Native solitary bee health in western Washington

Lila Rose Westreich

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Reading Committee:

Patrick C. Tobin

Julie K. Combs

Jonathan D. Bakker

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University of Washington

Abstract

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Lila Rose Westreich

Chair of the Supervisory Committee:

Patrick C. Tobin

School of Environment and Forest Sciences

Bees are a vital part of the terrestrial ecosystem, providing pollination services to plants and contributing to biodiversity. Spring-emerging solitary native bees face numerous challenges to survival, including inclement weather in early spring, harmful bacteria and parasites, and the need to accumulate nutritious pollen from potentially highly variable and fragmented landscapes to feed developing offspring. I examined the relationship between climate variables, plant richness, nutritional content of pollen, and presence of bacteria and fungi and the developmental success of the solitary cavity-nesting bee, *Osmia lignaria* Say, across 22 sites in western Washington located along an urban-to-rural gradient. Studies were conducted over two to four years, and during two distinct bee foraging periods: one that was synchronized with the phenologically-typical *O. lignaria* foraging period, and one to simulate asynchrony between *O. lignaria* and its phenologically-typical host plants. I used novel Next-Generation Sequencing (NGS) technology to perform DNA metabarcoding of plant pollen, bacteria, and fungi to quantify species richness, and used metabolomic techniques to quantify protein and lipid concentrations in pollen. To collect weather data, I used community science data in the form of temperature, wind speed, and rainfall from Weather Underground stations across King County, Washington. The results showed that *O. lignaria* larval development success and adult emergence increases with increasing hours with zero precipitation. NGS revealed that bees visited between 2-32 plants, consistently visiting certain genera including *Acer, Salix,* and *Rubus* species, and that plant species richness did not predict bee development success in either foraging period. The nutritional quality of pollen did not change with increasing plant richness, and in both foraging periods high lipid concentrations were associated with higher rates of development and emergence. Certain specific lipid classes were found to increase larval development success, including cholesterol ester and hexosylceramide. I also report on novel associations between fungi and bacteria and solitary bees. Bacterial richness increased during the second foraging period when the weather is conducive to bacterial growth and more plants are in bloom. Fungal analysis revealed that richness decreased with increasing plant richness, urging further microbial analysis in *O. lignaria* foraging behavior and development. Potentially harmful fungi for bee health were detected in sequencing analysis, including *Ascosphaera* spp. This study informs native solitary bee conservation efforts, especially in heavily urbanized areas, provides a basis for exploring in-depth relationships between lipid and protein content of pollen and bee development, and presents novel information on bacterial and fungal associations with solitary bee foraging activity and their potential role in bee health.

TABLE OF CONTENTS

List of Figures 6

List of Tables 7

Chapter 1. Weather and host plant selection affects native solitary bee developmental success 10

Introduction 11

Materials and Methods 15

Results 24

Discussion 26

Figures 33

Tables 39

References 45

Chapter 2. Bees needs: effect of protein and lipids in pollen on the developmental success of the native solitary bee *Osmia lignaria* Say 54

Introduction 55

Materials and Methods 56

Results 62

Discussion 64

Figures 72

Tables 75

References 79

Chapter 3. Symbiont associations with native solitary bees: an exploratory analysis of fungi and bacteria in bee-collected pollen provisions 86

Introduction 87

Materials and Methods 90

Results 96

Discussion 98

Figures 103

Tables 106

References 113

LIST OF FIGURES

Chapter 1. Weather and host plant selection affects native solitary bee development

Figure 1. Process of pollen provision sub-sampling prior to DNA sequencing 33

Figure 2. Effect of hours with zero rainfall on oviposition 34

Figure 3. Effect of plant richness on *O. lignaria* developmental success 35

Figure 4. Treemaps of plant genera identified in 2018, 2019, and 2020....................................36

Figure 5. NMDS plots of foraging period data for 2018, 2019, and 2020.................................37

Figure 6. NMDS plots of site classifications for 2018, 2019, and 2020....................................38

Chapter 2. Bees needs: effect of protein and lipids in pollen on the development of the native solitary bee *Osmia lignaria* Say

Figure 1. Lipid concentration on larval development and adult emergence success 72

Figure 2. Concentration of protein and lipids in pollen during the first and second foraging period 73

Figure 3. Heatmaps of lipid classes across year, foraging period, urban/rural site classification, and landscape use classifications...74

Chapter 3. Symbiont associations with native solitary bees: an exploratory analysis of fungi and bacteria in bee-collected pollen provisions

Figure 1. Larval developmental success by fungal richness and bacterial richness 103

Figure 2. Bacterial fungal richness by plant richness and foraging period...............................104

Figure 3. NMDS plots of fungal and bacterial richness by site classifications........................105

LIST OF TABLES

Chapter 1. Weather and host plant selection affects native solitary bee development

Table 1. Descriptions and locations of study sites from 2017-2020 39

Table 2. Three-week time periods used for weather data analysis for 2018-2020 42

Table 3. Linear mixed model results of all variables on *O. lignaria* development....................43

Table 4. Plant genera found in each foraging period from 2018-2020 44

Chapter 2. Bees needs: effect of protein and lipids in pollen on the development of the native solitary bee Osmia lignaria Say

Table 1. Lipid class and code identifiers 75

Table 2. Linear mixed model results of all variables on *O. lignaria* development....................76

Table 3. Linear mixed model results of plant richness on lipid and protein conc....................77

Table 4. Linear mixed model results of lipid class analysis on *O. lignaria* development 78

Chapter 3. Symbiont associations with native solitary bees: an exploratory analysis of fungi and bacteria in bee-collected pollen provisions

Table 1. Bacterial classes found in each foraging period from 2019-2020.................... 106

Table 2. Bacterial genera associated with bee health.................... 107

Table 3. Fungal genera found in each foraging period in 2019-2020 108

Table 4. Fungal genera found in 2019-2020 by site type and known associations 109

Table 5. Linear mixed model results of all variables on *O. lignaria* development.................... 111

Table 6. Linear mixed model results of plant richness on symbiont richness 112

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DEDICATION

For mom, dad, Olivia, and the bees.

Chapter 1: Weather and host plant selection affects native solitary bee developmental success

Abstract

Native spring-emerging bees depend upon the synchronized bloom times of angiosperms that provide a source of pollen and nectar for developing offspring. Insects and plants blooming in spring are linked to weather but can be phenologically mismatched. Annual weather variation in spring in the Pacific Northwest region can include frequent precipitation, high winds and storms, and fluctuating temperatures. I examined the relationship between weather and host plant selection on the native spring-foraging solitary bee, *Osmia lignaria* Say, across three years at 22 sites in and around Seattle, Washington. I used community science weather data to relate precipitation, wind, and temperature to *O. lignaria* oviposition and developmental success. I also collected pollen data over two distinct foraging periods, and used Next-Generation Sequencing to identify plant genera from pollen. I found that increases in the hours with no precipitation predicted larval developmental success and adult bee emergence success. I also observed, when using DNA metabarcoding, that foraging bees consistently visited certain genera during each foraging period, especially *Acer*, *Salix* , and *Rubus* spp., but that pollen collected by *O. lignaria* over different years varied in the number of total genera visited. Based on these findings, *O. lignaria* is sensitive to suboptimal spring weather during its 4-6 week foraging period, and visit a number of plants during this time. This study informs native solitary bee conservation efforts, particularly in urban landscapes.

Introduction

Insect pollinators are a vital part of the world's ecologic and economic ecosystem. The majority of angiosperms are reliant on pollinators, especially insects, for reproduction (Mitchell et al., 2009). The total economic value of pollination services worldwide was estimated at $172 billion in 2005, underscoring the importance of crop pollination to the stability of agricultural production (Settele et al., 2009). In addition to honey bees such as the European honey bee *Apis mellifera* L., wild bees also contribute significantly to crop pollination. For example, in a study of the leading crops planted around the world, 42% were pollinated by at least one wild bee species (Klein et al., 2007). Recent studies have also shown that wild bees improve fruit set in berry crops, yielding more uniform and larger berries than honey bee pollination alone (MacInnis & Forrest, 2019; Nicholson & Ricketts, 2019). Bees are undoubtedly an integral part of our landscape, important to ecological services worldwide, and remain at risk of decline and extinction (Potts et al., 2016).

Loss of insect pollinators is a growing concern for ecological conservation of insects and flowering plants. Alongside increased parasites, diseases, urbanization and development, and climate change, loss of habitat and flowering resources are a factor in the worldwide decline of pollinators (Fürst et al., 2014; Giannini et al., 2012; Koh et al., 2016; Lopezaraiza-Mikel et al., 2007). In an effort to combat these declines, restoration and conservation groups have focused on reducing chemical usage and promoting plantings of flowering resources for pollinators. An improved understanding of bee floral preferences and their specific nutritional needs is crucial to the success of these endeavors (e.g., Cook et al., 2003; Vaudo et al., 2015).

