA affected. The entirely stochastic process, drift, is the change in species abundance due to random births, deaths, and colonization. Below these ecological processes are discussed in the context of microbiome assembly within flowers, pollen provisions and bee hosts, with the assumption that drift is always an active ecological process (Table 1.1).

Bees acquire bacteria and fungi while visiting flowers for pollen and nectar. The chemistry of these floral resources enforces strong abiotic selection on microbial communities. Nectar contains antimicrobial proteins (Schmitt *et al.* 2018), plant secondary metabolites (Huang *et al.* 2012), reactive oxygen species (Thornburg *et al.* 2003), and high sugar osmolarity (Herrera *et al.* 2010) which only a few microbial species can tolerate. Moreover, early arriving microbial species can modify the niche by limiting resources (e.g., depletion of sucrose) for later arriving species, resulting in species-poor communities (Vannette & Fukami 2014). Animal visitation, however, can introduce variation in the nectar microbiome via the inoculation of transient microbes (McFrederick *et al.* 2012, 2017). Less in known about the pollen microbiome but early evidence suggests that plant species is a primary driver of community composition, yet

insect visitation seems to disperse microbes between conspecifics within close proximity (Manirajan *et al.* 2016, 2018). Variation in pollen microbiome composition among plant populations and across years, however, has yet to be uncovered.

It has been suggested that bee visitation patterns may establish transmission routes of abundant floral microbial taxa into bee pollen provisions (McFrederick *et al.* 2016, McFrederick & Rehan 2018, Rothman et al. 2019). Indeed, the *Apilactobacillus micheneri* (previously *Lactobacillus*) clade includes strains that have been found in both flowers and visiting solitary bees (McFrederick *et al.* 2016). However, Rothman and colleagues (2020) found minimal bacterial overlap within the provisions of congeneric bee species visiting the same flowers, suggesting that additional mechanisms may filter microbes during the collection of floral resources. Microbes vectored with imported nesting materials (e.g., soil for mason bees) also contribute to the brood cell microbiome (Keller *et al.* 2013, Rothman *et al.* 2018, Kapheim *et al.* 2021). In general, the microbiome composition of provisions seem to be driven by environmental features, such as floral resources and local pollinator community (McFrederick & Rehan 2018; Rothman *et al.* 2019, 2020; Cohen *et al.* 2020), as well as bee emergence phenology (Westreich *et al.* 2022). Taken together, most environmentally sourced microbes likely have transitory associations with bees, but potential mutualists may have developed mechanisms to ensure their transmission. Such mechanisms may include signaling their presence within flowers via microbial volatile organic compounds (Schaeffer *et al.* 2019) or the production of metabolites to provide information about the quality of floral resources (Schaeffer & Irwin 2014, Russell & Ashman 2019). The ability of bees to learn cues produced by floral microbes has only been demonstrated in social bees, however.

Upon completion of the brood cell, some vectored microbes may not tolerate the provision substrate and microbe-microbe competition could cause compositional differences between fresh and aged bee-collected pollen. Although the importance of the provision microbiome for bee health has been strongly suggested (Dharampal *et al.* 2019, 2020, 2022), experimental evidence demonstrating the functions of provision-associated microbial species and their interactions is currently lacking. For example, although it has been suggested, the ability of provision-associated microbes to modify the nutritional quality of bee-collected pollen (e.g., via pectic degradation) remains to be substantiated with *in vitro* studies. Recent research points to a promising mechanism for the liberation of nutrients from recalcitrant pollen by microbes, as the nectar-inhabiting bacterium *Acinetobacter pollinis* can release protein from pollen by prematurely germinating and rupturing the cell wall of the male plant gametophyte (Christensen *et al.* 2021). However, microscopic observation of honey bee stored pollen revealed that bacteria are found in low abundance and do not appear to aid in the pre-digestion of pollen (Anderson *et al.* 2014). Furthermore, a recent *in vitro* study suggests that not all imported floral bacteria may benefit bee health: megachilid bees reared on provisions supplemented with *A. micheneri* had the highest mortality rate compared to larvae reared on sterile pollen or microbe-rich provisions not supplemented with this bacterium (Brar *et al.* 2023). Larval-microbe interactions are just beginning to be uncovered in non-social bee hosts. Recently, larvae have recently been shown to reduce microbiome diversity in pollen provisions (Voulgari-Kokota *et al.* 2019, Kueneman *et al.* 2023). This may explain why similar microbiome composition between provisions and immature bees is often found

(Voulgari-Kokota *et al.* 2020, Nguyen & Rehan 2022, Kapheim *et al.* 2021), suggesting that larvae likely acquire their microbes while consuming the provision.

As holometabolous insects, bees restructure their anatomy during metamorphosis, requiring any symbionts to re-colonize their insect host after pupation (Hammer & Moran 2019). In fact, most social bees are colonized by their core hindgut bacteria after emergence (Powell *et al.* 2014). Here, the structure of the hindgut microbial community is shaped by host traits including pH and oxygen concentration (Callegari *et al.* 2021) as well as the physical structure of the gut (Engel & Moran 2013). Biotic interactions also contribute to the structure of the gut microbiota; for example, filtering by the host immune system restricts the colonization of non-native bacterial strains (Guo *et al.* 2023). Antagonistic interactions also contribute to microbiome composition, whether by outcompeting foreign species or promoting the coexistence among closely related strains via niche partitioning (Palmer-Young *et al.* 2018, Brochet *et al.*, 2021). Microbe-microbe competition led to the diversification among bacterial species and may provide a mechanism for the evolution of host-specific strain-level diversity (Powell *et al.* 2016; Kwong *et al.* 2017). Even environmental factors, such as seasonal thermal range, affects hindgut bacterial composition (Kešnerová *et al.* 2019). The study of microbiome assembly and function in non-social bee gut microbiota is just beginning to be understood.

Few studies have investigated microbiome acquisition in adult non-social bees after pupation. In a ground-nesting solitary bee, pre-pupae and newly emerged females had similar bacterial composition to that of their nesting materials (Kapheim *et al.* 2021). In contrast, bacterial diversity steadily declined in subsocial, stem-nesting bees after

pupation and low species richness was maintained throughout the rest of development (Nguyen & Rehan 2022). Collectively, these findings suggest that most bacterial genera cannot be maintained across the life cycle of non-social bees. An attempt to determine whether non-social bees have a core gut microbiota found three enterotypes in subsocial bees, but none in a facultatively eusocial bee species (Graystock *et al.* 2017). This finding implies that harboring a core gut microbiome is host-dependent, and that not all non-social bees can form these complex associations (Hammer *et al.* 2019).

Hosts, however, do not necessarily need to associate with a multispecies community to benefit from animal-microbe interactions. Some studies have detected vertically transmitted bacterial genera, including *Wolbachia*, *Spiroplasma*, *Sodalis*, and *Arsenophonus*, in solitary bees that likely can provide benefits to the host across development (Kueneman *et al.* 2023, Hettiarachchi *et al.* 2023, Drew *et al.* 2021, Saeed & White 2015, Gerth *et al.* 2015). The detection of vertically transmitted microbes in bees may depend on the tissues sampled, as many endosymbionts are housed in fat bodies and ovaries (Pietri *et al.* 2016), yet these tissues are typically not included in sampling efforts.

The first study of my thesis (chapter two) characterizes microbiome composition throughout the development of *O. lignaria*, a solitary mason bee. The next study (chapter three) aims to understand how temperature-mediated shifts in microbiome composition of *O. lignaria* pollen provisions affects larval health and development. These studies fill gaps in literature by enhancing our understanding of microbiome assembly and function in solitary, cavity-nesting bees. Finally, this thesis is concluded with commentary on how

bee-microbe interactions may change as a result of climate warming and also includes suggestions for future directions in non-social bee microbiome research.

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TABLES AND FIGURES

Table 1.1 Drivers of floral, provision and bee microbiome abundance and composition. Ecological processes shaping the floral microbiome are adapted from Vannette 2020.

Location	Process(es)	Mechanism	Reference(s)
Flower	Selection	Nectar and pollen chemistry: antimicrobial proteins, reactive oxygen species, sugar concentration, secondary metabolites	Hypothesized here, Schmitt et al. 2018, Thornburg et al. 2003, Herrera et al. 2010, Huang et al. 2012
Flower	Selection & Dispersal	Microbe-microbe interactions: niche modification	Vannette & Fukami 2014
Flower	Dispersal	Microbial species pool: environment (e.g., soil, rain), animal-vectored, other floral tissues	Morris et al. 2020, Zemenick et al. 2019, Junker et al. 2011, Manirajan et al. 2016
Provisions	Selection	Chemistry: reactive oxygen species, sugar concentration, pollen secondary metabolites	Hypothesized here
Provisions	Selection	Microbe-microbe interactions: competition, facilitation, etc.	Hypothesized here
Provisions	Selection	Environment: daily thermal range	Hypothesized here
Provision	Selection	Host-microbe interactions: modification by larvae	Voulgari-Kokota et al. 2019, Kueneman et al. 2023
Provisions	Dispersal	Microbial species pool: environment (e.g., nesting materials), floral specialists, bee-vectored, pathogens	Cohen et al. 2020, McFrederick et al. 2016, McFrederick et al. 2012, Rothman et al. 2018
Bee	Selection	Host traits: gut pH, oxygen concentration, gut structure, molting, pupation, immune system	Callegari et al. 2021, Engel & Moran 2013, Hammer & Moran, 2019, Kwong et al. 2017
Bee	Selection	Biotic interactions: competition, mutualism, host filtering	Palmer-Young et al. 2018, Brochet et al., 2021, Kwong et al. 2014
Bee	Selection	Environment: seasonal thermal range	Hypothesized here, Kešnerová et al. 2019
Bee	Dispersal	Mode of transmission: vertical, horizontal	Hypothesized here, Hettiarachchi et al.

			2023, Cohen et al. 2020
Bee	Diversification	Competition within hosts: host-specificity, strain-level diversity	Kwong et al. 2017, Powell et al. 2016

The developmental microbiome of *Osmia lignaria*: implications of *Arsenophonus* as a male-killing symbiont

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Abstract

Host-associated microbes are increasingly recognized as important drivers of bee health. Surveys of bee microbiomes have primarily sampled social bees, yet non-social bees constitute the majority of species. We employed 16S and ITS amplicon sequencing to describe the diversity and composition of bacterial and fungal communities across multiple developmental stages of *Osmia lignaria*, an important native and managed solitary mason bee. Bacterial and fungal diversity were not significantly different across bee development. However, the composition of bacteria and fungi significantly changed between larvae and fully pigmented adults, in synchrony with dramatic changes in host morphology during metamorphosis. Many of the microbial taxa found in provisions were also present in larvae, indicating that immature bees acquire their microbiome from food. Notably, the most prevalent bacterial genus was *Arsenophonus*, a symbiont with many recorded phenotypes, ranging from reproductive parasitism to beneficial endosymbiont. *Arsenophonus* was found in samples from provisions and eggs, yet reached higher read counts in larvae and fully pigmented adults. The *Arsenophonus* amplicon sequencing variants (ASVs) detected in this study had high sequence similarity with a symbiont that displays the son-killing phenotype, suggesting that the ASVs in *O. lignaria* are also reproductive parasites. The causative agent of chalkbrood disease in bees, *Ascosphaera*, was also detected in provisions and larvae. Most other taxa present were plant pathogens

or commonly found in soil. This study highlights that *O. lignaria* harbor horizontally and vertically transmitted microbial taxa with diverse consequences for bee fitness.

1 Introduction

For many multicellular organisms, host-associated microbes are important for their biology, ecology, and evolution. The advent of sequencing technologies has led to the rapid exploration of host-microbe interactions across many systems, thereby elucidating previously unknown symbioses. Microbial symbionts may provide a variety of services which can be categorized as pathogen resistance, mobility (e.g., dispersal), or nutritional and metabolic functions (e.g., detoxification or synthesis of essential nutrients) (Douglas 2010). Given their potential importance to influence host health, microbiomes are now being investigated as a new frontier to further our understanding of animal biology and aid in the conservation of species, including bees.

Early microbiome surveys of eusocial bees revealed the persistence of core bacterial species clusters (Martinson et al. 2011). Further investigation has determined that these core bacteria establish in the hindgut where they ferment carbohydrates, enzymatically degrade pectin (a major component of pollen), and synthesize amino acids (Zheng et al. 2019). In addition, infection assays comparing the load of an opportunistic pathogen in the guts of healthy versus dysbiotic honeybees clearly demonstrated the ability of resident microbes to exclude antagonists (Steele et al. 2021). The consistent transmission of symbionts between individuals is likely important for the health of eusocial bees, considering the nutritional and pathogen resistance functions that their core gut microbes carry out. Indeed, brief social interaction and exposure of newly emerged bees to freshly

stored pollen is sufficient for the transmission of core bacteria between generations (Anderson et al. 2022).

In the stored pollen of social bees, bacteria produce organic acids that prevent pollen spoilage and the establishment of pathogens, while others supplement the diet of developing queen bees with essential amino acids (Vásquez and Olofsson 2009; Vásquez et al. 2012; Parish et al. 2022). Fungi may also have a nutritive role: honey bees that consumed pollen with spores survived longer than their counterparts reared without spores supplemented in their diets (Parish et al. 2020). Several studies suggest that the food-associated microbiomes of solitary bees also influence larval health. For example, increased amounts of free fatty acids were found in *Osmia ribifloris* larvae reared on provisions with microbes compared to those reared on sterile provisions (Dharampal et al. 2019). These changes have fitness consequences for solitary bees: larvae reared on microbe-rich provisions are faster to develop, weigh more, and have lower mortality rates than those reared on microbe-deficient provisions (Dharampal et al. 2022). It has been hypothesized that these effects occur because microbes found within provisions are themselves consumed by larvae as a dietary resource, or that they aid in the catabolism of recalcitrant pollen, leading to the release of the bioavailable nutrients (Steffan et al. 2019; Mattila et al. 2012).

Most microbes found in association with solitary bees are horizontally transmitted from the environment, namely flowers and contents of their nest. There is little evidence to date that solitary bees consistently host a community of core gut bacteria unlike their

eusocial counterparts (Cohen et al. 2020; Hammer et al. 2019). Instead, foraging solitary bee adults have similar microbiome compositions to the flowers they visit, larvae to their pollen provisions, and non-feeding bees to their nesting materials (e.g., mud to separate brood cells) (McFrederick et al. 2016; Kapheim et al. 2021). While it is common for herbivorous insects to rely upon microbial symbionts for the catabolism of complex macromolecules or to supplement the diet of nutrient-deficient plant material (Hansen and Moran 2013), not all animals require multispecies microbial communities at every stage in the host life cycle for successful development (Hammer et al. 2019). For instance, the transient microbes found within the guts of caterpillars are found in low abundance, suggesting that these immature insects do not rely upon ingested microbes (Hammer et al. 2017). Moreover, the disparate life history stages of Holometabola may account for some of these compositional differences between developmental stages, as microbes may need to re-colonize their insect hosts after every molt or pupation (Engel and Moran 2013).

Nevertheless, most studies examining solitary bee microbiomes only sample one developmental stage, limiting our understanding of host-microbe interactions throughout their entire life cycle. Furthermore, many of these studies typically sequence bacteria only, although there are many notable insect fungal symbionts, such as the filamentous fungus *Zygosaccharomyces* provisioning an essential steroid to stingless bee larvae (Menezes et al. 2015; Paludo et al. 2018). Here, we characterize the microbiome of the solitary mason bee *Osmia lignaria*, an important wild and managed pollinator, at multiple stages in its development. Specifically, 16S and ITS amplicon sequencing was performed

to describe the bacterial and fungal composition of provisions, eggs, larvae, and the gut microbiomes of fully pigmented, emerged, and dead adults. Describing microbial taxa present at different developmental stages and examining shifts in microbiome composition are the first steps necessary to reveal microbes with consequences for host health. In addition, since commercial bee managers oftentimes source *O. lignaria* from wild populations for crop pollination (Bosch and Kemp 2001), characterizing the microbiome of native mason bees is important to understand whether any mutualists may be disrupted by chemicals applied in agricultural settings (Daisley et al. 2020) and to identify pathogens that may be transmitted between native and managed bees (Graystock et al. 2016).

2 Materials and methods

2.1 Bee life history

Found west of the Rocky Mountains, *Osmia lignaria propinqua* (Megachilidae) is a native solitary bee that nests in abandoned wood cavities previously excavated by long-horned beetles (Rust 1974). Populations of *O. lignaria* in northern Utah (USA) emerge in early spring. Females begin harvesting floral resources to create provisions, a mass of pollen and nectar provided singly to each offspring. The first 2-3 eggs laid on larger provisions at the rear of the nest develop into females, while the rest develop into males (Philips and Klostermeyer 1978). After hatching, bees proceed through five larval instars until the absence of food prompts cocoon spinning (Helm et al. 2017). Aestivation, a period of summer dormancy, begins once larvae, now termed prepupae, have completed cocoon production (Torchio 1989). Rather than overwinter as prepupae like most solitary bee species, *Osmia* pupates in mid to late summer, becoming fully pigmented adults prior

to the onset of cold temperatures (hereafter pre-wintering adults) (Levin 1966). Adults remain within their brood cells until the next spring when they chew out of their cocoons and emerge from their nests.

2.2 Trapping wild bees

Wild *O. lignaria* were trapped along Little Bear Creek in the Uinta-Wasatch-Cache National Forest in Logan Canyon, UT, USA (41.87834°N, 111.55782°W). This small canyon features a riparian environment dominated by aspen, willow, and subalpine forbs surrounded by uplands containing sagebrush and conifers. This study site is located at 1950 m a.s.l., nearing the upper bounds of the focal bee species' elevational distribution (Bosh and Kemp 2001). Bees were encouraged to nest within artificial trap nests constructed from dark navy, corrugated plastic boxes (21 x 21 x 25 cm) that faced approximately south (Artz et al. 2014). Trap nests contained two bundles of cardboard nesting tubes fitted with paper inserts (6 mm diameter; $N = 36$ per bundle; Crown Bees, Woodinville, WA) appropriate for capturing mason bees. Nesting tubes were sprayed with InvitaBee^{TM} Mason Bee Attractant (Crown Bees, Woodinville, WA) to encourage nesting within the artificial nests. Shortly after peak nesting activity, completed nesting tubes were transported to wooden dowels to mimic their natural nesting material. The wooden dowels (5 x 5 x 20 cm) contained three drilled holes (18 cm x 8 mm) and were hung from a branch on the same tree.

2.3 Microbiome sampling

For each sampling event, provisions and bees were only collected from the first three brood cells nearest the entrance of the nest containing developing males. Only this sex was collected because *O. lignaria* has a male-biased sex ratio, ensuring enough replicates were sampled at each targeted developmental stage and because prior work did not reveal any differences in provision microbiome composition between sexes in megachilid bees (Voulgari-Kokota et al. 2019). Dissection of nests were performed in a laminar flow hood and lab tools, including razor blades and forceps, were sterilized between brood cells by rinses in 5% bleach and then 70% ethanol. On June 13, 2022, prior to relocating nests to wooden dowels, a subset of nests (N = 10) was kept for the first sampling event – fresh pollen and egg microbiomes. Then, on July 2, 2022, nesting tubes (N = 15) were selected for dissection to sample the microbiomes of aged provisions and larvae. As described in Vojvodic et al. (2013), larvae were surface sterilized in three 75% ethanol washes prior to a final wash in sterile physiological saline solution (0.75 M NaCl, 0.1% Tween 80, 0.1% w/v peptone) to remove residual ethanol.

On September 17, 2022, nesting tubes (N = 10) were collected to sample the gut microbiomes of pre-wintering adults. After surface sterilizing bees by submersion in 95% ethanol, bees were dissected to isolate the digestive tract, from crop to hindgut, using methods adapted from Carreck et al. (2013). Each bee was submerged into a Petri dish containing sterile physiological saline. A dissecting needle was used to apply moderate pressure to the second and third abdominal teguments while viewing the ventral side of the bee under a microscope. Meanwhile, with forceps, the last abdominal tegument was grasped and gently pulled until the connective tissue between abdominal segments

separated. Micro-scissors were used to carefully cut each tegument away from the abdominal contents. Fat bodies and reproductive organs were teased apart from the gut. The last sampling event occurred on May 30, 2023 to collect the gut microbiomes of 2 emerged and 17 dead (time and exact cause of death is unknown) adult male individuals and followed the same methods detailed above.

2.4 DNA extractions

We adapted protocols from previously published solitary bee microbiome papers to extract microbial DNA from provision and bee samples (Dew et al. 2020; Graystock et al. 2017; Rothman et al. 2019, 2020; McFrederick and Rehan 2018) and followed suggestions for the preparation of pollen for metabarcoding to ensure sufficient lysis of provisions (Bell et al. 2016, 2018). We use Qiagen Blood & Tissue kits (Valencia, CA) with some adjustments from the manufacturer's instructions. Extra steps were taken to disrupt the cell walls of pollen, fungi, and gram-positive bacteria: samples were lysed during three cycles of 75 sec beating and 45 sec rest on a bead mill (Fisherbrand™ Bead Mill 24 Homogenizer, Fisher Inc., Waltham, MA) in autoclaved screw top microcentrifuge tubes containing approximately 100 µL 0.1 mm glass beads. In addition, 25 µL proteinase K (20 mg/mL) was added to each sample and treated overnight at 37°C. Afterward, lysed samples were centrifuged for 2 min at 6,000 rpm and then kit instructions were followed. Reagent controls and one extraction blank for every 17 samples were included.

We used a Quant-iT High-Sensitivity dsDNA Assay Kit (Invitrogen™, Thermo Fisher Scientific, Waltham, MA) to confirm ample DNA concentration and PCR to verify the presence of microbial DNA. Reactions to amplify the bacterial 16S V4-V5 rRNA gene region included 5 μL Phusion™ Plus DNA polymerase (2x; Thermo Fisher Scientific, Waltham, MA), 0.75 μL of the forward primer 515B-F (5 μM; 5'-GTGYCAGCMGCCGCGGTAA-3'), 0.75 μL of the reverse primer 926-R (5 μM; 5'-CCGYCAATTYMTTTRAGTTT-3'), and 1.25 μL pPNA clamps (5 μM; PNA Bio, Newbury Park, CA) to prevent the amplification of plastid and mitochondrial DNA. The bacterial PCR program included an initial denaturation step at 95°C for 3 min; 35 cycles of 95°C for 15 sec, 75°C for 10 sec, 50°C for 10 sec, and 72°C for 1 min; followed by a final extension step at 72°C for 10 min. Meanwhile, the eukaryotic internal transcribed spacer 2 region (ITS2) gene was amplified using 5 μL Phusion™ Plus DNA polymerase (2x), 0.5 μL of the forward primer ITS1-F (10 μM; 5'-CTTGGTCATTTAGAGGAAGTAA-3'), and 0.5 μL of the reverse primer ITS4-R (10 μM; 5'-TCCTCCGCTTATTGATATGC-3'). The fungal PCR program included an initial denaturation step at 95°C for 1 min; 35 cycles of 95°C for 1 min, 51°C for 1 min, 72°C for 1 min; and concluded with a final extension step at 72°C for 8 min. Of the 184 total samples collected, only the 98 total samples that had successfully amplified during PCR for either 16S only (15 samples), ITS only (9 samples), or both amplicons (78 samples) were submitted for sequencing.

2.4 Sequencing

Library preparation and Illumina MiSeq sequencing, generating 300 bp paired-end reads, was conducted at Integrated Microbiome Resource at Dalhousie University (Nova Scotia, Canada). During sequencing, PNA clamps and the same primers listed above were used, except the forward primer ITS86-F (5'-GTGAATCATCGAATCTTTGAA-3') was instead included to amplify the ITS2 region. Data were delivered as demultiplexed FASTQ files with adapters already trimmed. Sequencing resulted in 3,797,198 total 16S reads with a mean ± standard error (SE) of 17,827.2 ± 1,783.94 reads per file, while 2,182,060 total ITS2 reads were obtained with a mean ± SE of 10,965.1 ± 2,466.99 reads per file. 16S rRNA and ITS2 raw reads were inspected separately using the DADA2 pipeline which filters and trims reads, dereplicates identical sequences, performs sample inference, joins paired-end reads, and removes chimeric sequences (Callahan et al. 2016).

The bacterial forward and reverse reads were trimmed to 280 bp and 200 bp, respectively, where quality scores dropped below 30. Taxonomy of bacteria was assigned using the 16S rRNA Ribosomal Database Project v18 naïve Bayesian classifier (Callahan 2020). Sequences classified as cyanobacteria, chloroplast, mitochondria, Archaea, Eukarya, and the human pathogen *Legionella* were removed (Fields et al. 2002). ITS2 primers were removed from raw reads using cutadapt v4.6 (Martin 2011). Fungal taxonomy was assigned using the UNITE ITS v9.0 database (Abarenkov et al. 2023). We also removed reads assigned to *Pseudogymnoascus*, the causative agent of white-nose syndrome in bats, from the fungal dataset (Minnis and Linder 2013). Using the *decontam* package, contaminant reads were removed from samples informed by DNA concentration and prevalence of taxa in reagent and extraction blanks (McMurdie and Holmes 2013). This

action resulted in the retention of 67,635 total paired-end reads identified from 349 bacterial ASVs in 71 samples, including 8 fresh provisions, 8 aged provisions, 30 larvae, 14 pre-wintering adults, 0 emerged adults, and 11 dead adults. Meanwhile, 6,358 total paired-end reads were obtained from 207 fungal ASVs in 38 samples, including 5 fresh provisions, 5 aged provisions, 18 larvae, 5 pre-wintering adults, 1 emerged adults, and 4 dead adults. Mean reads per sample were 952.61 (± 461.61 SE) and 167.32 (± 36.89 SE) for bacteria and fungi, respectively.

2.5 Statistical analyses

All statistical analyses were performed in R 4.3.1 (R Core Team 2019). Provision samples were omitted from statistical analyses because mean read counts in fresh pollen and eggs (46.2 and 145 bacterial and fungal reads, respectively) and aged pollen (36 and 178 bacterial and fungal reads, respectively) were much lower than those found in previous studies (e.g., Rothman et al. 2019, 2020); thus, we opted to describe composition of pollen microbiomes only qualitatively. Shannon Diversity Indices and the observed richness of bee microbiomes were calculated using the 'estimate_richness' function in the *phyloseq* package (McMurdie and Holmes 2013). We examined the effect of bee sample type on alpha diversity using a linear mixed effects model (LME) with the *nlme* package, including bee nest as a random effect (Pinheiro et al. 2023). In addition, we used the *DESeq2* package to determine whether any taxa were differentially abundant across bee samples (Love et al. 2014). The initial DESeq analysis was performed using a rarefied dataset, then pairwise contrasts were used to examine whether any ASVs were

differential abundances across bee life stages. Significant differential abundances were identified using a BH-adjusted p-value < 0.05.

Given ongoing debates about the appropriate methods to normalize microbiome data, we performed beta diversity analyses on both relative abundances and rarified datasets (McMurdie and Holmes 2014; Schloss 2023). Bacterial and fungal communities in bee samples were rarefied at 16 and 20, respectively. Rarefying at these depths was deemed to be appropriate after observation of the rarefaction curves (Fig. A1). To test whether sample type affected bee microbiome composition, the 'adonis2' function in the *vegan* package was used to conduct permutational multivariate analyses (PERMANOVA) on a Bray-Curtis distance matrices from relative abundance and rarified datasets (Oksanen et al. 2020). When significant, the 'pairwise.perm.manova' function from the *RVAideMemoire* package was used to conduct pairwise post-hoc tests using a Benjamini-Hochberg (BH) correction of p-values (Herve 2023). We used the 'betadisper' function to test for violation of the PERMANOVA assumption that group dispersions are homogeneous. When appropriate, we followed up with pairwise comparisons using the Tukey's Honest Significant Difference method (Tukey's HSD). To visualize microbiome compositional differences between sample types, samples were viewed with principal coordinates analysis (PCoA) plots.

3 Results

3.1 Overall microbiome composition

Proteobacteria and Firmicutes were the most prevalent bacterial phyla across all samples. The top three bacterial genera found in pollen and bee microbiomes were *Arsenophonus*,

Enterococcus, and *Malikia*, accounting for 72.18%, 24.49%, and 1.12% of total reads, respectively. However, only two bacteria genera were found consistently at high relative abundances in each sample type: *Arsenophonus* and *Malikia* (Fig. 2.1, Fig. A2). Although *Enterococcus* was the second most abundant bacterial genus across all samples, it was only detected in one sample from a larva. Other bacterial ASVs accounted for less than 40 total reads across all samples and were found intermittently across developmental stages. When the total read count for an individual sample was greater than 125, most reads were attributed to ASVs from the genus *Arsenophonus*. This occurred in samples from one fresh pollen and egg, one larva, three pre-wintering adults, and one dead adult. The remaining samples had fewer than 125 total reads from all bacterial ASVs. In bee samples, reads from bacteria were extremely variable, with standard error values nearly as high as mean reads. Larvae had the lowest mean reads (587 ± 552 SE), followed by dead adults (1,152 ± 1,128 SE) and pre-wintering adults (2,622 ± 1,811 SE). ITS reads in bee samples were considerably lower: mean reads were reduced during the transition from larvae (258 ± 65.3 SE) to pre-wintering adults (8 ± 4.39 SE), then remained low in the single emerged bee (10 ± 0 SE) and dead adults (12 ± 3.76 SE).

Nearly all fresh pollen and eggs samples were dominated by *Arsenophonus* (Fig. 2.1, Fig. A2). The most common bacterial genus in aged pollen was *Malikia*, but a few samples were dominated by either *Arsenophonus* or *Stenotrophomonas* (Fig. 2.1, Fig. A2). Larvae were occupied by many of the same bacterial genera also found in pollen, including *Malikia* and *Arsenophonus*, among other less prevalent taxa such as *Stenotrophomonas*,

Acidovorax, Bacillus, and *Pseudomonas* (Fig. 2.1, Fig. A2). These same bacterial genera were also present in both pre-wintering and dead adults (Fig. 2.1, Fig. A2).

With respect to fungi, most taxa belonged to the phyla Ascomycota and Mucoromycota. The most dominant fungal genera included *Thamnidium*, *Penicillium*, *Sporormiella*, and *Mucor* (Fig. 2.1, Fig. A2), accounting for 16.03%, 12.78%, 11.95%, 11.08% of total reads, respectively. *Ascosphaera* was also among the top 15 fungal genera (Fig. 2.1), which accounted for 6.8% of total reads and were only found in a total of 8 samples from provisions and larvae. While five *Ascosphaera* ASVs were not identified to species, two were determined to be *A. pollenicola* and *A. fusiformis*. *Metschnikowia* and *Aureobasidium* were detected in the guts of dead adult bees, with the former accounting for less than 1% total reads but the latter in high relative abundance (Fig. A2). Most of the top 15 fungal genera were plant pathogens, such as *Botrytis, Valsa,* and *Alternaria,* as well as soil fungi, including *Cladosporium*, *Coniochaeta, Mucor*, and *Penicillium* (Fig. 2.1).

3.2 Diversity analyses

There were no significant differences in bacterial alpha diversity, calculated as Shannon's Diversity Index, across bee microbiomes (LME $F = 1.84$, $df = 2$, $p = 0.18$; Fig. 2.2). Likewise, there were no significant differences in the observed richness of bacteria ASVs among bee microbiomes (LME $F = 1.85$, $df = 2$, $p = 0.18$; Fig. A3). Alpha diversity of fungal ASVs were also not significantly different between bee samples, whether measured as Shannon diversity (LME $F = 1.51$, $df = 2$, $p = 0.27$; Fig 2.2) or observed

richness (LME F = 2.68, df = 2, p = 0.12; Fig. A3). Furthermore, DESeq analyses using rarefied datasets did not detect bacterial or fungal ASVs that had higher relative abundance in any particular sample type.

When using the relative abundance dataset, overall bacterial diversity was significantly different across sampled bee microbiomes (PERMANOVA $R^2 = 0.04$, $F = 1.20$, $p < 0.05$). Pairwise comparisons revealed that only larvae and pre-wintering adults had differences in bacterial composition (BH-adjusted $p < 0.05$), yet dispersion between these groups was violated (Tukey's HSD $p < 0.05$). There were no significant differences in bacterial composition between dead adults compared to both larvae (BH-adjusted $p = 0.08$) and pre-wintering adults (BH-adjusted $p = 0.23$). In addition, homogeneous dispersion was not violated when comparing dead adults to larvae (Tukey's HSD $p = 0.12$) and pre-wintering adults (Tukey's HSD $p = 0.96$). These findings were also observed while using the rarefied dataset: overall bacterial composition was significantly different across bee samples (PERMANOVA $R^2 = 0.06$, $F = 1.20$, $p < 0.01$). In particular, larvae and pre-wintering adults were significant different (BH-adjusted $p < 0.05$), while comparisons between dead adults and both larvae (BH-adjusted $p = 0.12$) and pre-wintering adults (BH-adjusted $p = 0.12$) were non-significant. However, heterogeneous dispersion was found between larvae and both pre-wintering (Tukey's HSD $p < 0.01$) and dead adults (Tukey's HSD $p < 0.01$), but not pre-wintering and dead adults (Tukey's HSD $p = 0.99$). Differences in bacterial composition by sample type for can be easily visualized in the PCoA plot, as dispersion in considerable (Fig. 2.3).

An overall significant difference was detected in fungal composition across bee samples when using a relative abundance dataset (PERMANOVA $R^2 = 0.15$, $F = 1.44$, $p < 0.01$). Pairwise comparisons indicated differences in fungal composition occur between larvae and both pre-wintering (BH-adjusted $p < 0.05$) and dead adults (BH-adjusted $p < 0.05$). Fungal composition did not differ between larvae and emerged adults (BH-adjusted $p = 0.21$), pre-wintering and emerged adults (BH-adjusted $p = 1$), pre-wintering and dead adults (BH-adjusted $p = 0.27$), as well as emerged and dead adults (BH-adjusted $p = 1$). Heterogenous dispersion was detected between emerged adults compared to larvae (Tukey's HSD $p < 0.001$), pre-wintering adults (Tukey's HSD $p < 0.001$), and dead adults (Tukey's HSD $p < 0.001$); however, fungal sequences were only found in one emerged adult, leading to differences in dispersion between these groups. While using a rarefied dataset, fungal composition was not significantly different between bee samples (PERMANOVA $R^2 = 0.13$, $F = 1.22$, $p = 0.08$). However, heterogeneous dispersion was detected between larvae when compared to pre-wintering adults (Tukey's HSD $p < 0.001$) and dead adults (Tukey's HSD $p < 0.001$), but not between pre-wintering adults and dead adults (Tukey's HSD $p = 1$). Visual inspection of samples in a PCoA plot confirms these statistical results: dispersion seems to drive differences in bee microbiomes (Fig. 2.3).

4 Discussion

We characterized the microbiome diversity and composition of *Osmia lignaria*, a native solitary mason bee that is also of economic importance. Examining multiple developmental stages provides insight into bee microbiome acquisition and maintenance,

as well as identifies key microbial taxa that may affect host health. Most bacteria and fungi were likely imported with nesting materials, such as soil microbes and plant pathogens. Our study found no significant differences in bacterial and fungal diversity across sample types. However, bacterial and fungal composition changed over the bee life cycle, with significant changes occurring between larvae and pre-wintering adults. This finding was anticipated, as drastic restructuring of bee anatomy happens between these developmental stages during pupation. These results are congruent with previous studies in both ground-nesting alkali and subsocial carpenter bees that have also described significant changes in microbiome composition between immature bees and adults prior to emergence from their natal nests (Kapheim et al. 2021; Nguyen and Rehan 2022).

Similar to other surveys of bees, the microbes associated with *O. lignaria* provisions and larvae were dominated by representatives from the Proteobacteria, Firmicutes and Ascomycota phyla. As in solitary ground-nesting bees, many of the same microbial taxa found in pollen provisions were also detected in larvae, suggesting that larvae acquire their microbiome while feeding (Kapheim et al. 2021). Despite their presence in provisions and larvae, most of the bacteria and fungi are not specific in their association with bees and are likely imported with nesting materials, including plant pathogens (e.g., *Acidovorax*, *Alternaria*, *Botrytis*, and *Valsa*) and soil microbes (e.g., *Cladosporium* and *Malikia*). Indeed, many of the fungal genera in *O. lignaria* pollen and larvae, including *Thamnidium* and *Mucor*, are often isolated from soil beneath conifers (Fresquez 1990). Although these soil microbes may be frequently transmitted into the brood cells of mason

bees, not all vectored microbes may be beneficial. For example, the soil bacterium *Bacillus* was consistently found in the larvae of deceased mason bees, implying detrimental effects on the survivorship of immature bees (Voulgari-Kokota et al. 2020). In contrast, some provision microbes may positively affect solitary bee health by modifying the nutritional quality of pollen, for example (Dharampal et al. 2019; Leonhardt et al. 2022). However, experimental studies providing direct evidence of microbial-mediated modification of pollen nutritional quality are currently lacking for most taxa found within the provision microbiota.