Access to an abundance of flowering plants is not the sole factor in bee success, but higher flowering species richness and diversity is thought to support pollinator success. In a meta-analysis of 109 observational and experimental studies looking at the relationship between plant species richness and pollinator species richness, insect pollinators increased with increased plant richness (Kral-O'Brien et al., 2021). However, the relationship between plant and bee richness can be more complex. In a meta-analysis of manipulated studies, Kral-O'Brien et al. (2021) observed a weak positive correlation between overall plant richness and pollinator richness, and they attributed this weak relationship to the fact that not all plants in the studies, such as grasses, provide nutritional benefits to pollinators. Increasing diversity of floral species has also been linked to higher bee species diversity, but the details and mechanism of this relationship do not transfer across variable landscapes (Sutter et al., 2017). A diverse planting of floral resources may offset the negative effects of other variables that are harmful to bee fitness, such as climate change and subsequent phenological asynchrony (Bartomeus et al., 2013). In other cases, a single non-native plant species with beneficial pollen for insects could lead to an increase in pollinator abundance (Fenesi et al., 2015). Increased plant richness may also increase available nutritional resources, with different species providing pollen with different levels of protein, amino acids, and lipids beneficial to bee development (Moerman et al., 2017; Vanderplanck et al., 2014). Without the knowledge of the specific nutritional requirements for developing bees, and the potential for bees to select from available pollen with variable nutritional quality, it remains difficult to define pollinator success in diverse landscapes (Vaudo et al., 2020).

In addition to the role that flowering plants play in influencing pollinator activity, in some bee species, variation in weather can be the difference between a successful or unsuccessful nesting year. Asynchrony between the emergence of spring pollinators, especially for species with short-lived adult stages, and the flowering cycle of the flowers they visit is a concern for conservation efforts. Moreover, climate change and the effect of increasing temperatures can cause a disruption in plant-pollinator interactions (Robbirt et al., 2014), which is likely to have a detrimental effect on the fitness of native pollinators (Memmott et al., 2007). Spring-emerging solitary bees are especially susceptible to early spring extreme weather events, such as rainfall or wind during foraging hours, and suboptimal temperatures that restrict foraging time and reduce immature developmental rate. For example, the use of delayed emergence and warming treatments in solitary bees has shown that an increase of ~1-2°C could lead to a 30-75% mortality rate in wild bee offspring, presumably due to disruption of physiological processes, and could lead to asynchrony with floral resources (CaraDonna et al., 2018). Increases in spring temperatures, a hallmark of climate change, can advance flowering times and insect emergence, but not necessarily at the same rate, which could lead to insect-plant asynchrony (Maglianesi et al., 2020; Radmacher & Strohm, 2011). Increased diversity of plant species is thought to help offset the harmful effects of floral asynchrony by providing alternative options for pollinators (Bartomeus et al., 2013).

Osmia lignaria Say (Hymenoptera: Megachilidae), the blue orchard mason bee, is a commercially available solitary bee that is used in orchard and fruit pollination. *Osmia lignaria* constructs nests in reeds as well as naturally occurring holes, in which they create individual brood cells. Adults emerge in early spring, and begin to locate suitable mates (Bosch & Kemp, 2000). Female bees, following mating, will then locate a suitable nest and begin amassing pollen provisions for their offspring. A study in cultivated apple estimated that a single female made >20,000 apple flower visits during a 15-day flowering period (Vicens & Bosch, 2000). When a female has collected a sufficient amount of pollen, she will begin to oviposit in an individual nest cell, using mud to separate the completed cell from the previous one. Past researchers have suggested that *O. lignaria* is an ideal system for understanding native solitary bees (Eckhardt et al., 2014; Haider et al., 2014; Williams & Tepedino, 2003). Blue orchard mason bees are also important as pollinators of native plants (Kraemer & Favi, 2009), and serve as a model species for quantifying environmental change impacts to solitary bee species due to their early spring emergence (Bosch and Kemp 2000). They have a relatively short foraging range (<600 m; Rust, 1990) and short 4-6 week life cycle, which makes them tractable in experimental studies involving the placement of bees.

Different methods have been used to identify pollen preferences of solitary bees, including capture and analysis of pollen grains using electron microscopy and the use of DNA metabarcoding (Bell et al., 2016; Klungness & Peng, 1984; Kraemer et al., 2014). Microscopy has varying levels of success; many pollen grains from the same plant families have similar visual exine structures, making it difficult to differentiate among genera or species within a family. Electron microscopy requires a highly intricate machine and a high financial cost. Due to limited availability of electron microscopy, its costs, and expertise needed to identify pollen to species, new methods have been developed to investigate bee foraging behavior. One method is the use of Next-Generation Sequencing (NGS) to identify plant DNA sequences from pollen samples. This method was initially used to identify pollen collected from honey bees in a study within a botanical garden in the United Kingdom (De Vere et al., 2017). Comparisons have been performed to test the ability of visual electron microscopic identification of plant species from pollen and the NGS in correctly identifying to genus, demonstrating the ability of NGS to identify taxon at a deeper richness than visual identification (Keller et al., 2015). In this paper, we studied the effect of landscape composition as a proxy for pollinator habitat, weather, and plant pollen sources, collected over 3 years following a one-year pilot study, on the developmental success of *O. lignaria* using novel technology for DNA metabarcoding of bee-collected pollen for plant analysis.

Materials and Methods

Field sites and Experimental Set-up

Field sites were established across a variety of urban and rural landscapes in King County, Washington. In 2017, a total of 14 sites were selected for preliminary placement of solitary bees to determine which site characteristics were conducive to nesting success based on whether the bees nested in the placed boxes or failed to nest. Of the 14 sites, nesting success was recorded from 12 sites. In 2018, these 12 sites were selected, and an additional 10 sites were chosen for 2019-2020. Sites were chosen based on 1) their proximity to flowering species, and 2) site characteristics conducive to solitary bee success. A brief description of sites is presented in Table 1. At each site, two bee houses constructed from 7.5 cm diameter PVC pipe, each containing 40 8mm diameter paper nesting tubes, were placed facing southeast. At each site and year, 100 locally purchased (Crown Bees, Woodinville, WA and Watts Solitary Bees, Bothell, WA) *O. lignaria* overwintering pupae were placed in emergence boxes in field sites once average temperatures had reached 10°C during peak foraging hours, which typically occurred in late March to early April in western Washington. Bee boxes and pupae were placed in the field slightly prior to minimum daytime temperature at >10°C to avoid missing the foraging season. Once adults had emerged, females were left to forage and nest in the boxes over 6 weeks, which was considered to be the first foraging period. The 6 weeks encompassed the life span of the adult bees (Bosch et al., 2000). The first foraging period was designed to be implemented during the phenologically-typical foraging period for *O. lignaria* based on prior work (Bosch & Kemp, 2000). Following the first foraging period, completed nesting tubes and remaining *O. lignaria* pupae were removed. New nesting tubes were then placed in bee houses, and 100 additional *O. lignaria* pupae were deployed and allowed to forage for another 6 weeks. The second foraging period was designed to simulate potential phenological asynchrony between *O. lignaria* and its phenologically-typical host plants. Following completion of the second foraging period, completed nesting tubes were removed. Nesting tube data from the two boxes at the same site were combined. In 2018, completed nesting tubes from each site were used to count the number of eggs, and allocated to molecular analysis to ascertain plant DNA identification. In 2019 and 2020, completed nesting tubes from each site were randomly assigned in approximate equal numbers to one of two groups: one group for molecular analysis to ascertain plant DNA identification, and one group to quantify developmental and emergence success. Nesting tubes used in molecular analyses were stored at -20°C until processed. Nesting tubes used to quantify developmental and emergence success were placed in Percival Scientific (Perry, Iowa) biological incubators under conditions that simulated average temperatures in King County, WA based on the recent 10 years (Weather Underground, 2021), and the photoperiod for the county. For each week, photoperiod was adjusted, and temperatures in the incubators were set to fluctuate between the average high and average low for the relevant week. The biological incubators were adjusted from mid-May to March-to-April of the following spring.

Larval Development and Emergence Success

In 2018, nesting tubes selected for identifying development and emergence success were removed from biological incubators in late fall (late October to mid-November) once *O. lignaria* had reached the pupal stage. Nesting tubes were dissected, and I counted segments inside the nesting tubes as a proxy for oviposition, as well as whole pollen provisions or 1st instars that had failed to develop to the late larval or pupal stage. I also counted the number of offspring that completed development from egg to 5th-6th instar or pupae as successful larval development. Pupae were washed in lukewarm water to remove frass and other debris, dried, and placed in containers, identified by site and foraging period, and placed in the biological incubators until spring in the following year under fluctuating weekly temperatures as described above. Once weekly temperatures had reached the minimum temperatures required for adult emergence (~10°C), adults were allowed to emerge for 6 weeks (Bosch et al. 2000). The total number of emerged adults, and number of pupae that failed to emerge were counted. This process was repeated in 2019 and 2020.

Compilation of Landscape Data

Landscape data was extracted from the National Land Cover Database (2011) within a 600-meter radius around each georeferenced field site in 2018. I used 2011 data because when this study was initiated, 2016 data were not available. Also, considering the resolution of National Land Cover Database (30×30m resolution), changes to the landscape over five years would likely not be visible at this scale, even with the introduction of non-native species, or landscape change from restoration projects and farm plantings. Landscape data were categorized as developed (open space, low intensity, medium intensity, high intensity), forest (deciduous, evergreen, mixed), scrub (dwarf, shrub), herbaceous (grassland, sedge), or wetlands (woody, emergent herbaceous). Landscape data categories were combined to represent the potential pollinator foraging habitat (forest, scrub, herbaceous, and wetland), and divided by the total area within a 600-meter radius around each nesting box (1.13 km^2) to calculate the proportion of potential foraging habitat given the estimated foraging distance for *O. lignaria*.

At each site for the first foraging period in 2018, all flowering resources within 50 meters of each nesting site were recorded between late March and mid-May. Plants were recorded to species when possible and used as a quality control in the molecular identification of plant data from pollen.