Metamorphosis results in pronounced changes to the abiotic environment of host-associated microbes; this unstable niche typically remains uninoculated until adult emergence and becomes colonized upon exposure to the external environment (Engel and Moran 2013). Bacterial and fungal reads in *O. lignaria* male pre-wintering adults were appreciably low, suggesting that solitary mason bees do not require gut microbes during this stage. This outcome is in accordance with the findings from subsocial carpenter bees: prior to emergence from nests, sclerotized adults had low bacterial read counts (508.7 ± 300.44 SE), with most bees containing less than 50 reads (Nguyen & Rehan 2022 Supplementary Table S1). Similar patterns of microbial acquisition are documented in eusocial bees: during pupation, bacterial read counts were significantly reduced, with the core bacterial community of worker females colonizing the gut after emergence (Hroncova et al. 2015; Dong et al. 2020). In solitary bees, the gut microbiomes of foraging adults are usually dominated by non-host specific microbes found in flowers and other plant tissues (Cohen et al. 2020; Kapheim et al. 2021; Nguyen and Rehan 2022;

Fowler et al. 2024), but also see Hettiarachchi et al. (2023) where endosymbionts were detected in the guts of solitary bees in addition to common environmental bacteria. Foraging patterns may establish transmission routes between floral microbes and visiting pollinators (McFrederick et al. 2016; McFrederick and Rehan 2018; Rothman et al. 2019), yet it remains to be determined whether these plant-associated microbes can establish within the guts of solitary bees and affect adult fitness and performance. In our study, the floral yeasts *Aureobasidium pullulans* and *Metschnikowia* spp. were absent or detected in low read counts in developing bees (Rering et al. 2017), but were also present in dead adult *O. lignaria*, suggesting that these particular taxa may incidentally associate with solitary bees rather than provide important symbiotic functions.

In addition to environmentally acquired microbes, some insect-specific taxa were also discovered. For instance, the most prevalent bacterial genus was *Arsenophonus* and observed in all sample types. The genus *Arsenophonus* includes members that display a variety of phenotypes, including obligate endosymbionts, horizontally transmitted mutualists with the ability to invade phylogenetically distant host lineages, and reproductive parasites (Nováková et al. 2009, Gherna et al. 1991; Thao and Baumann 2004). In this study, the 52 ASVs attributed to *Arsenophonus* had on average 98.61% sequence similarity to *A. nasoniae* when submitted to BLASTn (Table A1) (Altschul et al. 1990). The type species, *A. nasoniae*, is a son-killing extracellular symbiont first described associated with the parasitoid wasp, *Nasonia vitripennis* (Werren et al. 1986; Gherna et al. 1991). *Arsenophonus* has previously been detected in the microbiomes of honeybees and non-social bees (Drew et al. 2021; Gerth et al. 2015; McFrederick et al.

2014, 2016; Saeed and White 2015). Strains of *Arsenophonus apicola* isolated from honey bees were closely related to *A. nasoniae* and have been implicated with causing poor colony health (Nadal-Jimenez et al. 2022; Budge et al. 2016). Future studies should determine the transmission efficiency of *Arsenophonus* from mother to offspring in solitary bees.

Arsenophonus has been inventoried in seeds, flowers, as well as bee pollen, crops and midguts (Prado et al. 2020; McFrederick et al. 2016; Corby-Harris et al. 2014, Donkersley et al. 2018; Drew et al. 2021). However, *Arsenophonus* may not be able to replicate in environments outside of an insect host because of the loss of metabolic genes required for the uptake of glucose and catabolism of some amino acids (Darby et al. 2010). Experimental studies in honey bees revealed that *Arsenophonus* is transmitted from mother to offspring via the egg surface and then may cross the gut barrier after being acquired by feeding, reaching higher abundances as the host develops (Yañez et al. 2016; Gauthier et al. 2015). During *in vivo* tracking of *A. nasoniae* infection in a parasitoid wasp, Nadal-Jimenez et al. (2019) found that the symbiont was able to grow on the fly prey, host digestive tract, ovipositor, and exuviae. Accordingly, Darby et al. (2010) suggest that *Arsenophonus* may be able to use chitin as a source of carbon. We hypothesize that a similar infection process may occur in solitary bees because *Arsenophonus* was found in high frequency but low read counts in provisions and larvae, only reaching high read counts in pre-wintering and dead adult males of *O. lignaria*. In this population, obtaining sufficient sample sizes of surviving adult bees in this

population was difficult (BLC personal observation), further indicating that *Arsenophonus* resulted in the death of *O. lignaria* males in our cohort.

Notably absent in this survey of *O. lignaria* are fermentative lactobacilli, which are typically represented in bee samples. In honey bees, lactic acid bacteria function to prime the host immune system, prevent the spoilage of bee bread, and inhibit the establishment of pathogens (Kwong et al. 2017; Vásquez and Olofsson 2009; Forsgren et al. 2010). Indeed, genomic analyses revealed that members of the *Apilactobacillus micheneri* clade contain genes that would promote tolerance to life within nectar and bee gut environments (Vuong and McFrederick 2019). However, a recent *in vitro* study indicated that *A. micheneri*, in contrast to expectations, increased mortality rates of solitary bee larvae reared on diets supplemented with this bacterium (Brar et al. 2023). Regardless, bee-vectored microbes are likely ingested since *O. lignaria* larvae nearly consumes the provision in its entirety (Helm et al. 2017); rather, these microbial communities may contribute to bee health as an important dietary source (Steffan et al. 2019).

Concerningly, a subset of *O. lignaria* samples from provisions and larvae contained ASVs from the genus *Ascosphaera*, the causative agent of chalkbrood. These unique cyst-spore-producing fungi contain both pathogenic and non-pathogenic species (Klinger et al. 2013). In this study, two *Ascosphaera* ASVs were identified to species: *A. pollenicola* and *A. fusiformis*. In a recent genomic study, Maccaro et al. (2022) revealed similarities between *A. pollenicola* and the known bee pathogen, *A. aggregate*, including the presence of virulence genes. *A. fusiformis* was originally described in Japan, but this

pathogenic species was recently detected in native *Osmia* found in eastern North America (Skou 1988; LeCroy et al. 2023). Spores from pathogenic *Ascosphaera* are ingested before penetrating the larval midgut and finally germinating within the host, eventually causing death (Aronstein and Murray 2010). Filamentous fungi, such as *Penicillium*, found among the top fifteen genera in our samples, may have potential to prevent of the growth of disease-causing *Ascosphaera* (Disayathanoowat et al. 2020; Gilliam 1988). However, it remains to be discovered whether the provision microbiota has a protective effect against pathogens in solitary bees.

5 Conclusion

Our study described the microbial communities in association with *Osmia lignaria*, a native and managed solitary mason bee. The microbiomes of larvae had similar microbial taxa as provisions, suggesting that immature bees acquire their microbiome via consuming pollen. Although diversity was not significantly different between bee samples, bacterial and fungal microbiomes were significantly different between larvae and pre-wintering adults, coinciding with profound changes in host anatomy. In addition to finding environmentally acquired microbial taxa, this survey also detected bee pathogens, including *Arsenophonus* and *Ascosphaera*. We encourage future studies to determine the incidence and functions of vertically transmitted bacteria, including *Arsenophonus*, in solitary bee species.

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TABLES AND FIGURES

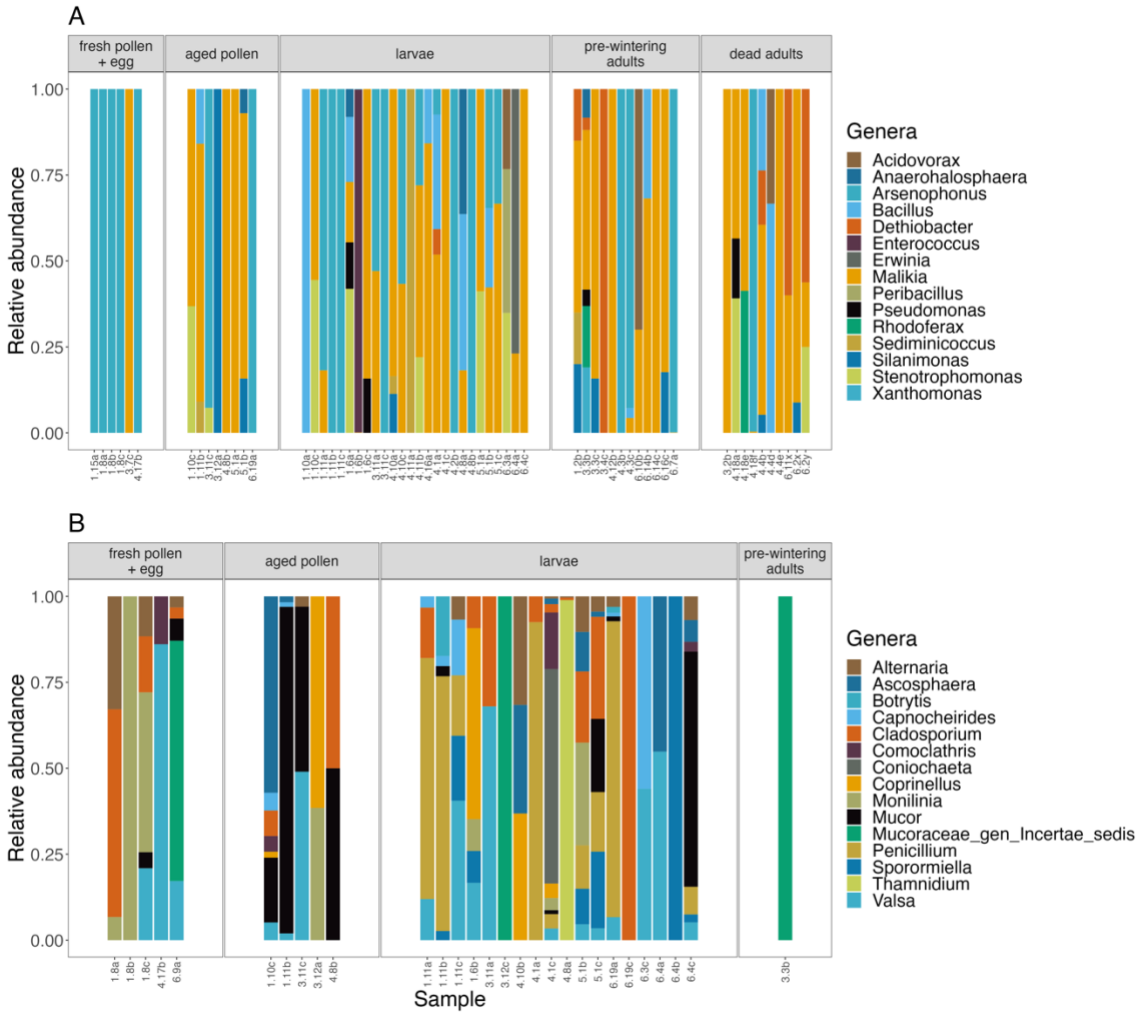

Fig. 2.1 Relative abundances (proportion of sequence reads) of the top 15 (A) bacterial and (B) fungal genera found in *Osmia lignaria* provisions, larvae, pre-wintering adults, and dead adults. See Fig. A2 for a relative abundance plot including all sample and genera.

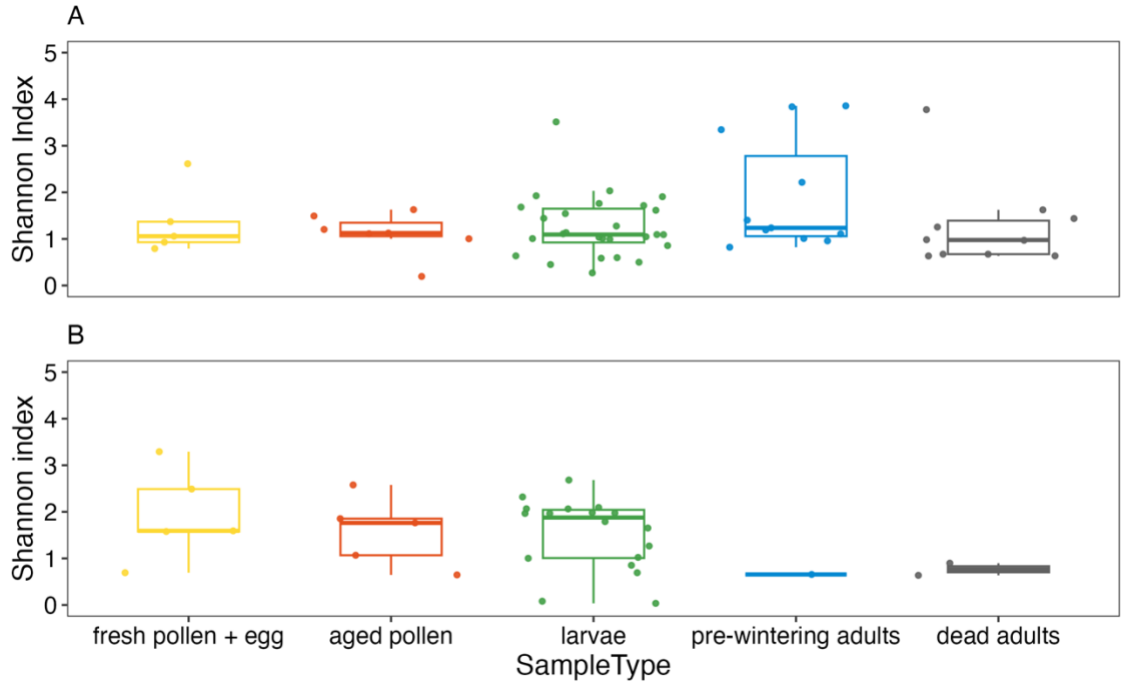

Fig. 2.2 Shannon diversity indices of provisions, larvae, pre-wintering adults, and dead adults using (A) bacterial and (B) fungal amplicon sequence variants (ASVs). Significant differences between sample types were not found for either kingdom.

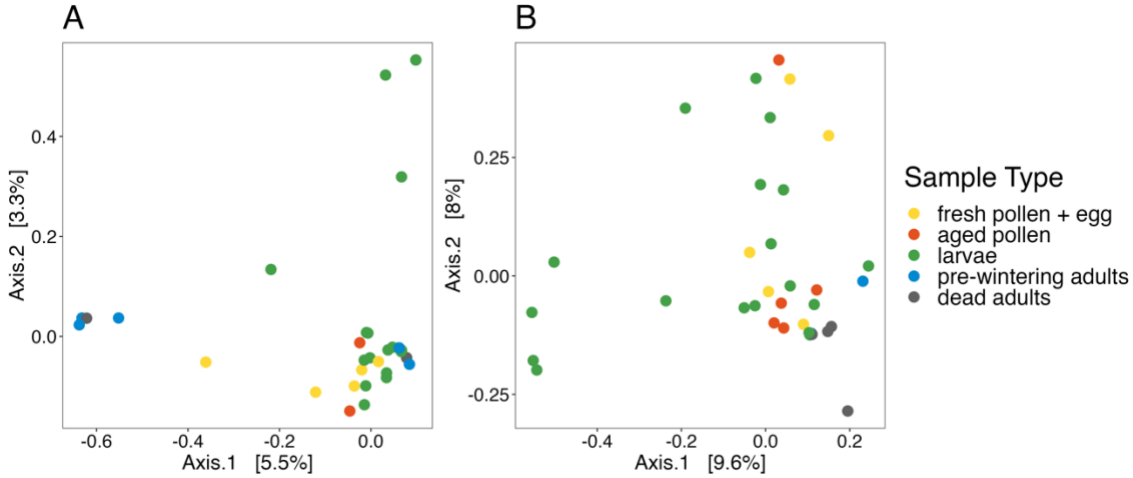

Fig. 2.3 Principal coordinates analysis (PCoA) of Bray Curtis dissimilarity across sample types of (A) bacterial and (B) fungal amplicon sequence variants (ASVs) using a relative abundance dataset. Overall significant differences were detected in bacterial ($p < 0.05$) and fungal ($p < 0.01$) community composition.

Bee microbiomes in a changing climate: investigating the effects of temperature on solitary bee life history and health

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Abstract

Climate change is rapidly warming thermal environments, an important abiotic stimulus governing bee development and species interactions. Increasing evidence suggests that solitary bees rely upon pollen provision microbes for successful development. The effects of heat stress on provision microbiota and resulting consequences for larval health and development, however, remain to be examined. We performed an *in vitro* study to investigate the effects of thermal environment on provision microbiome composition and measured fitness outcomes for *Osmia lignaria* larvae. Elevated temperatures moderately increased the relative abundance of *Arsenophonus*, a putative son-killing symbiont. While pollen sterilization removed *Arsenophonus* from microbe-rich provisions, larval survivorship did not significantly differ between bees reared on microbe-rich and sterile diets. In contrast to previous research in solitary bees, larvae reared on sterile provisions weighed more and had higher total fat contents, with temperature moderating the degree of difference. As anticipated, we observed a negative relationship between the duration of larval development and temperature. Our results indicated that an intact provision microbiota may not always improve bee fitness, and that bee-microbe interactions during larval development may contribute to the size-shrinking effect observed for cavity-nesting bees under warming conditions.

1. Introduction

Globally, pollinators face a multitude of threats, including habitat loss, the introduction of invasive species, climate change, or a combination thereof [1]. Climate warming, in particular, can have both direct and indirect consequences for pollinators. For example, pollinating insects, such as bees, can be directly impacted by changes in temperature, an abiotic cue important for the timing of complex life history traits, such as development and emergence [2,3]. Shifts in life history traits may indirectly cause phenological mismatches between entomophilous plants and their pollinators, as these mutualists respond differently to changes in temperature [4]. In sum, bee responses to warming temperatures can have implications for population persistence, as well as broader effects on the species with which they interact.