Most urban sites in Seattle, Washington were extremely diverse, with non-native plants and ornamental species common in urban gardens and privately-owned residential areas. In urban areas, there were several challenges in accurately measuring floral resources, including documenting floral resources on privately-owned land, sampling reliably within short bloom periods, and the infeasibility of sampling within a 600 m radius of all sites. For example, in 2018, all ground-surveyed sites were found to have >50 flowering plant species within a 50 m sampling radius, with likely hundreds of floral resources available within the full 600 m foraging distance of *O. lignaria*. Ground-surveying of sites in 2018 was both labor and time-intensive; moreover, by the completion of recording all flowering plants in the 50 m sampling radius of each site during the first foraging period, the flowering landscape had already shifted to the next phenological period. Originally, the goal of ground landscape composition data collection was to ground-truth molecular analysis results. However, in 2018, there was no clear connection between the plants identified in the field within 50 m of the nesting box and those found in the genetic data; for example, *Salix* spp. were found in both the ground landscape composition data and the sequencing data, but *Salix* was also present outside of the 50 m site radius for ground-truthing. Considering the labor and time involved in collecting ground-based floral data, and the subsequent success of genetic analyses in identifying floral resources visited by the bees, only genetic pollen analysis was used in subsequent years.

Compilation of Weather Data

Weather data were obtained from 48 weather stations available through Weather Underground (2021). Weather Underground stations are placed by community members, typically in backyards or attached to houses, and upload data through the Weather Underground online portal. The weather data accumulated for each site are publicly available and open access, making them an excellent option for ecological use as a source of community science data. All selected weather stations were <2 km from a nesting box to reflect weather conditions in the *O. lignaria* foraging area, and each field site was linked with the closest 2-4 weather stations. Weather station data were generally available in ~5-minute intervals. All weather data between April and July for each year, 2018-2020, were initially compiled. Precise dates were later selected for each year and foraging period (Table 2). Weather data collected between 0700 and 2000 were used, which encompassed foraging times for *O. lignaria*. Across all weather stations and years, this resulted in ~1.48 million weather measurements.

At each field site, weather data from linked weather stations were summarized into hourly measurements for each hour between 0700 and 2000. Hourly summaries included the mean temperature (°C), mean wind speed in meters per second ($m\ s^{-1}$), and total precipitation (mm). Within each foraging period, the total hours of available weather measurements were summed. Because 2-4 weather stations were used for each site, and due to the recording frequency of weather stations (~ every 5 min), missing hourly data were uncommon. However, to account for missing hourly data, the total hours of available weather measurements were used to calculate the proportion of hours with favorable temperature, wind speed, or precipitation. Favorable conditions for *O. lignaria* were defined as temperature >10 °C, wind speed < 1 $m\ s^{-1}$, and precipitation = 0 (Switanek et al., 2017). The proportion of hours with favorable temperature, wind speed, and precipitation for each foraging period and site was calculated by dividing the number of favorable hours for each weather metric by the total available hours of weather measurements at each site. Also, although bees were allowed to forage for up to six weeks, a three-week period of weather data was used for each foraging period (Table 2). This three-week period corresponded to peak *O. lignaria* flight based on field observations, and accounted for variation in adult emergence times for males and females, and the mating period.

Pollen removal and DNA extraction

Pollen samples consisted of single pollen provisions from unique nesting segments from each site. Four to 5 pollen provisions were randomly selected from the middle of nesting tubes from each site. The selected pollen provisions were homogenized using a micropestle to account for variability in plant pollen within the nesting tube and provisions. For DNA extraction, six sub-samples of 25mg were taken from homogenized samples at each site (Fig. 1). Extractions were performed on each of the six 25mg sub-samples for each site to obtain plant DNA. In summary, a total of six sub-samples were taken from each of the 12 sites for each of the 12 sites and foraging periods in 2018, and from each of the 22 sites and foraging periods in 2019 and 2020. A total of 288 DNA extractions in 2018, and 528 DNA extractions in 2019 and 2020 were performed.

A modified DNA extraction protocol from McFrederick and Rehan (2016) was used as a basis for thorough lysing of pollen. I added 180mL of ATL buffer (Qiagen, Germantown, Maryland) and a 5mm stainless steel bead to pollen samples. Samples were lysed for 3 minutes at 30hz using a Qiagen Tissuelyzer, then 20µl of proteinase K was added and vortexed to mix thoroughly. Samples were placed in a heat block at 57°C overnight. The next morning, 4µl of RNase A was added to each sample, and the remainder of the DNeasy Mini Plant Kit (Qiagen, Germantown, Maryland) was completed excluding the second spin column and elution steps. For the final elution, a total of 100µl AE buffer was pipetted directly onto the spin column membrane and incubated at ambient temperature for 15 minutes. The six sub-samples of DNA were also pooled into a single DNA sample to account for any bias in the DNA extraction kit. Pooled samples were vortexed, and a subsample of 200 µl DNA was removed and submitted for sequencing for each respective site. Extracted DNA samples were stored at -80°C prior to sequencing submission.

PCR and Sequencing

The *rbcL* region has been successfully used as a region of sequencing for plant identification to genus (De Vere et al., 2017; Galimberti et al., 2014; Lucas et al., 2018), and has an ample reference library available (Bell et al., 2017). For this study, I followed the previously established protocol and primer success of de Vere et al. (2012). The primers and adaptors used were the *rbcLaf* and *rbcLr506*:

(*rbcLaf*+ adaptor:
TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGATGTCACCACAAACAGAGACTAAAGC

rbcLr506+ adaptor:
GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAGGGGACGACCATACTTGTTCA)

Prior to submission of samples to the sequencing center, I performed a quality control check on ten randomly selected DNA extraction samples using the PCR protocol of de Vere et al. (2017) and primers described above. Amplified product from test PCR were run on a gel to determine whether further clean up steps were necessary; gel results showed clean DNA product with successful interaction with primers. Samples were submitted to the Integrated Microbiome Resource center (IMR) at Dalhousie University in Halifax, Nova Scotia for library preparation and Next-Generation Sequencing on Illumina MiSeq 2x300bp technology. Library preparation used Illumina Nextera XT kits and previously noted *rbcL* adaptors and primers.

DNA analysis

Sequenced samples in paired-end FASTQ format were merged using PEAR (Zhang et al., 2014). Quality control and removal of adaptor contamination was performed using Trimmomatic (Bolger et al., 2014) and clustering of reads was performed at 97% identity using CD-HIT (W. Li & Godzik, 2006). Clustered reads were aligned against an *rbcL* database developed by Bell et al. (2017) using BWA MEM for alignment (H. Li & Durbin, 2009). While databases can be dynamic, the *rbcL* database developed by Bell et al. (2017) was not updated during the 3-year period of this study, ensuring that identifications based on sequences did not change from year to year. Alignments were parsed with SAMtools (H. Li et al., 2009), and converted to BLAST b6 format. Custom Python scripting was used to combine individual results files into a merged table for statistical analysis at the genus level.

The sequence reads for plant genera in each sample were converted to proportions for simple downstream analysis. As a denoising step, all sequences totaling less than 0.1% of all sequences were removed prior to analysis. Subsequently, all sequences totaling less than 0.1% of individual sample sites (all sequences per sample) were also removed prior to analysis. DNA metabarcoding approaches are not an established method for quantification of plant taxa abundance of DNA due to the high potential for bias in amplification of different plant taxa and variations in plastid copy number, as well as bias in DNA extraction, PCR, and sequencing (Hawkins et al., 2015; Kraaijeveld et al., 2015). Consequently, plant richness was based upon the presence/absence of plant genera.

Statistical analyses

For *O. lignaria* developmental metrics, I considered the number of eggs oviposited (2018-2020), developmental success to the late instar or pupal stage (2019-2020), and adult emergence (2019-2020) as response variables for each site. All three response variables were transformed to meet the assumptions of normality. The number of eggs was transformed using $\log_{10}$. Developmental success to late instar/pupae was calculated as the proportion of eggs that successfully developed. Adult emergence was calculated as the proportion of adults that emerged from the late instar/pupal stage following overwintering. For the proportions, a value of 0.001 was added to each (due to the presence of 0 proportions), and a linearizing transformation was applied to the proportions, ln(proportion/(1-proportion)). I removed data on larval development success and adult emergence when these values were calculated from single digit counts of eggs to avoid bias in assessing development metric success. Therefore, proportional data on larval development success and adult emergence from sites with <10 eggs were excluded from analyses; across both 2019 and 2020, this included 2 sites in the first foraging period and 7 sites in the second foraging.

A linear mixed effects model was used to examine the effects of weather (temperature, wind, and precipitation), landscape composition (the proportion of potential foraging habitat), and plant richness based on DNA sequencing on each *O. lignaria* developmental metric. Analyses were conducted for each foraging period separately to reduce model complexity given differences in weather and flowering plants within each foraging period. Year and site were included as random effects. Some sites, due to their close proximity (< 600 m apart), were grouped; this resulted in 5 groups for the 12 sites used in 2018, and 16 groups for the 22 sites used in 2019 and 2020. Analyses were conducted using lme4 (Bates et al., 2015) and lmerTest (Kuznetsova et al., 2019) in R (R Core Team 2020).

In addition, I used non-metric multidimensional scaling (NMDS) to visually examine DNA sequencing data from each site and year. Plant richness data were standardized using the Jaccard index. I used the vegan package in R (Oksanen et al., 2020) to calculate the distance matrix for 2018 (dimensionality=2, stress=0.19), 2019 (dimensionality=2, stress=0.20), and 2020 (dimensionality=2, stress=0.19). I constructed hulls to illustrate compositional similarities between points based on distance matrices. Hulls were calculated using the grDevices package in R (R Core Team, 2020). Post-hoc, foraging periods during each year were identified using shapes and colors, and sites from each ordination (2018, 2019, 2020) were visually classified using shapes and colors into rural farms, urban farms (e.g., community gardens, urban gardening plots known locally as 'P-patches,' on-campus learning farms), restoration sites (e.g., areas where active restoration projects were ongoing), and city parks.

Results

Weather analysis

The proportion of hours during which rainfall was zero was negatively associated with the number of eggs in the first foraging period, but not in the second foraging period (Fig. 2). No other weather variables were significant in predicting oviposition (Table 3).

Increases in the proportion of hours during which temperature was >10°C were significantly associated with decreased developmental success to late instar/pupae during the first foraging period, while reduced precipitation was significantly associated with increased developmental success during the first foraging period (Table 3). No other weather variables were significant predictors for developmental success in either foraging period (Table 3).

Reduced precipitation during the first foraging period was significantly associated with increased adult emergence success, but no other weather variables were significant predictors for adult emergence in either foraging period (Table 3).

Landscape Composition

The proportion of the potential pollinator foraging habitat within a 600m radius of sites taken from NLCD data did not significantly predict any of the *O. lignaria* developmental metrics (Table 3).

Next Generation Sequencing Data and Plant Richness

Plant richness was not a significant predictor for oviposition, larval developmental success, or adult emergence in either foraging period (Table 3). However, because of the interest in understanding the effect of plant richness on solitary bee health, relationships between plant richness and *O. lignaria* developmental metrics for each foraging period are presented in Figure 3. Visually, there are no trends between plant richness and oviposition (Fig. 3A), and plant richness and larval developmental success (Fig. 3B). However, there was a non-significant trend showing a positive relationship between plant richness and adult emergence in both foraging periods (Fig. 3C).

DNA metabarcoding revealed that *O. lignaria* visited as few as two unique plant genera and up to 32 unique plant genera at a single site during the three years of data collection. Certain genera, including *Salix* spp. and *Rubus* spp., were found in all sites during the first and second foraging periods, respectively, indicating their presence within the *O. lignaria* foraging distance of each site (Table 4). The highest number of plant genera was found in 2019 during which 83 genera were detected in the first foraging period, and 80 genera in the second foraging period (Table 4). In 2018, 45 plant genera were detected in the first foraging period, and 53 in the second foraging period. Data from 2020 revealed only six plant genera in the first foraging period and seven in the second foraging period.

The plant genera reflected in the sequencing data showed that across all sites, there was high visitation to *Acer* spp., *Rubus* spp., and *Salix* spp. (Fig. 4). Pollen from *Pinus* spp. was detected in 2018 and 2019, but not in 2020, which could be a result of incidental pine pollen dispersal. In 2020, we detected *Stauntonia* spp. at all 22 sites. *Stauntonia* contains several ornamental species that are commonly planted in the Seattle area. No *Stauntonia* spp. were detected in 2018. In 2019, *Stauntonia* spp. were identified in the sequencing data at extremely low sequence counts, and subsequently filtered out through denoising procedures. The presence of *Stauntonia* spp. in 2020 highlights the importance of multi-year studies to accurately ascertain the plants upon which solitary bees, such as *O. lignaria*, forage given year-to-year variation in plant availability on the landscape and flowering times.

Using NMDS, clusters of sites were observed in the first and second foraging periods in 2019, but were visually less clustered in 2018 and 2020 (Fig. 5). When visualizing site classifications using NMDS, there were no apparent clusters across sites (i.e., city parks, restoration sites, and rural or urban farms) (Fig. 6).

Discussion

Solitary bee species often require considerable pollen and nectar resources for their offspring. Consequently, foraging and offspring success can be dependent on biotic conditions, such as the availability of suitable host plants, subject to environmental stochasticity arising from year-to-year variation in weather, and potential weather-induced asynchrony between foraging mothers and flowering times. Unseasonable cold spring temperatures, including those as a result of false springs, and other unfavorable weather conditions can play an important role in the reproductive success of species such as *O. lignaria* that have relatively short adult foraging periods. However, the analyses suggested that increases in the number of hours without precipitation led to decreased *O. lignaria* oviposition, likely due to other factors affecting the ability of foraging bees to oviposit in large numbers in the nest, such as increased predation, temperature change, or lack of available floral resources. Insects, including bees, are able to fly in light rain, which is relatively common in spring in the Pacific Northwest. Increases in precipitation could also lead to increased flowers, and thus an increase in the floral resource for *O. lignaria* foraging and subsequently, higher oviposition rates. However, less precipitation was linked to increased larval developmental success and adult emergence during the first foraging period (Table 3). Precipitation during larval development could affect success by facilitating mold growth, which was observed to be an issue. Generally, spring weather in the Pacific Northwest is characterized by frequent precipitation. However, in 2020, and between late April and late June, there were several major rain events and cooler than average temperatures. Similarly, there was reduced nesting success in 2020 during both the first and second foraging periods, and increased mold growth in nesting tubes. The weather analyses reported here also demonstrate the use of fine-scale community science weather station data in studies of insect population dynamics.

The use of a larger scale metric of landscape composition, such as when using data from the National Land Cover Dataset, was not a significant predictor of *O. lignaria* success (Table 3), which could suggest the need for ground level landscape data collection. However, when quantifying plant richness based on plant DNA, increases in plant richness did not increase *O. lignaria* developmental success (Table 3). Given that *O. lignaria* females dedicate nearly their entire 4-6 week adult stage to collecting pollen and creating pollen provisions on which to oviposit, it seems intuitive that flower availability, which is independently affected by weather conditions, affects *O. lignaria* success. However, in this case, other variables besides plant richness may be important in determining the larval development success, such as plant diversity. Increased richness of floral resources can ensure that foraging mothers acquire their preferred nutrients, while also buffering against any negative effects such as asynchrony with specific floral resources. In this study, plant richness varied by year, suggesting the *O. lignaria* females selectively forage for certain genera during different foraging periods.

The use of NGS provided new information regarding the number of plant genera visited by *O. lignaria* across urban and rural landscapes, highlighting both consistency in visitation and the importance of a relatively small number of genera used by *O. lignaria*. Visitation to plant genera shifted between foraging periods, with reliance on certain flowering plant genera during the first foraging period in comparison to plant genera visited during the second foraging period. In 2018, *Salix* and *Rubus* spp. were found in all sampled sites in the first and second foraging periods, respectively, while *Acer* spp. were found in all samples in the first foraging period (Fig. 4). *Salix* spp. were present across most sites, with native and non-native species common in wet, disturbed environments throughout Seattle city parks. Outside the city, *Salix* spp. were present on the edges of rural restoration areas and working farms, and provide a source of pollen and nectar for spring pollinators. In the second foraging period, bee visits were dominated by *Rubus* spp., with *Salix* spp. and *Acer* spp., reflecting the overlap in bloom time between the first and second foraging period and highlighting preference for these plants by *O. lignaria* (Fig. 4). In 2019, *Rubus* spp. were found in all sampled sites in both foraging periods, while *Salix* spp. were found in all sites but one in the first foraging period. Generally, across all sites and years, and across both foraging periods, *Acer, Rubus*, and *Salix* spp. were among the most dominant sources of pollen collected by *O. lignaria* (Fig. 4). However, 2019 hosted the highest number of plant genera in the pollen during both foraging periods, almost doubling the total number of genera visited in 2018 (Fig. 4). In contrast, 2020 hosted single digit plant richness values, a fraction of previous visits, potentially due to a lack of flowers available to foraging bees. A lack of floral resources in 2020 may have driven *O. lignaria* to switch from previously available genera to *Stauntonia* spp.

The presence of *Pinus* spp. pollen at sites was not surprising considering the number of species that release pollen in spring and their proximity to all sites. Conifers are widespread throughout the city of Seattle, in urban sites as well as rural sites near forested areas. Conifers are wind-pollinated, and pollen from *Pinus* spp. may have blown into pollen provisions or onto foraging bees, or accumulated from the surface of the flowers the bees were visiting. The possibility of accumulating other wind-pollinated species was present across all of the sites, but accumulation was low. For example, grass pollen can be widespread during spring and summer, but was not found in pollen from foraging bees across any years following denoising of sequencing data. Pollen from *Pinus* spp. was found at a high enough level to show up following denoising, indicating it may be more dominant in samples than other wind-pollinated species. Bees have been shown to visit conifers, but this relationship is not known to affect bee development (Rust, 1987). High presence of *Pinus* spp. pollen in 2018 and 2019 may indicate a relationship between foraging bees and conifer species.

Multiple genera that contain species considered to be non-native and in some cases invasive were found during the first and second foraging periods (e.g., *Crataegus monogyna, Ilex aquifolium*, *Sorbus aucuparia*, and *Rubus armeniacus*), underlining the potential importance of the floral resources of non-native or invasive plant species for native bees in urban environments. *Stauntonia* spp. presence in 2020 was surprising, considering the genera had not been present in 2018 or 2019. Ornamental species of this vine, which as a genus is native to Asia, produces large numbers of flowers in early spring, and potentially could provide a floral resource for bees when emergence occurs prior to or following their preferred floral resources. The increased precipitation and unseasonably cool temperatures in 2020 (between late April and late June) could have potentially affected the bloom time of plants that *O. lignaria* typically forage on during that period. *Stauntonia* spp. typically bloom in April in their native range in Japan, and may have had an extended blooming season during the spring of 2020.