An overwhelming majority of bee species are solitary, and they display considerable variation in functional traits, including their nesting preferences (ground vs. cavity) and overwintering stage (prepupae vs. fully pigmented adults). These functional traits influence how solitary bees are affected by variation in temperature [5]. For example, a recent study observed that the body size of cavity-nesting bees has declined more quickly than that of ground-nesting bees over the same 20-year period in response to rising temperatures [6]. Furthermore, recent experimental studies have found that cavity-nesting solitary bees experiencing elevated temperatures had increased fat body consumption, reduced longevity, and altered emergence phenology [7-11]. Remaining relatively unexamined is how temperature interacts with bee-associated microbiomes to affect host health.

Previous research has demonstrated that microbes associated with provisions (larval food) positively impact solitary bee health and development. For instance, a diet

manipulation study found that, when fed sterile diets, solitary bee larvae grew slower, weighed less, and had reduced survivorship compared to their counterparts reared on microbe-rich provisions [12,13]. Upon further investigation, analyses comparing larvae reared on sterile and non-sterile provisions found more free fatty acids in bee in the latter treatment [14]. Although the mechanisms underlying these effects are unclear, early research suggests that microbes may permit larval access to bioavailable protein through degradation of recalcitrant pollen, or by serving as a dietary resource in and of themselves [15,16]. Thus, examining factors that may affect provision microbiome composition and function is essential to understanding drivers of bee health.

The activity of provision-associated microbes may be altered by abiotic factors, including temperature, which have potential downstream consequences for bee development and health. In some cases, increased temperature may be beneficial for the host through the indirect actions of symbionts. For example, high temperatures alter the symbiotic community of bumblebee gut microbes, resulting in reduced optimal growth temperatures for parasites and increased symbiont-pathogen competition [17]. However, destabilization of a symbiosis can also occur under thermal stress. For instance, the abundance of an obligate symbiont population was greatly reduced in a pest insect experiencing simulated warming and resulted in reduced fitness of the host [18]. While changes to the activities and functions of insect symbionts experiencing heat stress are still being uncovered, describing shifts in microbiome composition is a necessary step to elucidate these host-microbe interactions.

Here, we examined whether temperature shifts provision microbiome composition and the resulting effects on bee development and health outcomes using the solitary

mason bee, *Osmia lignaria*. We implemented an *in vitro* diet manipulation experiment with a fully crossed 2 x 3 factorial design. The first factor, provision microbiome, consists of two levels - natural or sterile. Meanwhile, the second factor, temperature, includes three levels - cool, ambient, or warm. The simulated treatments emulated historic, current, and projected (2040-2099) temperatures for the Great Basin region (USA), where wild *O. lignaria* reside [19,20]. We used amplicon sequencing to describe shifts in provision microbiome composition and quantify bee fitness outcomes as larval body mass, total proportion of fat content, and development time (2nd to 5th instar). This experimental design allows us to examine how interactions between immature solitary bees and symbionts may have been altered during the past several decades of warming, and to predict how these relationships may change with continued increases in temperature.

2. Methods

(a) Study system

Osmia lignaria propinqua (Megachilidae) is a native solitary mason bee found west of the Rocky Mountains (USA) [21]. In the Great Basin region, *O. lignaria* emerge between late May and early June as adults. During the following weeks, females create nests within pre-existing wooden cavities, using mud to separate brood cells. Provisions are constructed from harvested pollen and nectar, upon which an egg is deposited. Female offspring are laid in the 2-3 brood cells at the rear of the nest, while the rest develop into males [22]. Mother bees allocate larger provisions to female compared to male offspring. Hatched eggs proceed through five larval instars, which develop through early summer until food absence signals the start of cocoon spinning [23]. Bees residing in the Great

Basin region have experienced between 0.3-0.6°C increase in temperatures over the past 100 years, and the western United States is expected to warm 2-5°C in the coming decades [19,20].

(b) Rearing

O. lignaria offspring were collected from wild females nesting in artificial trap nests in Logan Canyon, Utah (UT), USA (41.89827°N, 111.57591°W), located in the northeast corner of the Great Basin region [24]. The number of active and completed nests were regularly monitored to determine peak nesting activity. Once peak nesting activity was reached, plugged nesting tubes were transported back to the lab and aseptically dissected in a laminar flow hood to reveal developing bees. For our diet manipulation study, we used a rearing protocol developed specifically for *O. lignaria* [25]. First, bees were grafted onto 1% agarose gels using sterile lab spatulas that were first submerged in sterile ringer's solution [25]. We predominately grafted male *O. lignaria* because the species has a male-biased sex ratio. Male bees were selected from the first three brood cells (i.e., nearest the nest entrance), while females were only chosen from the last two brood cells that contained larger provisions. We only grafted *O. lignaria* that were identified as eggs or first instar larvae, as bees in these early stages have not yet consumed any of the maternally allocated provision [26,27].

Provisions from randomly selected nests ($N = 8$) were subsampled to describe microbiome composition at the nest level. Then, provisions from all collected nests were homogenized, divided in half, and assigned to a microbiome treatment: microbe-rich or sterile. We homogenized provisions allocated to males and females together because

there is no evidence of pollen microbiome composition between sexes [28]. To sterilize provision material, pollen was exposed to ethylene oxide (EO) gas overnight with one ampule of 17.5 g Anprolene in a benchtop fumigation chamber (Anderson Sterilizers Inc., Haw River, NC) [29]. Sterilized pollen was then rehydrated with sterile DI water to the initial weight prior to EO exposure. While it is unknown how this treatment affects the nutritional quality of the provision, previous research has demonstrated that bumblebees consume equal amounts of EO-treated and untreated pollen, suggesting that there is no effect on the palatability the pollen [29].

The following day, nesting tubes were used like cookie-cutters to weigh 190 ± 2 mg and 351 ± 2 mg of pollen for male and female bees, respectively. The amount of pollen allocated to immature bees was calculated as the average of 10 randomly selected provisions for each sex. These artificial brood cells were held upright in sterile 24-well plates to ensure bees do not fall off the provision, guaranteeing immobile larvae have access to food [25]. Male (N = 180) and female (N = 66) bees were randomized into treatments and grafted onto their respective provisions in the same manner as described above. To minimize the amount of handling time, all male bees were grafted within 24 h of nest dissection [25]. Immature females, however, were grafted 2 days after being removed from their natal nest. Bees were then placed into incubators (Percival I-30L, Geneva Scientific, Fontana, WI) with thermal environments approximating historic, current, and projected climates (hereafter cool, ambient, and warm) throughout the duration of their larval development. The ambient temperature treatment followed mean daily temperature fluctuations of June 2022 collected from data loggers (iButtonLink DS1925L-F5#, Whitewater, WI) located near the field site. Simulated cool and warm

microclimates were programmed to be -1°C and +2°C relative to the ambient temperature treatment, respectively. Incubators across treatments were set at 60% relative humidity throughout the duration of the experiment. This experimental design produced a total of six different treatment combinations: cool-sterile (CN), cool-natural (CN), ambient-sterile (AS), ambient-natural (AN), warm-sterile (WS), and warm-natural (WN). Microbe-rich provisions without bees (351 ± 2 mg; N = 8 per treatment) were also placed within incubators to provide a baseline understanding of microbiome dynamics in response to temperature without potential modification by feeding larvae [30].

(c) Bee response variables

Bees were monitored daily to track their development. Once bees reached the fifth instar, indicated by the production of frass [27], they were removed from their artificial brood cell and both the larva and provision weighed before being stored at -20°C until microbiome and lipid extractions were performed. To determine the total body fat content of fifth instar larvae, bees were first lyophilized and then submerged in 20-fold the volume of the tissue sample in 2:1 (v/v) chloroform-methanol solution. The chloroform-methanol solution was replaced every 24 h for two more subsequent days before larvae were once again lyophilized and reweighed. The proportion of total body fat was calculated as the difference between initial dry mass and dry mass following lipid extraction divided by the initial dry mass [11,31]. Of note, this method measures both fat bodies and non-storage lipids. However, in adult *O. lignaria*, this overestimation of storage lipids is negligible when using a methyl-chloroform extraction protocol [10,32].

(d) DNA extractions

We prepared provisions for amplicon sequencing using DNA extraction protocols adapted from solitary bee microbiome papers [33-37] and incorporated advice for pollen metabarcoding [38,39]. First, samples were added to screw top microcentrifuge tubes containing about 100 µL of 0.1 mm glass beads, including reagent controls and 1 extraction blank for every 17 samples. To disrupt the cell walls of gram-positive bacteria, fungi, and pollen, samples were lysed with three cycles of 75 sec beating and 45 sec rest using a bead mill (Fisherbrand™ Bead Mill 24 Homogenizer, Fisher Inc., Waltham, MA). Lysed samples were then processed using Qiagen Blood & Tissue kits with some slight adjustments: samples were treated overnight with 25 µL proteinase K (20 mg/mL) before centrifugation for 2 min at 6,000 rpm. Thereafter, kit instructions were followed.

We confirmed DNA concentration and the presence of microbial DNA in our samples using a Quant-iT High-Sensitivity dsDNA Assay kit (Invitrogen™, Thermo Fisher Scientific, Waltham, MA) and PCR, respectively. The bacterial 16S V4-V5 rRNA gene region was amplified using 5 µL Phusion™ Plus DNA polymerase (2x; Thermo Fisher Scientific, Waltham, MA), 0.75 µL of the forward primer 515B-F (5 µM; 5'-GTGYCAGCMGCCGCGGTAA-3'), 0.75 µL of the reverse primer 926-R (5 µM; 5'-CCGYCAATTYMTTTRAGTTT-3'), and 1.25 µL pPNA clamps (5 µM; PNA Bio, Newbury Park, CA) to prevent the amplification of plastid and mitochondrial DNA. To amplify DNA from bacteria, the PCR program included an initial denaturation step at 95°C for 3 min; 35 cycles of 95°C for 15 sec, 75°C for 10 sec, 50°C for 10 sec, and 72°C for 1 min; ending with a final extension step at 72°C for 10 min. PCR reactions to amplify the eukaryotic internal transcribed spacer 2 region (ITS2) of fungi included: 5 µL

Phusion™ Plus DNA polymerase (2x), 0.5 μL of the forward primer ITS1-F (10 μM; 5'-TCCTCCGCTTATTGATATGC-3'), and 0.5 μL of the reverse primer ITS4-R (10 μM; 5'-TCCTCCGCTTATTGATATCG-3'). The fungal PCR program began with an initial denaturation step at 95°C for 1 min; 35 cycles of 95°C for 1 min, 51°C for 1 min, 72°C for 1 min; followed by a final extension step at 72°C for 8 min. Samples that successfully amplified were submitted for amplicon sequencing.

(e) Sequencing

Library preparation and Illumina MiSeq sequencing, producing 2 x 300 bp reads, was performed at Integrated Microbiome Resource at Dalhousie University (Nova Scotia, Canada). During sequencing, pPNA clamps and the same primers listed above were used, except the forward primer ITS86-F (5'-GTGAATCATCGAATCTTTGAA-3') was alternatively selected for the amplification of the ITS2 region. Samples containing DNA from bacteria was sequenced on a single run, while fungal samples were separated into two sequencing runs. The 16S and ITS2 raw reads were delivered as demultiplexed FASTQ files with adaptors already trimmed. Sequencing resulted in 794,418 total 16S reads with a mean ± standard error (SE) of 5,715.24 ± 1,145.43 reads per sample, while 428,074 total ITS2 reads were obtained with a mean ± SE of 2,218 ± 303.66 reads per sample. In R 4.3.1 [40], we inspected sequences using the DADA2 pipeline which filters and trims reads, dereplicates identical sequences, performs sample inference, joins paired end reads, and removes chimeric sequences [41]. Reads from the two fungal sequencing runs were separately passed through the DADA2 pipeline before being merged into a single dataset.

To ensure quality reads, bacterial sequences were trimmed at 250 bp and 200 bp for the forward and reverse reads, respectively, where the quality scores dropped below thirty. Taxonomy of bacteria was assigned using the 16S rRNA Ribosomal Database Project v18 naïve Bayesian classifier [42]. We removed sequences classified as cyanobacteria, chloroplast, mitochondria, Archaea, and Eukarya from the bacterial dataset. ITS2 primers were removed from raw reads using cutadapt v4.6, and we filtered ASVs that were not identified to at least family [43]. We used the UNITE ITS v9.0 database for taxonomic assignment of fungi [44]. Using the *decontam* package, contaminant reads were removed from samples based on prevalence in reagent and extraction blanks [45]. After controlling the quality of sequences, we retained a total of 1,930 paired-end reads from 138 ASVs in our bacterial dataset containing 30 samples. In the fungal dataset, we preserved 10,478 paired-end reads from 266 ASVs in our 68 samples. Mean reads per sample were 64.33 (± 7.35 SE) and 154.09 (± 21.25 SE) for bacteria and fungi, respectively. Albeit low, our rarefaction curves suggest that extraction methods sufficiently captured the diversity of the provision microbiome (Figure B1). While other studies find higher read counts per provision in solitary bee species [33-37], the age of pollen is typically not reported. We sample provisions when larvae were fifth instars; the viability of microbial taxa in aged solitary bee pollen has yet to be examined. In fact, the microbiota of honey bee stored pollen undergoes drastic reductions in diversity and culturable microbes, ultimately becoming dominated by osmotolerant bacteria [46].

(f) Statistical analyses

All statistical analyses were performed in R 4.3.1 [40]. Health and life history data from female bees were excluded from analyses because they died rapidly, likely because of delayed grafting – females were off their provisions for 48 h (BLC personal observation). We implemented linear mixed effects (LME) models using the lme4 package to assess the effectiveness of simulated climatic treatments and to analyse the effects of treatments on bee response variables [47]. In the models assessing differences in mean temperature and relative humidity, we used day of year as a random intercept to account for multiple daily temperature readings. Meanwhile, grafting stage (egg or 1st instar) was used as a random intercept in models testing the effects of microbiome and temperature treatments on the biomass, proportion of fat content, and development time of male larvae. Residuals for larval development time were not normally distributed; thus, this response variable was analysed using a generalized mixed effects model (GLMM) with a gamma error distribution. We estimated the *p*-values of linear mixed effects models using the Kenward-Rogers approximation. We used the emmeans package to examine pairwise comparisons, adjusting *p*-values with the Tukey's Honest Significant Difference method (Tukey's HSD) [48]. Prior to analysing survivorship data, we removed male bees that died within 48 h of grafting, which we attribute to physical damage inflicted during handling. Log rank and Gehan-Breslow generalized Wilcoxon tests were used to compare median survival time of male bees across treatments using the survival package [49]. In addition, we used the survreg function to fit an accelerated failure time (AFT) model to determine the effects of temperature and microbiome treatments on survival time, including grafting stage as a fixed effect.

We qualitatively describe the microbiomes of provision samples that were not consumed by larvae but exposed to temperature treatments given the small number of these samples. Provisions containing developing bees (males and females) were statistically analysed. We removed the only cool-sterile sample from our bacterial dataset prior to statistical analysis because it contained two reads. Alpha diversity measures, including Shannon's Diversity Indices and observed richness, were calculated using the estimate_richness function from the phyloseq package [45]. We assessed the effects of bee sex and temperature treatment on alpha diversity using a linear mixed effects model (LME) with the nlme package, including grafting stage as a random effect [50]. In addition, we quantified Pielou's evenness to examine the effect of bee sex, thermal environment and microbiome treatments on the distribution of microbial ASVs amongst our treatments. We also performed a Kruskal-Wallis test *a posteriori* to evaluate shifts in mean rank relative abundance of the dominant bacterial genus, *Arsenophonus,* in response to our climatic treatments.

To test the effects of microbiome treatment and thermal environment on the composition of bacterial and fungal communities, we performed permutational multivariate analyses (PERMANOVA 999 permutations) using the adonis2 function from the vegan package [51]. Beta diversity analyses were applied to Bray-Curtis distance matrices generated from relative abundance datasets. When an overall significant effect was found, pairwise comparisons with Benjamini-Hochberg (BH) correction of *p*-values were performed using the RVAideMemoire package [52]. The PERMANOVA assumption of homogenous group dispersion was inspected with the betadisper function. When significant, we followed up with pairwise comparisons using the Tukey's HSD

method. Differences in microbiome composition between treatments were visualized with principal coordinates analysis (PCoA) plots.

3. Results

(a) Effectiveness of climatic and microbiome treatments

The mean thermal environment in the warming treatment was 2.2°C higher than the ambient treatment; meanwhile, the mean temperature of the cool treatment was 0.8°C below the mean ambient temperature (LME, $F_{2,10227} = 117.65$, $p < 0.001$; figure 3.1). The maximum temperatures bees experienced in each microclimate were 23.2°C, 25°C, and 27.2°C for the cool, ambient, and warm treatments, respectively. These climatic manipulations approximate historic (*c*. 1900s), current, and conservative projected (*c*. 2050-2100) mean daily temperatures for the Great Basin region [19,20]. Although relative humidity was programmed to remain the same across incubators regardless of simulated microclimate, this variable significantly differed between thermal environments (LME, $F_{2,11183} = 343.15$, $p < 0.001$; figure 3.1). However, mean relative humidity was 61.74%, 60.60%, and 61.33% for the cool, ambient, and warm microclimates, respectively. These minimal differences in relative humidity between treatments likely did not affect bee fitness outcomes and shifts in microbiome composition.

Nest-level sampling of pollen prior to homogenization revealed that the bacterial genus assigned the most read counts, *Arsenophonus*, did not appear consistently in natal nests; however, after mixing, our mass of pollen contained this genus as a dominant member of the provision microbiota (figure B2). The fungus, *Cladosporium*, was found in nearly all nest-level samples of provisions, while other genera appear more

sporadically and in low relative abundances (figure B2). Inspection of our pollen mass after homogenization suggests that we sufficiently mixed provisions prior to reallocating larval food to *O. lignaria* offspring, as *Cladosporium* remained the most dominant fungal genus and other taxa were found in low relative abundances (figure B2).

Pollen sterilization was effective at eliminating bacteria, as amplicon sequencing did not detect reads from this kingdom in EO-treated pollen. Fungi were still detected in sterilized pollen, but analyses indicated that our method reduced species richness (LME, $F_{1, 38} = 9.90$, $p = 0.003$) and had significant effects on provision composition (PERMANOVA, $R^2 = 0.12$, $F = 5.76$, $p = 0.001$). Upon examining Pielou's evenness, we found a marginal effect of temperature on the distribution of bacterial taxa in microbe-rich provisions (LME, $F_{1,11} = 3.58$, $p = 0.09$; figure B3), suggesting that thermal environment may affect the relative abundance of certain genera. In contrast, temperature did not have an effect on the evenness of fungal taxa (LME, $F_{2, 31} = 0.33$, $p = 0.72$; figure B3) but pollen sterilization had a marginal effect (LME, $F_{1,31} = 3.55$, $p = 0.07$; figure B3).