Conservation of solitary bees requires time and effort. Despite the lack of a link between plant richness and bee developmental success, higher plant richness can still positively benefit bees by providing increased potential floral resources. Restoration projects typically feature annual and perennial plants and shrubs, which can provide large numbers of flowers for pollinators. However, restoration projects in western Washington and similar ecological environments could benefit by focusing on the planting of the dominant plants visited by *O. lignaria* in this study. This includes flowering trees such as *Salix* spp. and *Acer* spp. to provide blooms during the early foraging season, and *Rubus* spp. to provide floral resources later in the foraging season. *Rubus* spp. typically take a bushy form, and many species grow well in previously disturbed environments. Similarly, *Salix* spp. grow well in wet, disturbed environments such as those in city parks, drainage areas, and historic landfills. Consideration of flowering plants that collectively provide blooms throughout the spring could serve as a bridge resource during disruptive weather and other stochastic events. Because *O. lignaria* serves as a model species for understanding native solitary bee behavior, the findings could serve to inform the conservation of other solitary bee species. It is important that future studies consider other solitary bee species to broaden our understanding of native pollinators. It is also necessary to take into account plant abundance to better inform restoration planting and pollinator gardens.

Landscape composition is also known to affect foraging success of solitary bees, and the availability of flowering plants has been identified as a potential cause of bee population decline (Cameron et al., 2011). Bees can also vary their foraging behavior in response to factors such as the presence of invasive plants or in the presence of diverse urban gardens (Lopezaraiza-Mikel et al., 2007; MacIvor et al., 2014; Williams et al., 2011). To acquire the necessary nutritional components, generalist solitary bees visit a variety of floral resources, and can prefer larger, more diverse flowering displays (Jha & Kremen, 2013). Plants also vary in nutrient content, such as in lipids and proteins, which can be important in bee development (Vaudo et al., 2016, 2020). Collectively, the observations from this study highlight potential effects of weather on the developmental success of an exemplar solitary nesting bee. This research also shows the utility of NGS as a tool in exploring pollinator forage choices across highly variable landscapes such as those in urban environments. Future research that experimentally measures the consequence of plant-pollinator asynchrony in *O. lignaria* would inform conservation management in the presence of changing climatic conditions.

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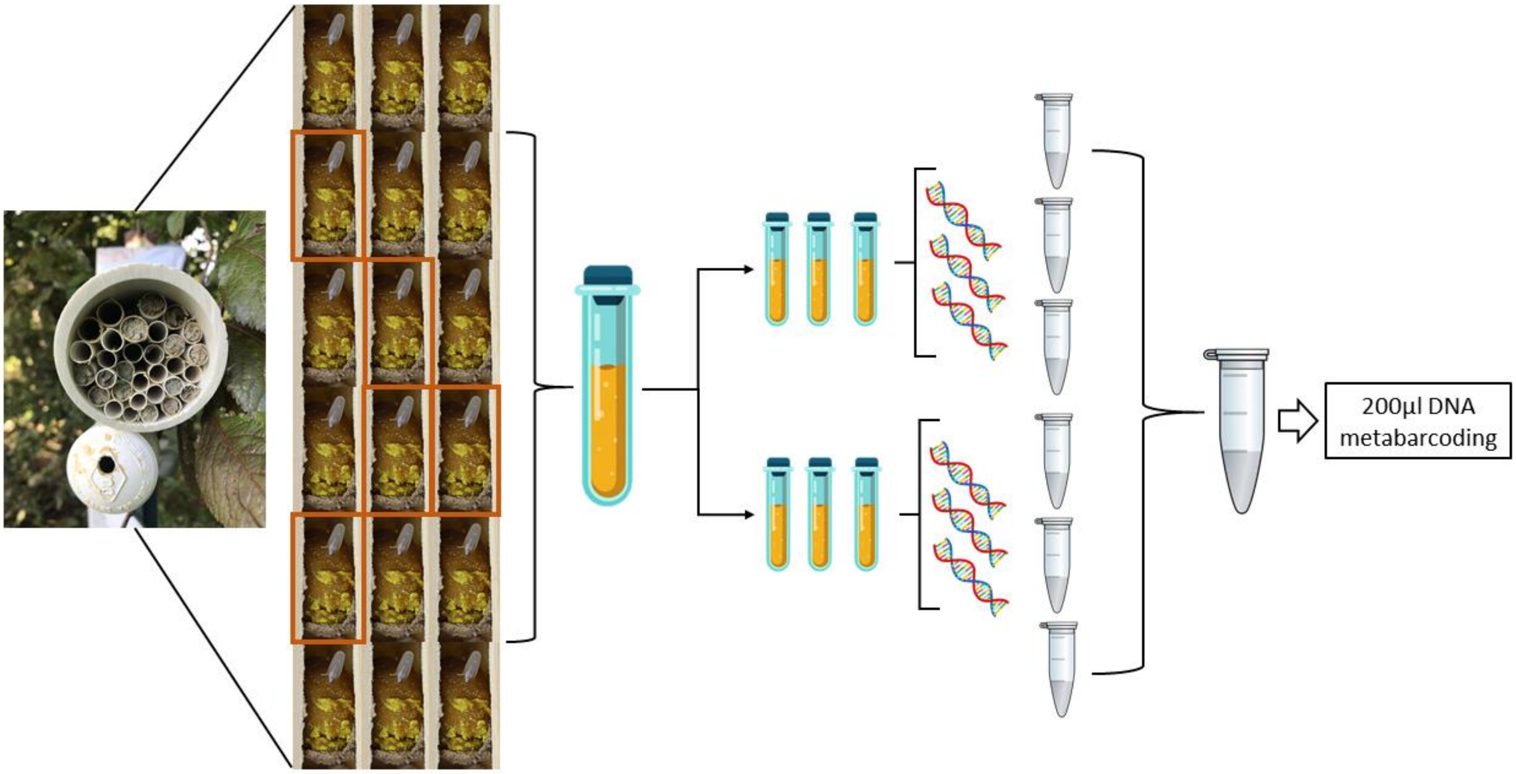

Figure 1. Process of pollen provision sub-sampling prior to DNA sequencing (library preparation and metabarcoding). Initially, nesting tubes from boxes at each site were divided into two groups: one for pollen analysis and one for developmental analysis. Nesting tubes used in pollen analysis were dissected, and five pollen samples taken from the middle of the tube were selected for genetic analysis. The five pollen samples were homogenized in a single tube using a micropestle to reduce the potential for inaccurate sampling; six sub-samples were taken from the homogenized sample and used for DNA extraction. Extracted DNA was pooled into a single sample, representing the plant DNA from a single site (and respective foraging period and year). Following vortexing to ensure unbiased sub-sampling, 200 µl was pipetted from the pooled DNA sample for submission to a DNA sequencing facility.

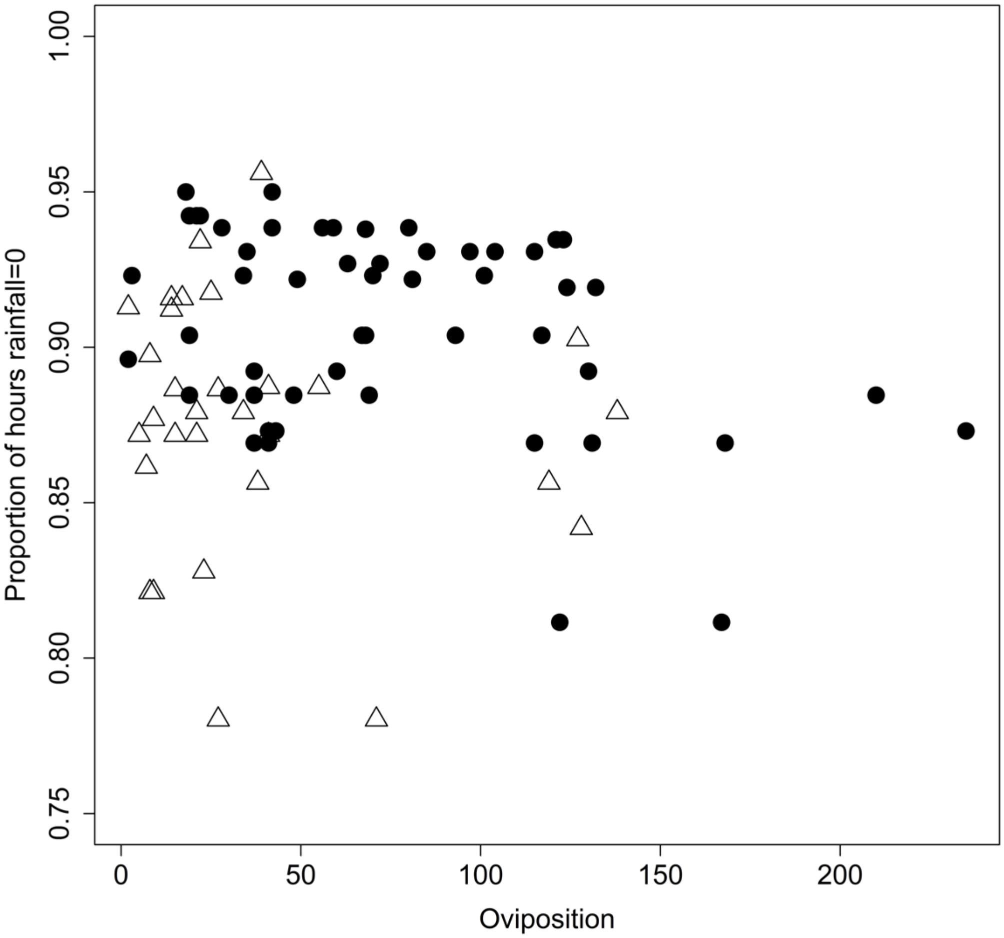

Figure 2. Effect of the proportion of hours with zero rainfall on total number of eggs laid. Symbols represent a single site between 2018-2020 during the first foraging period (closed circles) and second foraging period (open triangles).

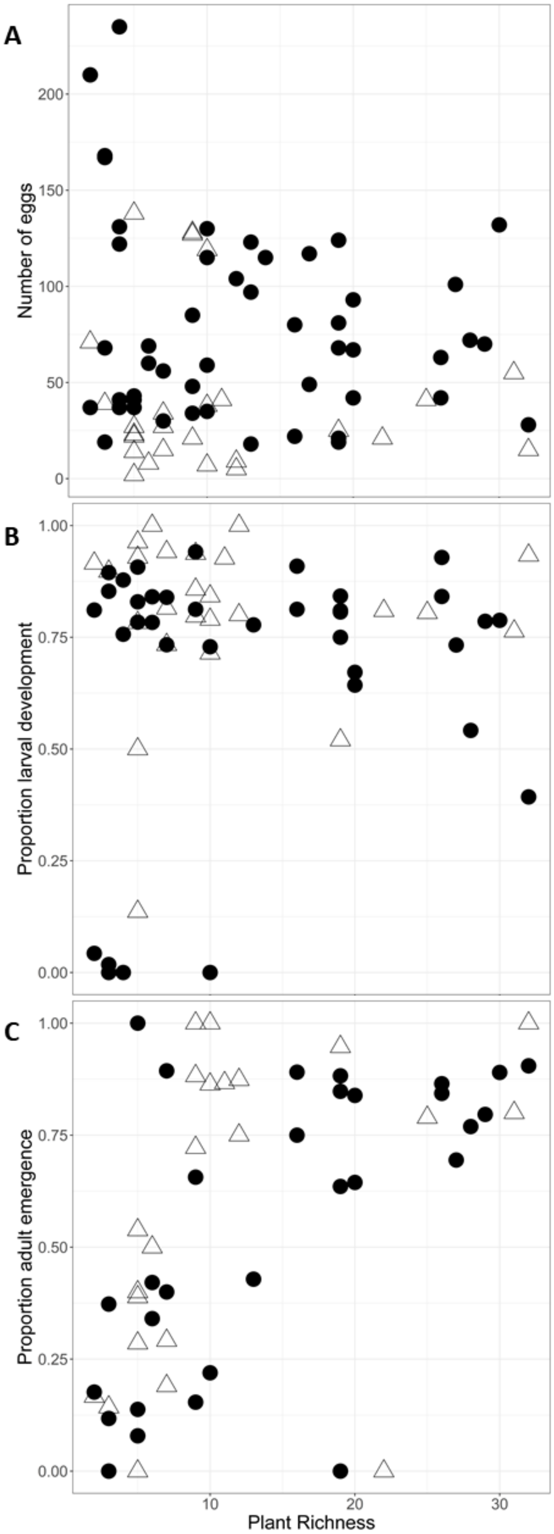

Figure 3. Effect of plant richness, as ascertained from genetical analysis of pollen, on (A) total number of eggs oviposited, (B) proportion of successful larval development, and (C) proportion of adult emergence. Symbols represent a single site between 2018-2020 during the first foraging period (closed circles) and second foraging period (open triangles). Total number of eggs laid per site includes 2018 data, which was not used for larval development or adult emergence; therefore, there are more data present in (A) than in (B) or (C).

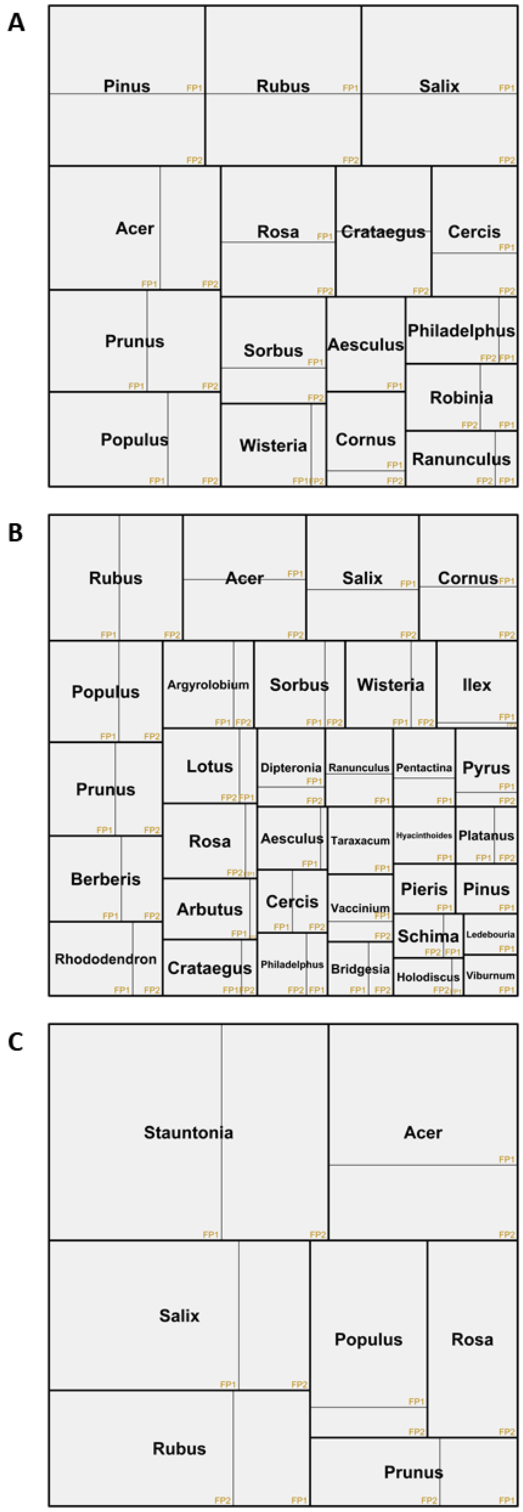

Figure 4. Tree maps showing all genera found at ≥5 sites for (A) 2018, (B) 2019, and (C) 2020. Foraging period is indicated in the bottom right corner of each box (FP1 = first foraging period, FP2 = second foraging period). The overall size of the boxes in each treemap reflect the number of sites at which that unique genus was found. Within a treemap, the size of subgrouping boxes for each genus reflects the number of sites at which the unique genus was found in each foraging period.

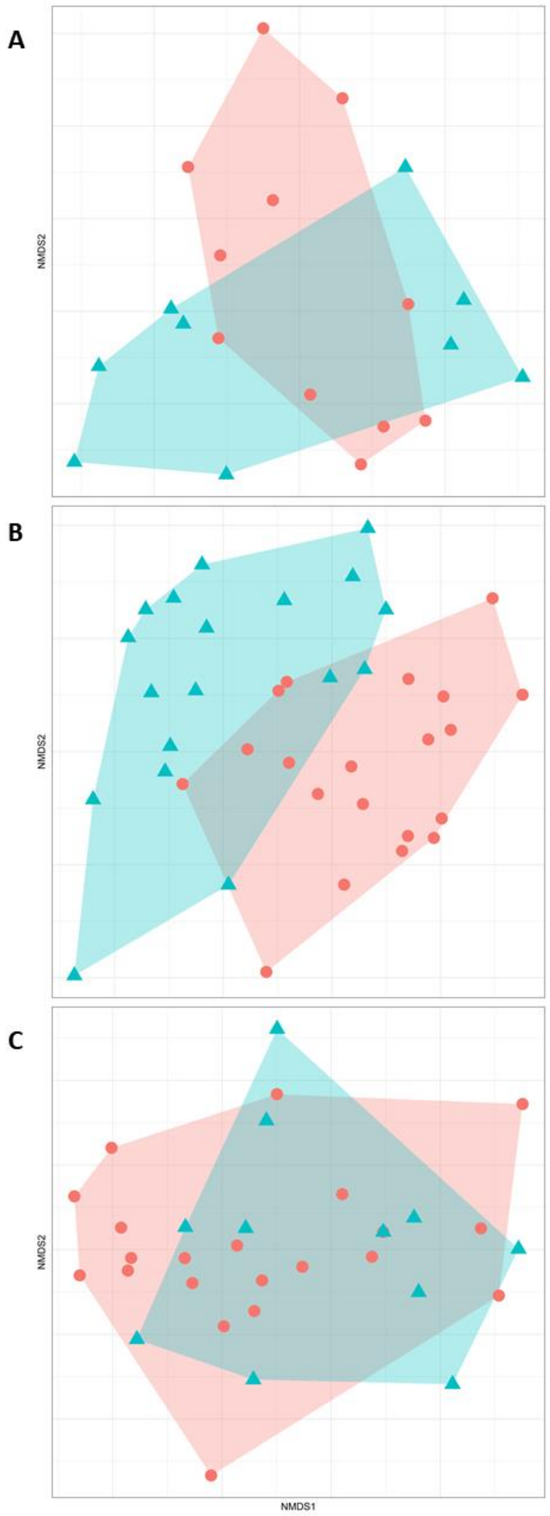

Figure 5. Non-metric dimensional scaling of scores taken from ordinations of (A) 2018 (dimensionality=2, stress=0.19), (B) 2019 (dimensionality=2, stress=0.20), and (C) 2020 data (dimensionality=2, stress=0.19). Hulls illustrate the similarities between points based on distance matrices from ordinations by year. The first (red) and second (blue) foraging periods are represented for all sites in each year. Points are taken from the distance matrix calculated for each foraging period and year. Shaded hulls represent a subset of points which lie on the convex hull of the set data from each foraging period.