(b) Overall microbiome composition

Most of the taxa present in provisions were represented by common soil-borne microbes and plant pathogens, such as Bacillaceae, Bradyrhizobiaceae, Comamonadaceae, Cladosporiaceae, Valsaceae, and Pucciniaceae. In addition, two insect-associated genera were present: *Arsenophonus* and *Ascosphaera*, accounting for 43.26% and 0.07% of total bacterial and fungal reads, respectively. In nearly all provision samples with feeding larvae present, *Arsenophonus* and *Cladosporium* were found in high relative abundance (figure 3.2, figure B4). Provisions without feeding larvae had lower mean bacterial read

counts (36 ± 12.32 SE) compared to those with actively feeding larvae (82 ± 21.16 SE). Similar patterns were also observed with fungi: provisions had higher fungal read counts when larvae were present (205.68 ± 63.49 SE) than absent (56.82 ± 20.09 SE). Nearly all bacterial and fungal genera detected in pollen without actively feeding larvae were also found in provisions containing developing bees (figure B4, figure B5). Indeed, overall bacterial (PERMANOVA, $R^2 = 0.04$, $F = 1.00$, $p = 0.42$) and fungal (PERMANOVA, $R^2 = 0.02$, $F = 0.99$, $p = 0.41$) composition in provisions did not differ depending on the presence or absence of larvae. However, provisions with actively feeding larvae contained more bacterial and fungal genera that were not found in pollen without bees, albeit in low relative abundances (figure B4, figure B5).

(c) Impact of thermal environment on microbiome diversity and composition in provisions with actively feeding larvae

Thermal environment had no effect on the alpha diversity of bacteria, quantified as Shannon's Diversity Index (LME, $F_{2,11} = 0.24$, $p = 0.79$; figure B6) and observed richness (LME, $F_{2,11} = 0.43$, $p = 0.66$; figure B7). Although we did not find a significant effect of temperature on the mean rank relative abundance of *Arsenophonus* (Kruskal-Wallis, $H_2 = 0.78$, $p = 0.68$), the warm treatment trended toward a higher mean (0.81 ± 0.07 SE) than both the cool (0.78 ± 0.06 SE) and ambient (0.68 ± 0.10 SE) microclimates (figure 3.3). Thermal environment did not have an effect on the observed richness of fungal ASVs across treatments (LME, $F_{2,38} = 0.88$, $p = 0.42$; figure B6), but a marginal effect was found for Shannon's Diversity Index (LME, $F_{2,38} = 2.82$, $p = 0.072$; figure B5). Bee sex was a significant predictor in the model of the observed richness of bacteria

(LME, $F_{1,11} = 5.63$, $p = 0.037$), however there were no significant pairwise comparisons. Likewise, bee sex was a significant covariate in the model of fungal Shannon's diversity (LME, $F_{1,38} = 6.26$, $p = 0.016$), but the only significant pairwise comparison detected were between sterile and natural provisions.

Both temperature (PERMANOVA, $R^2 = 0.13$, $F = 0.126$, $p = 0.086$) and bee sex (PERMANOVA, $R^2 = 0.07$, $F = 1.38$, $p = 0.052$) had a marginal effect on the composition of bacterial ASVs in provisions with actively feeding larvae. Dispersion between groups was not violated due to temperature treatment (ANOVA, $F = 0.02$, $p = 0.98$) nor bee sex (ANOVA, $F = 0.19$, $p = 0.66$). Temperature (PERMANOVA, $R^2 = 0.04$, $F = 0.97$, $p = 0.47$) and bee sex (PERMANOVA, $R^2 = 0.02$, $F = 1.19$, $p = 0.29$) did not have a significant effect on fungal composition. In assessing the PERMANOVA assumption of fungal composition, homogeneous group dispersion was not violated in pairwise comparisons between microbe-rich provisions. Upon visual inspection in ordination space, the provision microbiome did not cluster by temperature treatment (figure B8).

(d) Larval health and development outcomes

Microbiome treatment had an overall effect (LME, $F_{1,53.11} = 8.02$, $p = 0.007$) on the biomass of male fifth instar larvae. Pairwise comparisons revealed a moderate effect (Tukey's HSD $p < 0.08$) when comparing sterile and microbe-rich provisions within each temperature treatment as well as between WN versus both CS and AS. While the thermal environment did not have an overall effect on larval body weight (LME, $F_{2, 53.22} = 1.28$, $p = 0.29$), the differences in mean biomass between larvae reared on sterile versus

microbe-rich provisions was incrementally greater with increasing temperatures. Specifically, bees reared on sterile pollen were 3.88%, 22.3%, and 28.7% larger on average than larvae consuming microbe-rich provisions in the cool, ambient, and warm microclimates respectively (figure 3.4*a*).

Similarly, the presence or absence of microbes in the larval diet had a significant effect on the proportion of total fat content in male immature bees (LME, $F_{1,53.10} = 10.42$, $p = 0.002$). Thermal environment did not have an overall effect (LME, $F_{2,53.19} = 0.82$, $p = 0.44$) on the fat content of fifth instar larvae; however, increasing temperatures exacerbated the differences in mean total fat content between bees reared on sterile and non-sterile provisions. In particular, larvae reared on sterile provisions had 6.45%, 30.7%, and 49.8% higher total fat content on average than bees fed microbe-rich provisions (figure 3.4*b*).

The duration of larval development was significantly affected by nest temperatures (GLMM, $X^2 = 20.08$, $p < 0.001$) but was unaffected by microbiome treatment (GLMM, $X^1 = 2.58$, $p = 0.11$). Further investigation revealed significant differences in all pairwise comparisons between larvae reared in the warm nest environment compared to those in the cool and ambient microclimates, irrespective of microbiome treatment (figure 3.4*c*). Median survival time was not significantly different between treatments (log rank test: $X_5^2 = 2$, $p = 0.8$; Gehan-Breslow-Wilcoxon test: $X_5^2 = 2.4$, $p = 0.8$) (figure 3.5). These findings are supported by the AFT model ($X^5 = 4.78$, $p = 0.44$): neither temperature ($p = 0.40$) nor microbiome ($p = 0.66$) treatments had a significant effect on larval survivorship.

4. Discussion

Solitary bees are thought to derive benefits from the provision microbiota, either as dietary resources or by pre-digesting pollen [12-14,16]. Differences in thermal optima between larvae and symbionts have unknown consequences for developing solitary bees. We performed an *in vitro* study to investigate the effects of temperature on provision microbiome structure and determined the outcomes for the health and development of male *O. lignaria* larvae. We found trends that point to temperature-mediated effects on the mean relative abundance of *Arsenophonus*, a putative son-killing bacterium in solitary bees. In contrast to expectations, we did not observe differences in survivorship between larvae reared on microbe-rich and sterile diets, although pollen sterilization was effective at eliminating *Arsenophonus*. Upon examining the effects of microbiome treatment on larval biomass and fat content, we found that larvae reared on sterile provisions weighed more and had higher total fat contents. Within each thermal environment, the differences in mean biomass and fat content between bees reared on sterile versus microbe-rich provisions increased with warming temperatures. We also found a negative relationship between temperature and the duration of larval development. Our data presents evidence that the maternally provided provision microbiota may not be beneficial to bee health in all circumstances. In addition, this study highlights that bee-microbe interactions may be mediated by temperature, with potential consequences for larval fitness such as the decrease in bee body size observed over the past several decades of warming climates [6,53].

While most microbial taxa were not specific to *O. lignaria*, the most dominant bacterial genus detected, *Arsenophonus*, is a known symbiont of plants and insects. The *Arsenophonus* genus is monophyletic group contains symbionts which exhibit

exceptionally diverse phenotypes, including phytopathogenicity, obligate endosymbiosis, and reproductive parasitism [54-56]. The 16 ASVs assigned to *Arsenophonus* in this study had on average 98.92% sequence similarity to the son-killer, *A. nasoniae* (Table B1) [57-59]. Surveys of social and non-social bees have detected *Arsenophonus* in pollen, bee body surfaces, crops, and midguts [60-68]. The *A. apicola* strains isolated from honey bees have also been implicated as a male-killing symbiont [69,70]. Although *A. nasoniae* originally described in wasps lacks genes for the uptake of glucose and metabolisms of key amino acids [71], *A. apicola* has the ability to utilize more carbon sources [72], likely the reason why the bee associate can be found in flowers and stored pollen. Laboratory studies discovered that *Arsenophonus* is transmitted from mother to offspring via inoculation of the egg surface, eventually localizing in the midgut of infected individuals and then may cross the gut epithelium [66,73,74]. Similar infection processes may occur in solitary bees: while characterizing the development of *O. lignaria* in a recent microbiome survey, *Arsenophonus* was found in low relative abundance in early developmental stages, only reaching higher read counts in the guts of adults [67].

Rises in temperature increased the mean relative abundance of *Arsenophonus*, suggesting that our temperature manipulations were below the thermal optimum of this reproductive parasite. However, some larvae successfully developed in the presence of the putative son-killing bacterium. While investigating the transmission of *Arsenophonus* between honey bee nestmates, Drew et al. (2021) documented that a subset of adult bees lost the infection over the course of the experiment, likely by shedding the parasite in faeces [66]. We speculate that the surviving bees in our study voided *Arsenophonus* in their digestive tract once they reached the fifth instar stage, or shed the parasite while

moulting. When tracking *A. nasoniae* infection processes *in vivo*, the parasite was found on the host digestive tract, ovipositor, and exuviae of infected male wasps [75], suggesting that this bacterium has the ability to metabolize chitin. Indeed, genes for the production of a novel chitinase and chitin-binding proteins have described in *A. nasoniae* and are likely responsible for the ability of the parasite to occupy this niche [71]. Future studies should investigate larval-parasite interactions in more extreme warming scenarios.

Although our sterilization method eliminated *Arsenophonus* and other bacteria from microbe-deficient pollen, larvae reared on sterile provisions had comparable survivorship to bees consuming microbe-rich diets. These results contradict previous research strongly suggesting that larval survivorship depends on the presence of microbes in larval food [12-14]. Preliminary data from a recent *in vitro* study, however, also indicates that provision microbes can have adverse consequences for solitary bee fitness: the addition of *Apilactobacillus micheneri* (previously *Lactobacillus*) in the diet of megachilid larvae increased mortality [76], an unexpected result given the frequent detection in solitary bees [62,77,78] and known functions of lactobacilli in eusocial bees (e.g., preventing the spoilage of stored pollen and defense against pathogens) [79,80]. Furthermore, Anderson et al. (2014) used microscopy and culture-dependent methods to challenge the long-held belief that microbes in the stored bee bread of honey bees aid in the pre-digestion pollen: upon visual inspection, microbes were greatly outnumbered by uncompromised pollen grains, and counts of viable microbes markedly fell after 96 h of storage [46]. Collectively, these findings suggest that the maternally allocated provision microbiome may not always benefit larval health. Future research should leverage

experimental studies to investigate the functions of exosymbionts found within solitary bee provisions.

We found contradictory evidence regarding the importance of the provision microbiota for larval fitness: immature bees reared on pollen without microbes weighed more and had higher total fat content that those feeding on microbe-rich provisions. The thermal environment affected the difference in mean biomass and fat content between larvae consuming sterile and microbe-rich diets, where bees in the warmest microclimate had the largest difference in means for each response variable. Past research found that larvae acquire fatty acids (and amino acids) from microbial prey within provisions, resulting in heavier bees than those reared on sterile pollen [12-14]. These opposing results may be explained by differences in pollen sterilization methods: in choice assays, EO exposure does not affect the palatability of pollen to bumblebees [29], but it is unknown whether soaking provisions in ethanol [12-14] affects pollen consumption in solitary bees, perhaps leading to differences in biomass, fat content, and trophic positions. Of note, while not statistically significant, bumblebees consumed less pollen per day when fed EO exposed pollen compared to the no treatment control [29]. This likely contributed to slight differences in biomass and fat content between bees reared on sterile versus microbe-rich provisions, though most pollen consumption occurs while fifth instar larvae in *Osmia* spp. Indeed, *O. lignaria* body size is correlated with the weight of the provision [81]. *Arsenophonus* has been detected in the fat bodies of bumblebees co-infected with *Apicystis bombi* [82], a parasitic protist clearly documented to cause fat body depletion [83]. However, to-date, *Arsenophonus* had not been shown to metabolize the fat bodies of bees. Moreover, mean relative abundances of *Arsenophonus* was not

markedly different in provisions from the cool and ambient microclimates, yet we observed striking changes in larval biomass and fat content when comparing means between sterile and microbe-rich provisions for each of these response variables at these temperature treatments. The lower total fat content measured in larvae reared on microbe-rich provisions is concerning, as lipids obtained early in bee development are converted into fat reserves used during diapause. In overwintering adults, warmer temperatures elevate metabolism and lead to increased fat body depletion [7,9,10]; these effects may be intensified if larvae do not acquire enough fat storage prior to pupation. Furthermore, emerged adults with reduced fat stores have shorter lifespans and vigor [7-9], which may result in negative consequences for the reproductive success of female bees by impacting ovarian development [84].

Most research studying the effects of temperature on development in solitary bees have focused primarily on the mature stages given the importance of timing emergence phenology with flowering periods and adult vigor in managed species. However, bees have stage-specific temperature requirements. For instance, *O. cornifrons* has a narrow thermal window for successful development of immature bees: eggs only remained viable between 13-29°C and larvae were sensitive to temperatures outside of 21-29°C [85]. The consequences of a warmer nest environment on larval survivorship depends on the frequency and intensity of thermal stress. For example, exposure to consistently elevated temperatures accelerated larval development [86] but brief exposure to heatwaves slowed growth in *Osmia* spp. [87], suggesting that temperatures above a certain thermal tolerance can inflict physiological damage that affects the uptake of food by immature bees. Our treatments fell within the thermal window of *Osmia* spp. [85], and thus were not likely

the cause of the high larval mortality observed in this study. We found that increases in nest temperatures shortened the duration of *O. lignaria* larval development. Other studies have shown that warming also shortens the length of development from the pupal to adult stages in *Osmia* spp. [88]. Entering the adult stage early and continuing to experience elevated temperatures while overwintering has adverse consequences for bee fitness, including reduced biomass and increased mortality [7-10, 89]. In addition, warming can have non-linear effects on nest emergence – either advancing [8,89] or delaying [11] emergence. Shifts in bee emergence can lead to phenological mismatches with floral resources [90], which may be especially detrimental for early-spring bees, such as *Osmia* spp.

Here, we found that the duration of larval development shortened with warming temperatures. Simulating a warmer climate increased the mean relative abundance of the putative son-killer *Arsenophonus*, yet larval survivorship did not differ between bees reared on microbe-rich versus sterile provisions. By comparing the difference in larval biomass and fat content between bees reared on pollen with and without microbes, we provide evidence that an intact provision microbiota may not always be beneficial; however, the degree by which larval biomass and fat content differed was moderated by temperature. These results suggest that provision exosymbionts may have contributed to the decrease in body size observed during the past several decades of warming.

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TABLES AND FIGURES

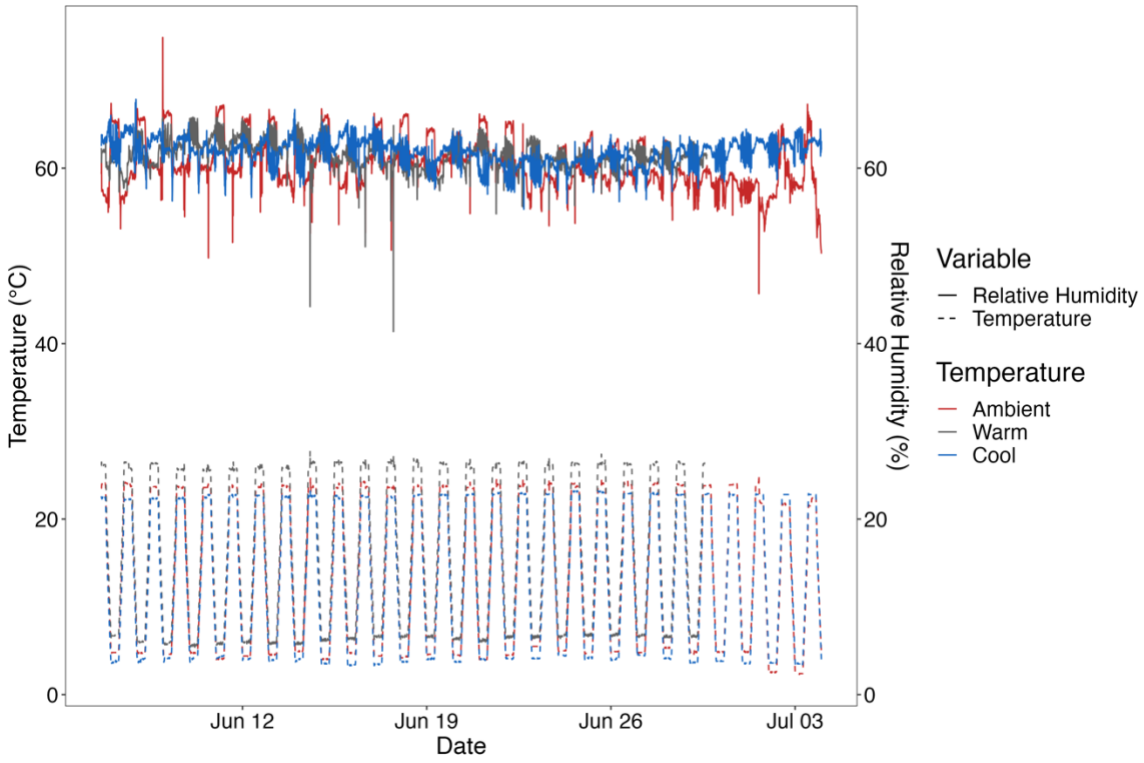

Figure 3.1. Simulated temperature treatments emulating past (*c.* 1900), current, and projected (*c.* 2050-2099) temperatures for the Great Basin region. Mean temperature for the cool, ambient, and warm treatments were 12.9°C, 13.7°C, and 15.9°C, respectively. Relative humidity was approximately 60% for all treatments across the length of the experiment. Temperature and humidity loggers were stopped once all bees within the treatment had reached the fifth instar.