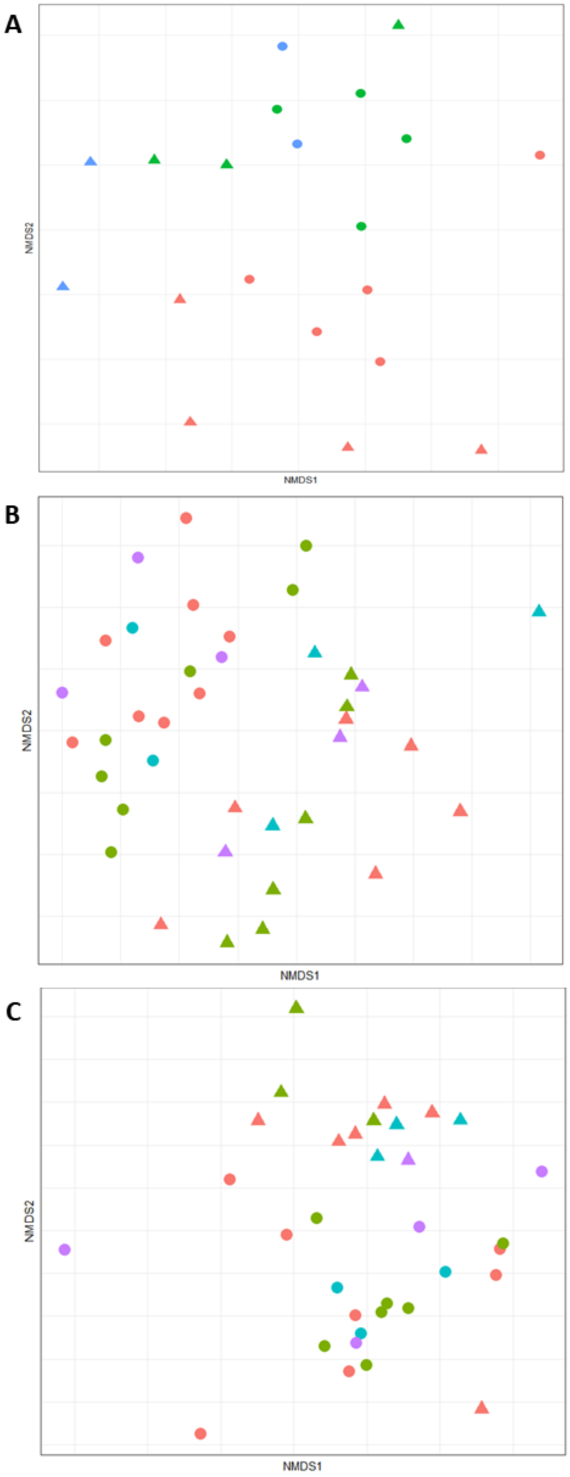

Figure 6. Non-metric dimensional scaling ordinations of all sites from (A) 2018, (B) 2019, and (C) 2020; in the top row, symbols indicate the first (circles) or second (triangles) foraging period. Symbols are also classified by color as city park (orange), restoration site (green), rural farm (blue), and urban farm (purple). Dimensionality and stress are equivalent to those reported in Fig. 5.

Table 1. Description and location of study sites, 2017-2020. Landscape data refers to the proportion of land use categories that represent the potential pollinator foraging habitat (forest, scrub, herbaceous, and wetland) out of the total 600-meter radius area around each site for 2018-2020 sites; landscape data were not available for all sites (denoted as NA).

Site Name	Site Description	Longitude (°W)	Latitude (°N)	Years Studied	Landscape data	Size (km^2)
21 Acres Reserve	Rural non-profit community farm and reserve	-122.1560	47.74801	2018-2020	0.1871	0.0849
Washington Park Arboretum	Urban Public Arboretum in Seattle, WA	-122.2962	47.63440	2017-2020	NA	0.9307
Beacon Hill Food Forest	Urban community farm	-122.31278	47.567686	2018-2020	0.0662	0.028
Center for Urban Horticulture	Urban botanic garden	-122.28985	47.656948	2017-2020	0.1037	0.0647
Discovery Park East	Urban park	-122.40948	47.664408	2017-2020	0.4699	2.16
Discovery Park West	Urban park	-122.42851	47.660915	2017-2020	0.3293	2.16
Genesee Park	Urban park	-122.27847	47.567763	2019-2020	0.0031	0.233
Green Line	Urban community restoration stewardship project	-122.26796	47.50587	2019-2020	0.0867	0.00809

Lee Memorial Forest	Suburban Research Forest	-122.12036	47.82226	2017	NA	0.6474
Magnuson Park North	Urban park	-122.24932	47.679742	2017-2020	0.0301	1.416
Magnuson Park South	Urban park	-122.25002	47.678069	2017-2020	0.0143	1.416
Oxbow Farm North	Rural working farm	-121.97849	47.69001	2019-2020	0.2887	0.971
Oxbow Farm South	Rural working farm	-121.9719	47.69277	2019-2020	0.4789	0.971
Residential Home	Urban residential home in Seattle, WA	-122.29000	47.67000	2017	NA	0.00404
Seward Park North	Urban park	-122.25032	47.551622	2017-2020	0.5304	1.214
Seward Park South	Urban park	-122.25254	47.550034	2017-2020	0.3909	1.214
Union Bay Natural Area 1	Urban public wildlife and restoration area	-122.29096	47.657451	2017-2020	0.1099	0.299
Union Bay Natural Area 2	Urban public wildlife and restoration area	-122.29171	47.656438	2017-2020	0.1323	0.299
UW Farm East	Urban farm	-122.2926	47.6584	2017-2020	0.0806	0.00607
UW Farm West	Urban farm	-122.293	47.658115	2017-2020	0.0927	0.00607
Vashon Island Farm	Rural community farm	-122.46661	47.43165	2019-2020	0.2034	0.0404

Vashon Island North	Rural natural restoration area/preserve	-122.46525	47.46776	2019-2020	0.7347	0.7689
Vashon Island 2	Rural natural restoration area/preserve	-122.4775	47.42139	2019-2020	NA	0.7689
Vashon Island 3	Rural natural restoration area/preserve	-122.47496	47.41526	2019-2020	NA	0.627

Table 2. Three-week time periods during which weather data were analyzed for each foraging period, 2018-2020.

Year	**Foraging period**	
	1	**2**
2018	April 15-May 4	May 25-June 13
2019	April 28-May 17	June 2-June 21
2020	April 12-May 1	May 24-June 12

Table 3. Effects of plant richness ascertained using NGS, landscape data ascertained using NLCD data, and weather variations on *O. lignaria* oviposition, larval developmental success, and adult emergence in each foraging period. Values in bold indicate significance ($P \leq 0.05$).

First Foraging Period	**Oviposition**	**Larval developmental success**	**Adult emergence**
Plant Richness	t=0.56; df=40; P=0.58	t=-0.24; df=32; P=0.81	t=1.56; df=16; P=0.14
Landscape data	t=0.50; df=10; P=0.63	t=-0.28; df=31; P=0.78	t=0.82; df=10; P=0.43
Temperature >10°C	t=-1.44; df=40; P=0.16	**t=-2.05; df=31; P=0.05**	t=-1.53; df=21; P=0.14
Rainfall = 0	**t=-2.08; df=39; P=0.04**	**t=4.36; df=31; P<0.01**	**t=2.77; df=14; P=0.02**
Wind speed <1 m s^{-1}	t=-1.20; df=15; P=0.25	t=-0.53; df=31; P=0.60	t=-1.19; df=14; P=0.26
Second Foraging Period	**Eggs**	**Larval development**	**Adults**
Plant Richness	t=0.04; df=17; P=0.97	t=1.21; df=8; P=0.26	t=0.42; df=10; P=0.69
Landscape data	t=-0.42; df=14; P=0.68	t=-1.83; df=11; P=0.09	t=-0.29; df=8; P=0.78
Temperature >10°C	t=1.60; df=18; P=0.13	t=-0.64; df=11; P=0.54	t=0.90; df=10; P=0.39
Rainfall = 0	t=-1.41; df=16; P=0.18	t=-0.88; df=14; P=0.39	t=-0.04; df=7; P=0.97
Wind speed <1 m s^{-1}	t=-0.49; df=12; P=0.63	t=-0.05; df=14; P=0.96	t=0.97; df=7; P=0.36

Table 4. All plant genera found in each foraging period from 2018-2020 based upon Next-Generation sequencing on collected pollen.