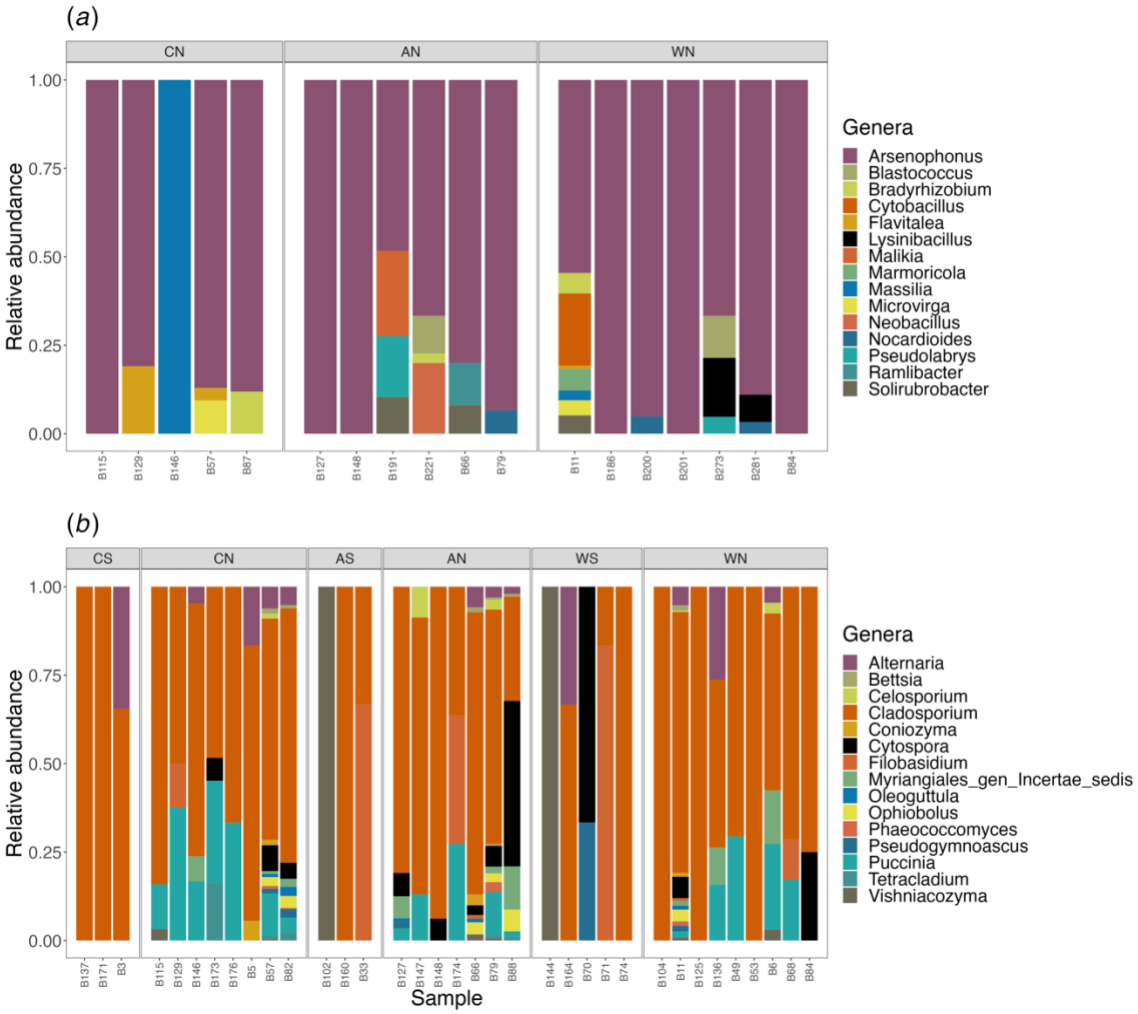

Figure 3.2. Relative abundance (proportion of sequence reads) of the top 15 (*a*) bacterial and (*b*) fungal genera present in *Osmia lignaria* provisions with male and female bees. See Fig. B4 for a relative abundance plot including all sample and genera.

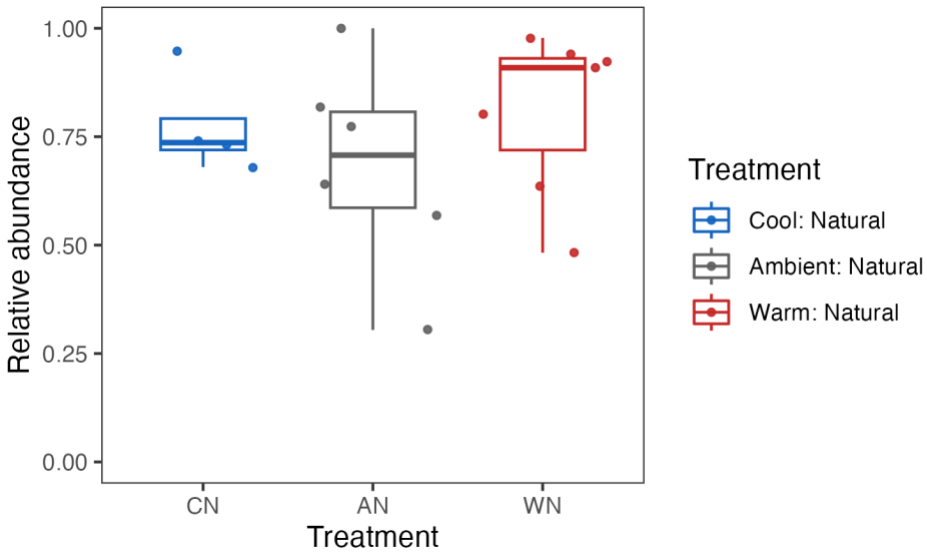

Figure 3.3. Mean rank relative abundance of *Arsenophonus* in provisions with bees. Kruskal-Wallis tests did not indicate any significant differences between treatments due to temperature treatment nor sex.

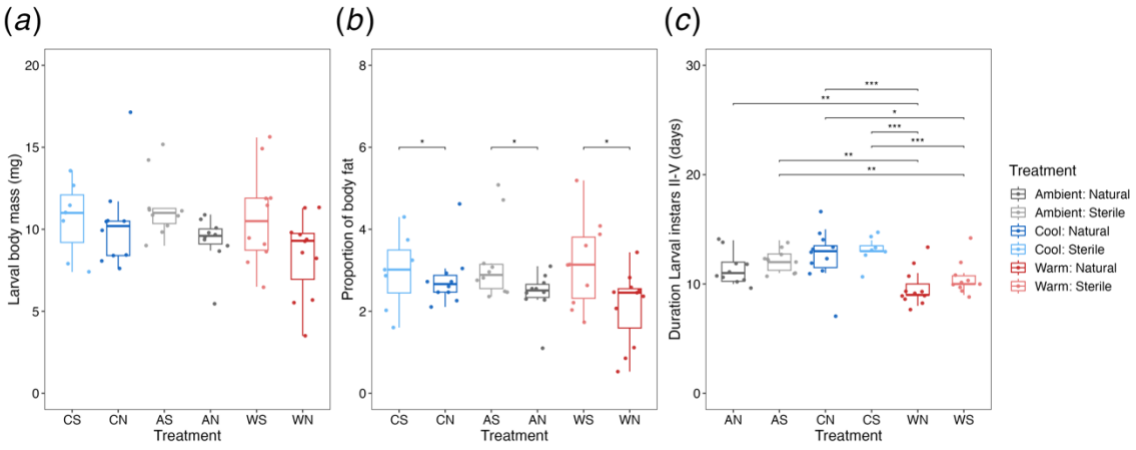

Figure 3.4. Male bee health and life history outcomes, including (*a*) body mass, (*b*) proportion of body fat, and (*c*) development time from the second to fifth larvae in response to temperature and microbiome manipulations. Fifth instar larvae were used to determine body mass and the proportion of body fat. Asterisks represent significant differences, where '*' indicates a p-value < 0.05, '**' $p < 0.01$, and '***' $p < 0.001$.

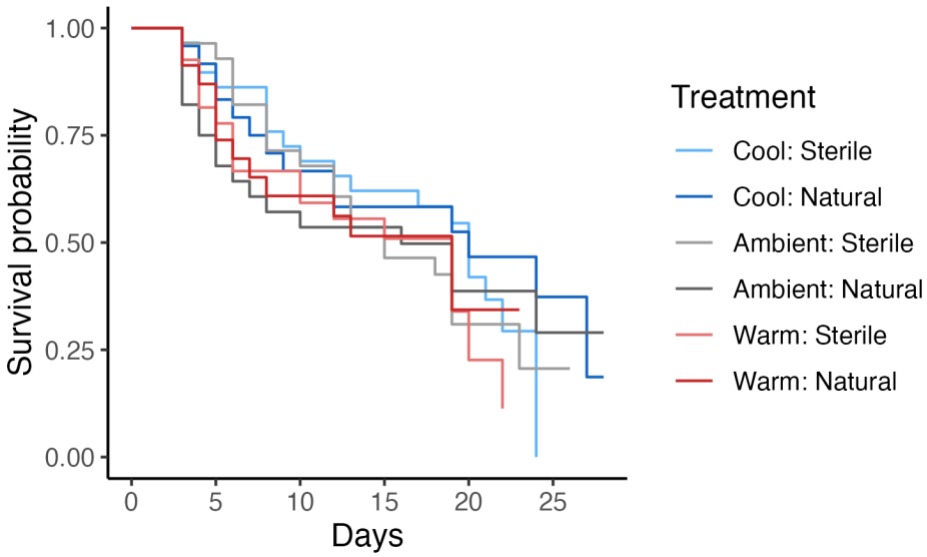

Figure 3.5. Kaplan-Meier survival plot of male *Osmia lignaria* larvae across treatments. Bees that died within 48 h of grafting were excluded. An accelerated failure time model did not reveal significant differences in survivorship across treatments due to microbiome nor temperature treatment.

Conclusion

This thesis explored the assembly and function of microbes associated with the solitary mason bee, *Osmia lignaria*, with both *in vivo* and *in vitro* studies. During the first project, I developed bioinformatic skills to characterize changes in the microbiome of *O. lignaria* throughout its development. This research project was the first to examine the ecological processes driving microbiome structure in a wild solitary cavity-nesting mason bee across its development. In my second project, I gained experience rearing solitary bees, a non-model host system. Here, I aimed to understand how shifts in microbiome diversity and composition of larval food impact bee development and health under temperature change. This study addressed a knowledge gap in our understanding of the interactions between solitary bees and their associated microbes when challenged by temperature, an abiotic stressor. The conclusion of this thesis summarizes the findings of both research projects, considers the ways climate warming may alter bee-microbe interactions, and finally recommends experiments for future studies.

The first study of this thesis (chapter two) described changes to *O. lignaria* microbiome diversity and composition across host development in a cohort of wild male bees. Using amplicon sequencing, we revealed that bacterial and fungal diversity was not significantly different between larval and adult bee samples. As expected with drastic changes in host morphology during pupation, the composition of both bacterial and fungal communities was significantly different between larvae and pre-wintering adults. Many microbial taxa were documented in both provisions and larvae, suggesting that immature bees acquire their microbiome while ingesting pollen. *Arsenophonus*, the bacterial genus found in the highest incidence and counts had high sequence similarity to

a reproductive parasite, suggesting that the ASVs detected in this study also display the son-killing phenotype. Other microbial taxa, such as plant pathogens and common soil inhabitants, were likely acquired from environmental sources. This research highlighted that *O. lignaria* associates with horizontally and vertically transmitted microbes that may have diverse consequences for pollinator fitness.

In the second study (chapter three), I examined changes to the microbial community of *O. lignaria* provisions in response to temperature and the resulting effects on larval health (biomass and fat content) and development. Immature bees were reared *in vitro* at climatic treatments approximating historic, current, and projected temperatures. Unsurprisingly, warmer temperatures led to shorter larval development times. Regardless of treatment, larval survivorship was comparable across microclimates. Considering that only male *O. lignaria* were used in this study, no observed effect on larval survivorship could be explained by the presence of the candidate son-killing pathogen, *Arsenophonus*. In contrast to previous research, larvae reared on provisions depleted of microbes had better health outcomes than those reared on microbe-rich provisions: fifth instar larvae had higher biomass and total fat content when consuming provisions without microbes. However, the effect of microbiome treatment on larval biomass and fat content was mediated by microclimate, such that bees in the warmest thermal environment experienced the greatest reduction in biomass and fat content. This finding indicates that the provision microbiota may not benefit larval health and potentially contributed to the decrease in bee body size observed over the past several decades of warming temperatures.

Besides directly affecting the activity or abundance of bee-associated microbes, climate warming may also affect the timing of the interactions themselves. In particular, changes to the thermal environment are predicted to shift the phenology of plants and pollinators, albeit not congruently, leading to mismatches in species interactions (Gérard *et al.* 2020). Briefly, I extend the framework proposed by Rafferty and coauthors (2014) – that phenological shifts will affect the intimacy, duration, and specificity of mutualisms – to the cross-kingdom interactions of angiosperms, bees, and microbes. I primarily focus on the microbial taxa found within solitary bee provisions, as the establishment and function of non-social bee gut microbes has yet to be confirmed (Hammer *et al.* 2019).

Shifts in bee emergence and flowering periods can lead to mismatches in plant-pollinator mutualisms and the microbial communities therein. Bee-vectored microbes will likely experience new selection pressures due to changes in the chemistry of floral resources, as plants also responds to abiotic stress by changing their metabolism. For example, during droughts, plants alter the ratio of sugars in nectar (Rering *et al.* 2020), leading to an increase in osmolarity and a different resource niche for colonizing microbes. In addition, changes to bee emergence may result in the overlap with novel plant communities that harbor microbial species not previously encountered. The degree to which these interactions change, however, may depend on the extremity of phenological shifts in both bee and plant communities (Pardee *et al.* 2022, CaraDonna *et al.* 2014). As such, the duration of the established tripartite interactions could also be affected. Furthermore, pollinator community networks are also expected to change in future climates as a result of these phenological shifts (Hegland *et al.* 2009), likely affecting the microbial species pool at flower hubs. Thus, temperature-mediated shifts in

phenology are predicted to affect not only the duration of established mutualisms but also lead to the formation of new interspecific interactions.

The outcomes of these cross-kingdom mutualisms may also be dependent on the function and specificity of the associations. For example, changes to interactions with microbial taxa that are environmentally acquired or sparsely occur will not likely have detrimental outcomes for bee fitness. Of consequence to pollinator health are mismatches with symbionts and pathogens. Phenological disruptions to bee symbioses with floral microbes is expected to result in poor pollinator fitness (Russell & McFrederick 2022), however mismatches with pathogens may benefit bees by disrupting infection processes (Nürnberger *et al.* 2022). In contrast, if phenological shifts lead to the increased connectedness of pollinator communities, bees could also be exposed to novel pathogens or flowers with greater pathogen loads (Proesmans *et al.* 2021).

While there is much to learn about the interactions between non-social bees and their microbes in future climate scenarios, fundamental knowledge on the biology and ecology of these cross-kingdom interactions also have yet to be examined. Table 4.1 outlines some of these knowledge gaps and proposes experiments to address these research questions. This table does not consider the evolutionary processes that shape bee-microbe interactions, but these are also open-ended questions that need to be addressed. For example, candidate beneficial symbionts were found to consistently associate with the solitary bees *Ptiloglossa arisonensis* and *Anthophora bomboides* (Hammer *et al.* 2023, Christensen *et al.* 2023). These are likely the first solitary bee symbioses discovered, prompting investigation into the evolutionary history of these lineages: When in evolutionary history were these candidate symbionts acquired? Have

non-social bee symbionts within host genera been acquired via multiple routes, or were these associations formed in a single event and followed by subsequent losses? Learning about the evolutionary history of non-social bees may direct us to other species that also host novel symbionts, advancing our understanding of bee biology and aiding in the conservation of pollinators.

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TABLES AND FIGURES

Table 4.1. Outstanding questions in plant-microbe-bee interactions.

Question	**Suggested experiment(s)**
Floral microbiome	
Does pollen chemistry structure the associated microbiome?	Inoculate synthetic microbial communities on pollen from different plant species. Quantify shifts in microbiome composition and abundance.
Transmission	
Are there bee behaviors (e.g., egg smearing, reinoculation during emergence) that ensure the transmission of symbionts to the next generation?	Sample the surface of bee eggs. Compare microbial ASVs before and after bee emergence. Sample nesting materials (e.g., Dufour's glad secretions).
Can non-social bees detect cues (e.g., microbial volatile organic compounds, fermentation products) from floral microbes?	Perform olfactometer and electroantennogram assays with microbes found in nectar and pollen.
Symbiont incidence and function	
Which microbes survive the lifespan of the provision? How do these microbes affect provision nutritional quality?	Inoculate sterile pollen microcosms with only one floral microbial species. Quantify microbial abundance over time. Describe changes to the quantity and profile of essential amino acid, fatty acid, and sterols.
Do pollen-associated microbes prevent the spoilage of non-social bee provisions?	Measure the pH of aged bee-collected pollen. Determine whether fermentation products are present. Inoculate aged bee-collected pollen with spoilage-causing microbes and determine their ability to colonize the provision.
Do provision-associated microbes prevent the growth of bee pathogens?	Compare microbiomes of healthy and diseased bees. Competition assays between microbes and bee pathogens in provision microcosms or agar plates supplemented with pollen.
What is the prevalence of endosymbionts in non-social bee species?	Sample fat bodies surrounding the gut and the reproductive organs of females.
How does the ratio of pollen to nectar in provisions affect microbial growth?	Compare microbial growth in pollen with an increasing amount of a nectar analog.
Can microbes establish in the hindgut of non-social bees?	Fluorescent in-situ hybridization of microbes detected in the gut of foraging non-social bees. Genomic analysis of

	candidate microbes to determine presence of host-adherence factors.
Bee physiology	
What digestive capability do larvae and foraging adults have to degrade pollen without the assistance of microbes?	Feed bees sterile provisions. Compare the number of ruptured pollen grains in the provision, bee gut, and feces.

APPENDICES

Appendix A

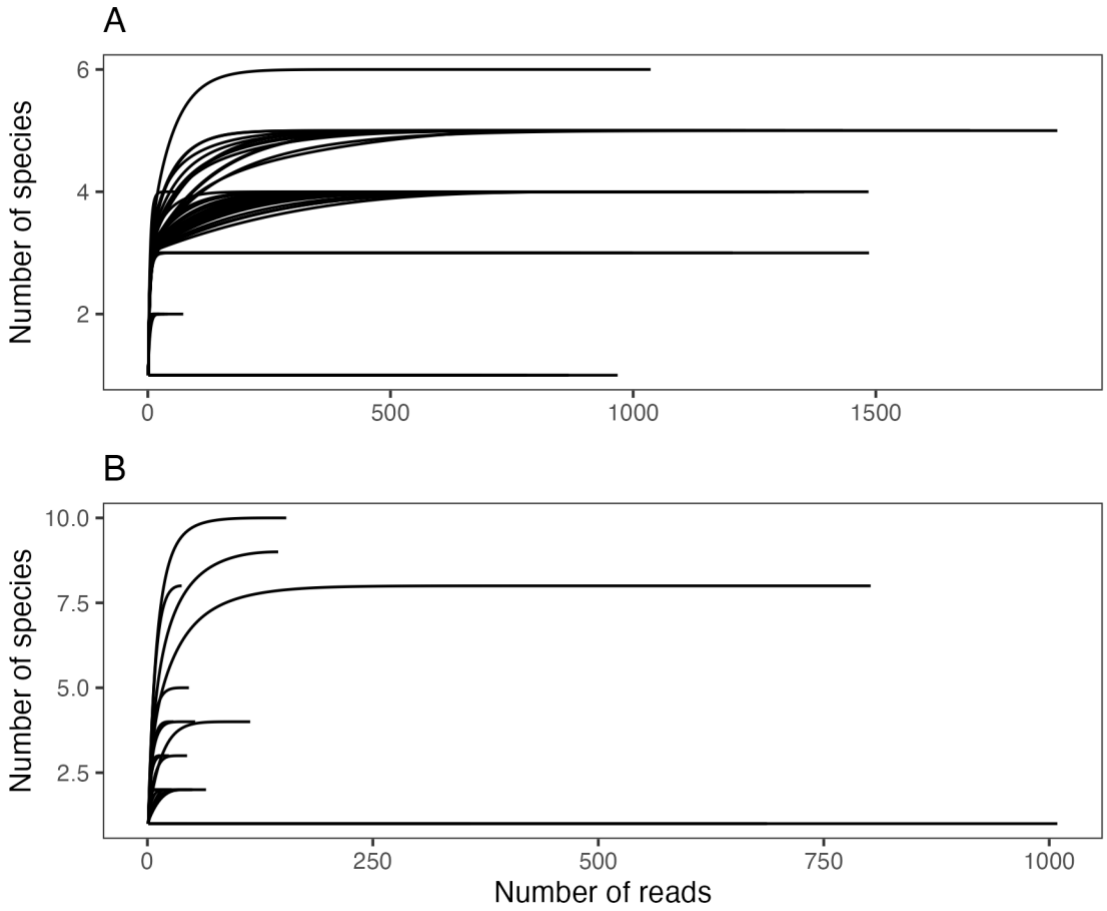

Fig. A1 Rarefaction curves showing observed bacterial (A) and fungal (B) species richness in bee samples, including larvae, pre-wintering adults, emerged adults, and dead adults.

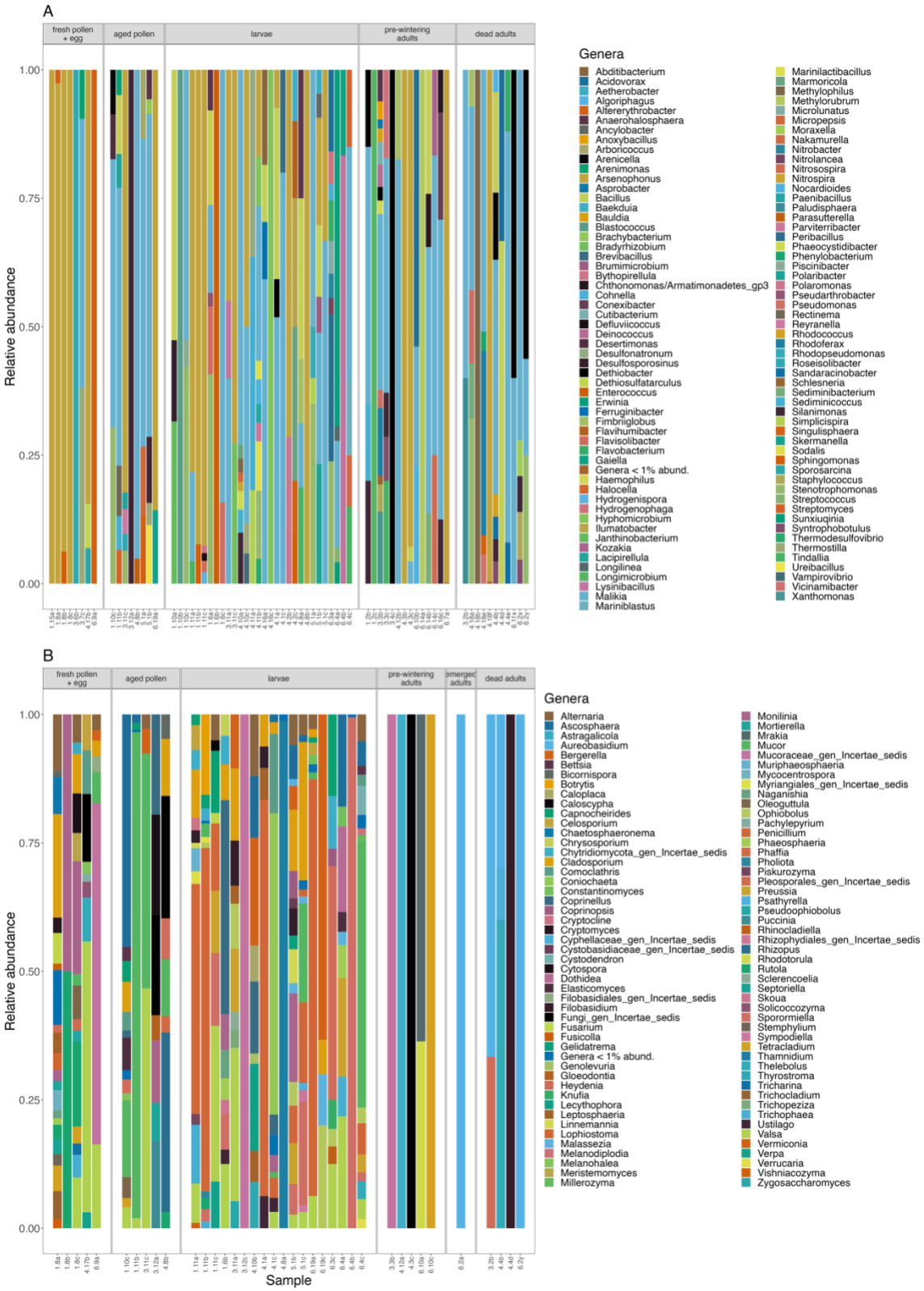

Fig. A2 Relative abundances (proportion of sequence reads) of all (A) bacterial and (B) fungal genera found in *Osmia lignaria* provisions, larvae, pre-wintering adults, emerged adults, and dead adults using a relative abundance dataset.

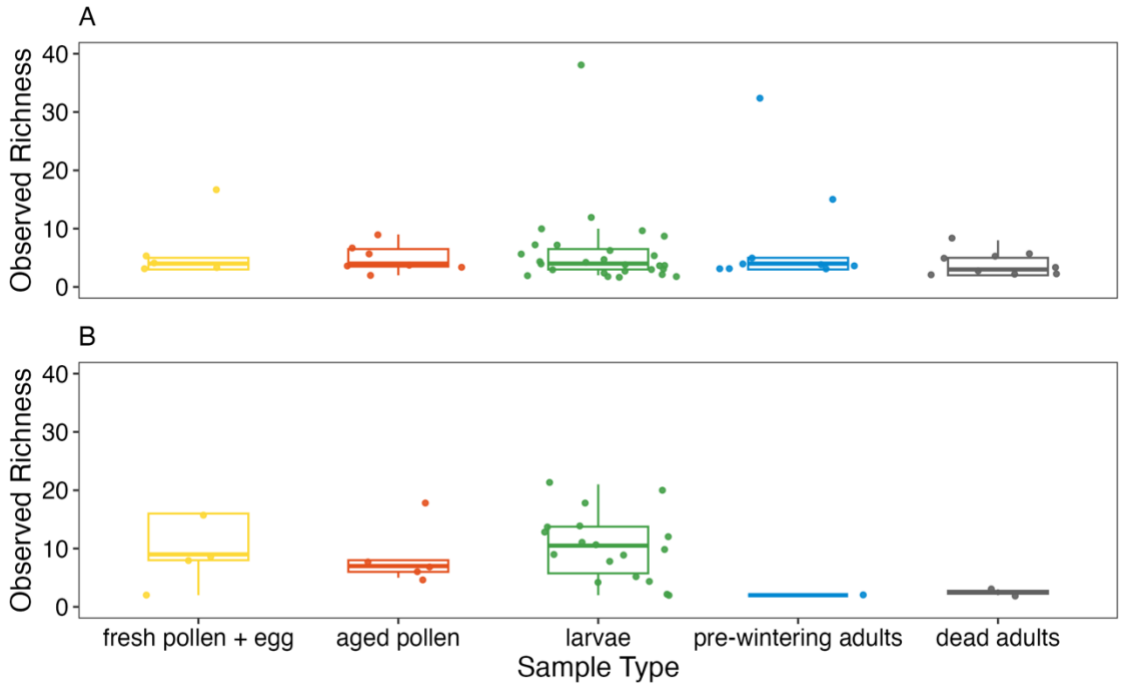

Fig. A3 Observed richness of provisions, larvae, pre-wintering adults, and dead adults using (A) bacterial and (B) fungal amplicon sequence variants (ASVs). Significant differences between sample types were not found for either kingdom.

Table A1 BLASTn results for *Arsenophonus* amplicon sequence variants (ASVs) found across the life cycle of *Osmia lignaria*. All 52 ASVs had high sequence similarity to *A. nasoniae* (accession: NR_042811.1), with each having a query cover of 100%. These ASVs accounted for a total of 49,437 bacterial reads in the dataset (73.12% total reads).

ASV	Total Reads per ASV	Percent Identity	ASV	Total Reads per ASV	Percent Identity
ASV1	1874	99.03%	ASV27	905	98.79%
ASV2	1694	98.79%	ASV28	903	98.54%
ASV3	1523	96.12%	ASV29	900	98.79%
ASV4	1486	98.79%	ASV30	887	98.54%
ASV5	1432	99.27%	ASV31	874	99.27%
ASV6	1352	99.51%	ASV32	871	98.79%
ASV7	1333	99.03%	ASV33	842	99.03%
ASV8	1317	99.27%	ASV34	821	98.79%
ASV9	1297	99.03%	ASV35	804	98.30%
ASV10	1293	99.03%	ASV36	741	98.79%
ASV11	1261	98.79%	ASV37	735	98.54%
ASV12	1231	99.27%	ASV38	723	98.79%
ASV13	1229	99.03%	ASV39	704	98.54%
ASV14	1205	98.54%	ASV40	680	98.54%
ASV15	1203	99.03%	ASV41	677	98.79%
ASV16	1162	99.03%	ASV42	675	99.03%
ASV17	1144	99.27%	ASV43	670	98.54%
ASV18	1134	98.79%	ASV44	663	98.79%
ASV19	1105	99.03%	ASV45	635	98.79%
ASV20	1074	98.79%	ASV46	632	98.54%
ASV21	1038	98.79%	ASV47	615	98.79%
ASV22	1036	99.03%	ASV48	584	98.54%
ASV23	1011	98.54%	ASV49	541	98.54%
ASV24	991	99.03%	ASV50	2	94.66%
ASV25	969	98.79%	ASV51	2	93.93%
ASV26	955	99.03%	ASV52	2	94.90%

Appendix B

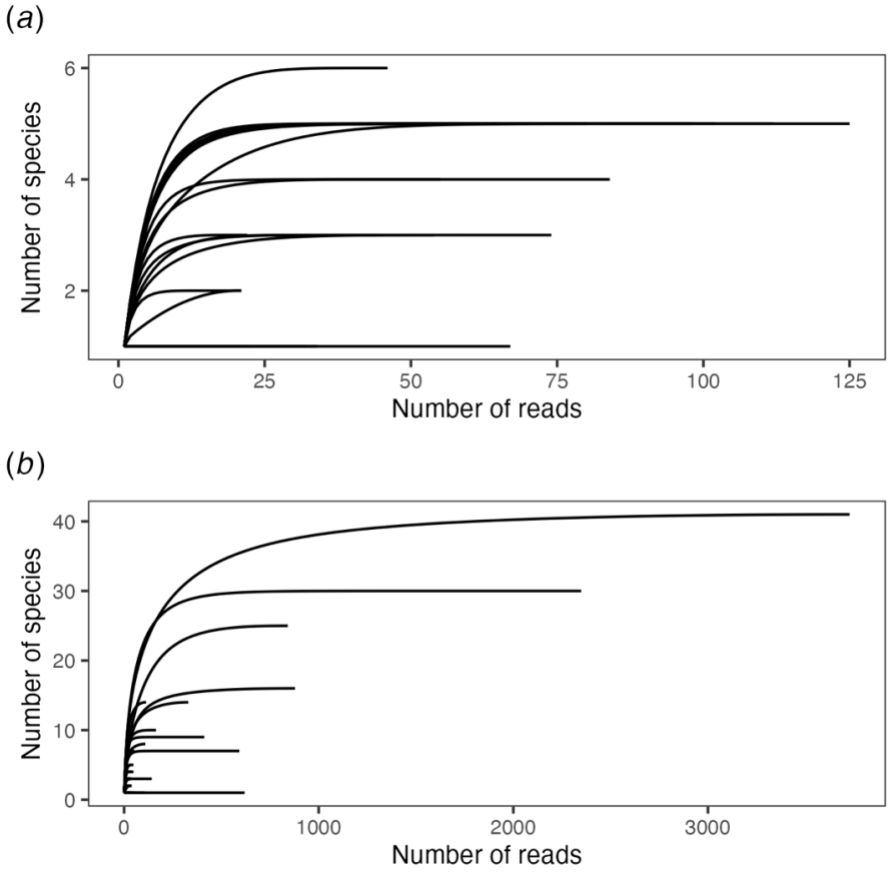

Figure B1. Rarefaction curves showing observed (*a*) bacterial and (*b*) fungal species richness in provisions with bees.

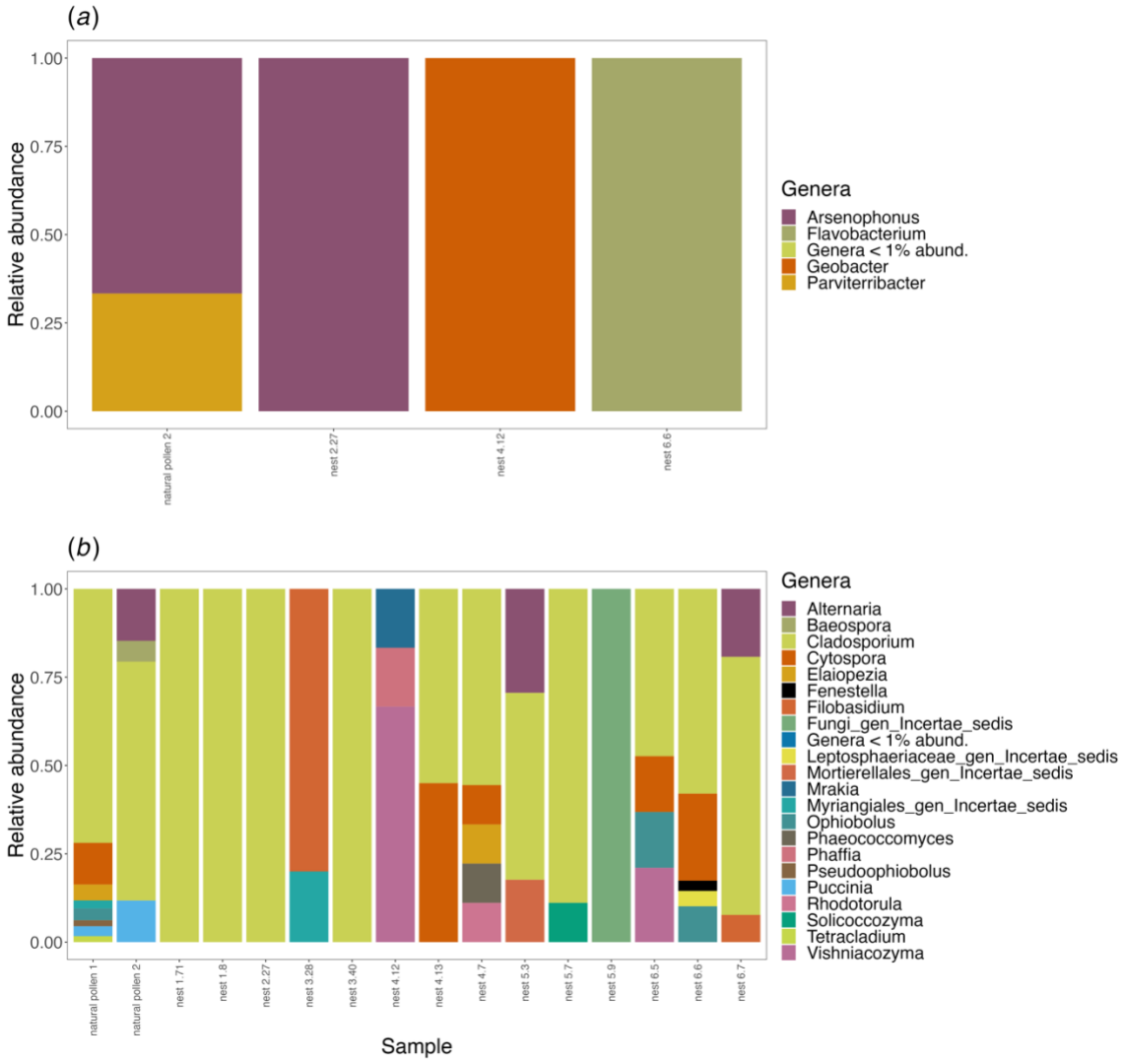

Figure B2. Relative abundances (proportion of sequence reads) of (*a*) bacterial and (*b*) fungal genera present in provisions before and after homogenization.

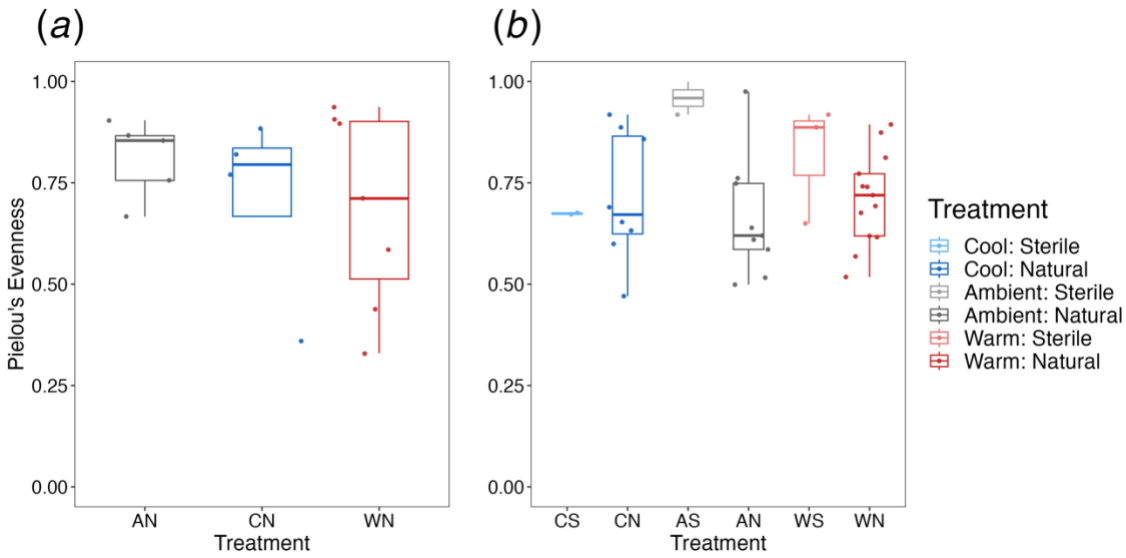

Figure B3. Pielou's evenness of provisions with male bees using (*a*) bacterial and (*b*) fungal amplicon sequencing variants (ASVs). Both temperature and microbiome treatments were not significant predictors in neither the bacterial nor fungal model.

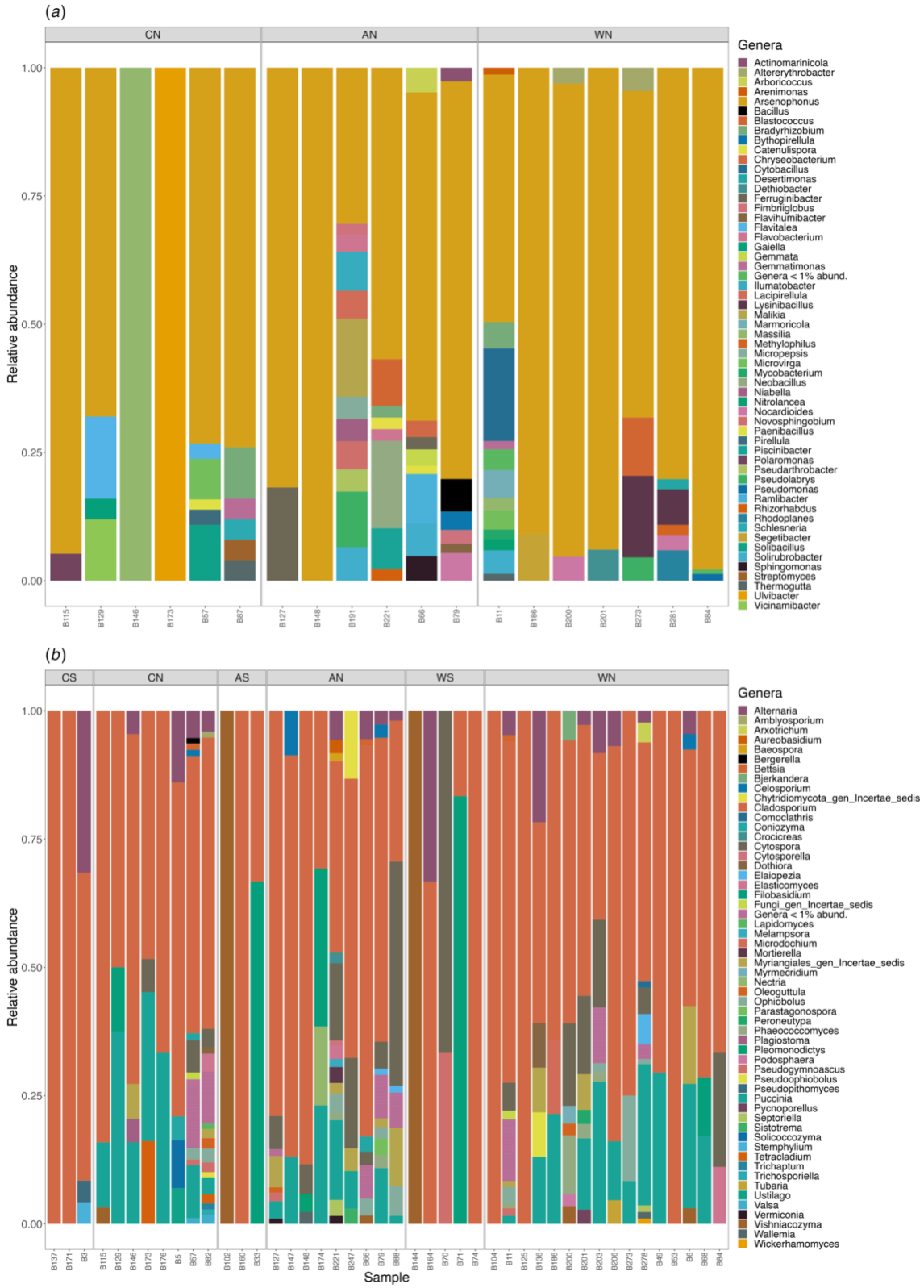

Figure B4. Relative abundance (proportion of sequence reads) of (*a*) bacterial and (*b*) fungal genera present in provisions with bees.

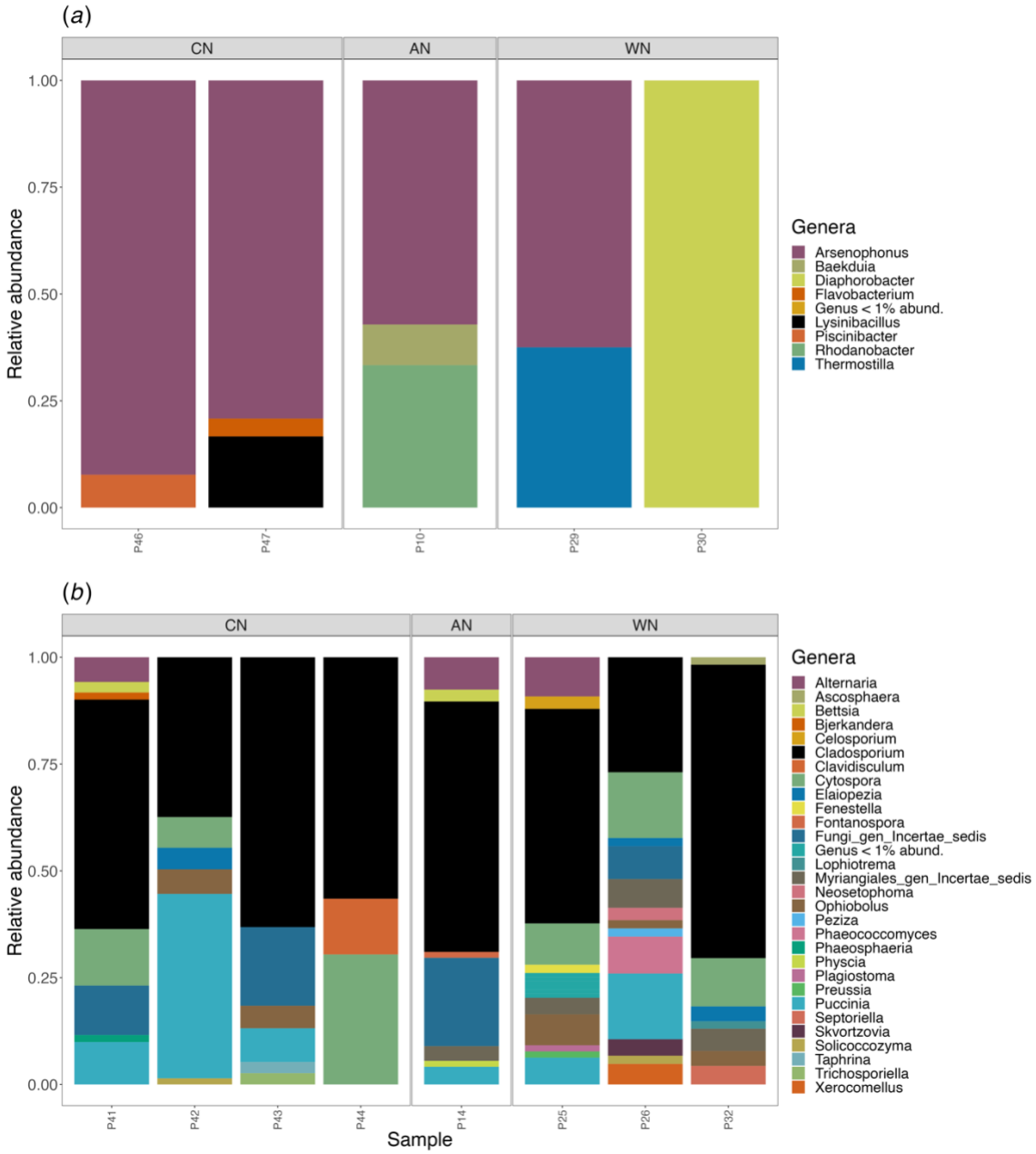

Figure B5. Relative abundance (proportion of sequence reads) of (*a*) bacterial and (*b*) fungal genera present in provisions without bees.

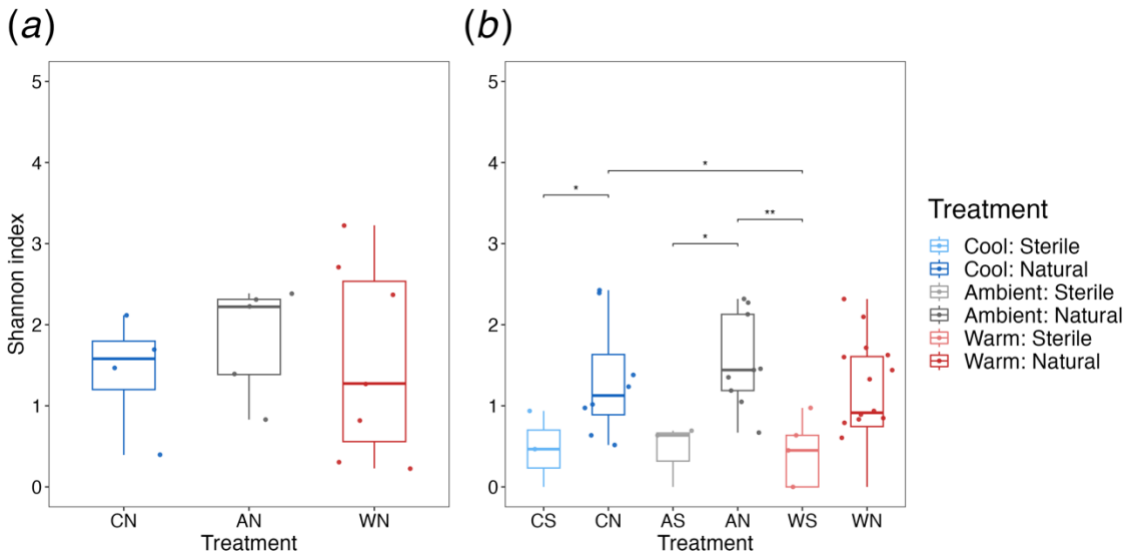

Figure B6. Shannon diversity indices of provisions with male and female bees using (*a*) bacterial and (*b*) fungal amplicon sequencing variants (ASVs). Temperature treatment was not a significant predictor in the bacterial model. However, microbiome treatment was a significant covariate ($p = 0.0001$) in the fungal model. Asterisks represent significant differences, where '*' indicates a p-value < 0.05, '**' $p < 0.01$, and '***' $p < 0.001$.

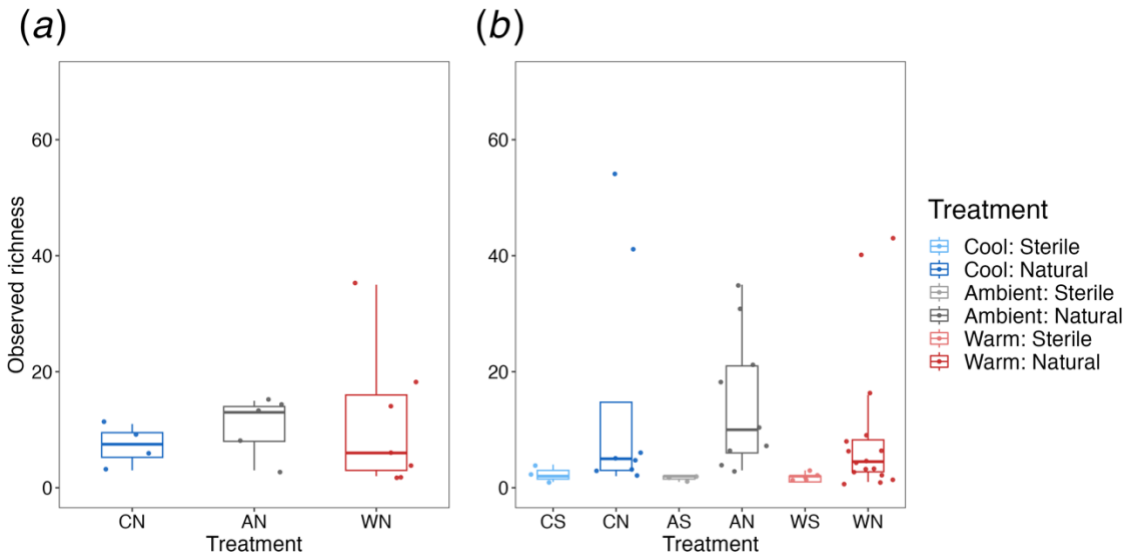

Figure B7. Observed richness of provisions with male and female bees using (*a*) bacterial and (*b*) fungal amplicon sequencing variants (ASVs). Temperature treatment was not a significant predictor ($p = 0.66$), yet bee sex had a significant effect on the observed richness of bacteria ($p < 0.05$). However, post-hoc tests did not reveal any significant pairwise comparisons. In the fungal model, both bee sex ($p = 0.41$) and microclimate ($p = 0.42$) did not affect observed richness. Rather, microbiome treatment had an effect on the observed richness of fungi ($p < 0.05$).

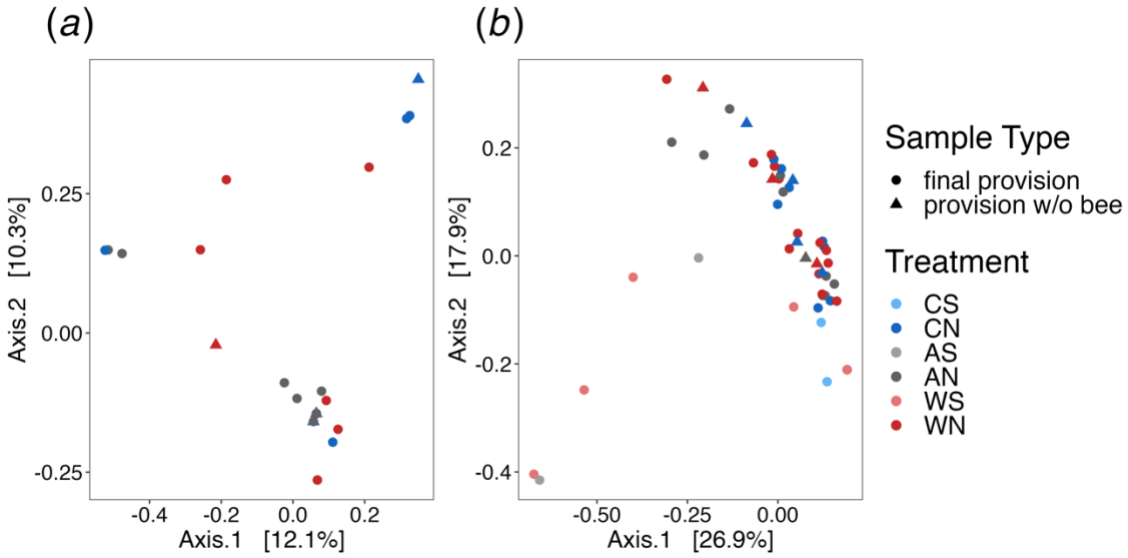

Figure B8. Principle coordinates analysis (PCoA) of Bray Curtis dissimilarity in provisions with and without bees of (*a*) bacterial and (*b*) fungal amplicon sequencing variants (ASVs). Bacterial composition was not significantly different. Microbiome treatment (p = 0.001) was a significant predictors of fungal composition.

Table B1. BLASTn results for *Arsenophonus* ASVs found with *Osmia lignaria* provisions. All 16 ASVs had high sequence similarity to the same accession (NR_042811.1), with each having a query cover of 100%. These ASVs accounted for a total of 835 bacterial reads (47.02% of total bacterial reads in all provision samples).

ASV	Total Reads per ASV	Percent Identity
ASV1	120	98.79%
ASV2	95	98.79%
ASV3	87	99.03%
ASV4	74	98.79%
ASV5	68	98.79%
ASV6	66	99.02%
ASV7	59	99.51%
ASV8	57	98.54%
ASV9	36	99.03%
ASV10	34	99.27%
ASV11	32	99.27%
ASV12	31	99.03%
ASV13	28	99.03%
ASV14	27	98.79%
ASV15	12	98.30%
ASV16	9	98.79%