Year	Foraging Period	Genera Present
2018	1	*Acacia, Acer, Aesculus, Allium, Arbutus, Argyrolobium, Aronia, Astragalus, Bauhinia, Callerya, Carex, Cercis, Cornus, Cotinus, Crataegus, Cupaniopsis, Dasiphora, Dipteronia, Euphorbia, Fragaria, Genista, Indigofera, Malus, Mangifera, Nyssa, Ozoroa, Philadelphus, Photinia, Pinus, Populus, Prunus, Pyrus, Ranunculus, Rhododendron, Rhus, Robinia, Rosa, Rubus, Salix, Schinus, Senna, Sorbus, Toxicodendron, Vitis, Wisteria*
2018	2	*Acacia, Acer, Allium, Amelanchier, Arbutus, Argyrolobium, Brucea, Camellia, Ceanothus, Cercis, Chaenomeles, Cistus, Cordyline, Cornus, Cotoneaster, Crataegus, Dasiphora, Dipteronia, Euphorbia, Fendlera, Fragaria, Garrya, Heteromeles, Hydrangea, Ilex, Kirengeshoma, Luzula, Malus, Mentzelia, Oxytropis, Ozoroa, Phanera, Philadelphus, Photinia, Pimelea, Pinus, Populus, Prunus, Pyracantha, Pyrus, Ranunculus, Rhododendron, Rhus, Robinia, Rosa, Rubus, Salix, Sambucus, Schinus, Senna, Sorbus, Stewartia, Wisteria*
2019	1	*Acacia, Acer, Aesculus, Alopercurus, Arabidopsis, Arbutus, Argyrolobium, Baptisia, Berberis, Brassica, Bridgesia, Cassinopsis, Ceanothus, Cercis, Choisya, Clematis, Comoranthus, Cornus, Crataegus, Crotalaria, Davidia, Dicentra, Dimocarpus, Diospyros, Diplopeltis, Dipteronia, Drypetes, Elaeagnus, Exbucklandia, Forsythia, Fragaria, Frangula, Franklinia, Fraxinus, Geranium, Glycine, Heuchera, Holodiscus, Hyacinthoides, Ilex, Ionopsidium, Laccopetalum, Landolphia, Ledebouria, Ligustrum, Litchi, Lonicera, Lotus, Melolobium, Nandina, Olea, Oncosiphon, Pediomelum, Pentactina, Phaseolus, Philadelphus, Pieris, Pinus, Platanus, Populus, Prunus, Pyrus, Ranunculus, Rehderodendron, Rhaphiostylis, Rhododendron, Rhodoleia, Rosa, Rubus, Salix, Sambucus, Schefflera, Schima, Sorbus, Syringa, Taraxacum, Toechima, Trifolium, Vaccinium, Veronica, Viburnum, Viola, Wisteria*
2019	2	*Acacia, Acer, Aesculus, Arabidopsis, Arbutus, Argyrolobium, Baptisia, Berberis, Boehmeria, Brassica, Bridgesia, Camellia, Cardamine, Cassinopsis, Cercis, Clematis, Comoranthus, Cornus, Crataegus, Crotalaria, Cucurbita, Davidia, Dimocarpus, Diospyros, Diplopeltis, Dipteronia, Drypetes, Fragaria, Frangula, Franklinia, Hesperelaea, Hesperis, Heuchera, Holodiscus, Hybanthus, Hypericum, Ilex, Lathyrus, Lepidium, Ligustrum, Litchi, Lotus, Nandina, Olea, Ozoroa, Pachycladon, Pentactina, Phaseolus, Philadelphus, Platanus, Populus, Prunus, Pyrus, Ranunculus, Raphiostylis, Rhododendron, Rhodoleia, Rhus, Rosa, Rubus, Salix, Sambucus, Schefflera, Schima, Shrebera, Solanum, Sorbus, Spiraea, Syringa, Tilia, Toechima, Torminalis, Trifolium, Ulmus, Vaccinium, Veronica, Vigna, Viola, Vitis, Wisteria*
2020	1	*Acer, Populus, Prunus, Rubus, Salix, Stauntonia*
2020	2	*Acer, Populus, Prunus, Rosa, Rubus, Salix, Stauntonia*

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Chapter 2: Bees needs: Effect of protein and lipids in pollen on the developmental success of the native solitary bee *Osmia lignaria* Say

Abstract

The health of solitary bees is dependent on the pollen they consume, driving their development from larvae to pupae. Nutrients such as proteins and lipids play an important role in bee development and could be the key to supporting declining populations. I examined the concentration of protein, total lipids, and 13 individual lipid classes in pollen provisions collected from the native solitary nesting bee, *Osmia lignaria* Say, across 22 sites in western Washington over three years and during two distinct foraging periods. The results indicate that while protein did not significantly affect larval development, higher lipid concentrations in the second foraging period were associated with increased larval developmental success and adult emergence success. Individual lipid classes may also play a role in the successful development of *O. lignaria*, as hexosylceramide and cholesterol ester were positively associated with *O. lignaria* larval developmental success, which emphasizes the need for additional studies on the role of individual lipid classes on bee development. Lipid levels in pollen were higher during the second foraging period, and because plant richness did not differ significantly between the two foraging periods, the results suggest that *O. lignaria* foraging behavior is not specifically driven by the nutritional content of the pollen. This study informs bee conservation plans and highlights the role of pollen protein and lipids in bee development.

Introduction

Pollen is a vital food source for a number of living organisms. Collected and consumed by many insect species, including a majority of pollinator species, pollen has a rich history in shaping nutrition, even among humans. Navajo tribes collected pollen from crops and wild flowering plants, kneading it into bread, and other Native American civilizations collected and consumed pollen as a common food source (Linskens & Jorde, 1997). Among insects, nearly all species of bees (Hymenoptera: Anthophila) rely on nutrients obtained from pollen and nectar for reproduction, development, and overall survival. Bees spend a majority of their adult life cycle visiting floral resources in search of pollen to bring back to their offspring, and nectar to provide carbohydrates and energy for flight. The bee's sole source of protein, lipids, and micronutrients come from foraged pollen, which is fed to offspring to support larval development and adequately prepare for diapause.

The outermost layer of the pollen wall, the pollenkitt, consists of lipids, terpenoids, carotenoid pigments, and hydrocarbons (Dobson, 1988). Inside, the exine and intine contain nutrient rich cytoplasm, necessitating the digestion of pollen grains to receive the full benefit of their nutrient value. In some species of insects, the pollen grains are thought to be broken down in the midgut, such as in the case of the beetle *Cyclocephala amazona* Oliver (Coleoptera: Scarabaeidae) (Rickson et al., 1990), while others grind pollen using the mandibles. Species of thrips feed on pollen by piercing and sucking out the contents of the inner pollen grain (Kirk, 2009). In bees, pollen digestion is extremely intricate. Nectar and pollen combine in the adult bee crop, where it is digested using combinations of osmotic pressure changes and submersion in sugar solution (Roulston & Cane, 2000). In larval bees that lack a crop, pollen is digested solely in the midgut. Social bees store pollen in the hive, adding secretions and microbial organisms that can change the digestibility of pollen grains (Klungness & Peng, 1984).

Protein is a necessary building block for immature bee development. The basic structures that comprise proteins are amino acids, which are used for cellular repair and growth. Lipids include fats and oils and are used in the body as a source of energy storage, insulation, and used to build cell membranes. Lipids break down into fatty acids, waxes, sterols, vitamins, and other chemical forms- each used in different ways to benefit physiological processes and development of cellular structures in larvae (Manning, 2001). Certain combinations of protein and lipid levels are thought to provide the best nutritional benefit to bee species, with the protein-to-lipid ratio of interest to researchers in terms of providing the best method of quantifying and qualifying nutrient value of bee pollen (Vaudo et al., 2020).

Previous work on identifying the nutrient needs of bee species have focused almost entirely on social species such as honey bees or bumble bees (Baloglu & Gurel, 2015; Brodschneider & Crailsheim, 2010), and only a few studies have explored the quantitative requirements of protein and lipids for generalist solitary bees. For example, recent diet studies analyzed the reproduction rates of solitary bees when fed an altered diet, which resulted in fewer oocytes in females fed less protein-rich pollen (Cane, 2016). While foraging, solitary bees visit both plants close to their nesting site as well as those further away, suggesting bees mix high and low quality pollen due to extrinsic factors (Williams & Tepedino, 2003). Studies of solitary bees in the genus *Osmia* found that some pollen is better digested than others, depending on the thickness of the pollen walls of different plant species (Suarez-Cervera et al., 1994).

Collection of pollen from bees is vital to exploring its nutritional content. Solitary bees such as *Osmia lignaria* Say (Hymenoptera: Megachilidae), the blue orchard mason bee, present an opportunity for collecting pollen collection across a range of habitats for subsequent nutritional bioassays, which can furthermore be linked to larval developmental success. *Osmia* spp. are important spring pollinators, and contribute to cherry, apple, and almond crop production (Bosch et al., 2006). *Osmia lignaria* are reared commercially across the United States, and nest successfully in human-made structures. Adults emerge from overwintering pupae in early spring and locate suitable mates. Following mating, females will locate a suitable nest and begin amassing pollen for her offspring (Bosch & Kemp, 2000). Total nesting and foraging time for *O. lignaria* is only about 6 weeks, and foraging distances are generally limited around 600 m from the nest (Rust, 1990). Due to their availability and feasibility in research, and their importance as pollinators of cultivated crops and native plants, *O. lignaria* is an ideal system for exploring native solitary bee behavior and biology (Eckhardt et al., 2014; Haider et al., 2014; Kraemer & Favi, 2009). In this chapter, I examined the effect of total protein and lipid concentrations in pollen provisions collected from *O. lignaria* over 3 years, and across two distinct foraging periods in western Washington, on *O. lignaria* development. I also considered the concentrations of 13 unique lipid classes commonly used in nutritional analyses, but not previously analyzed in studies of solitary bee health, and their effect on *O. lignaria* development. Plant richness data ascertained from DNA sequencing performed as part of Chapter 1 was also used to assess relationships between plant richness over two distinct foraging periods, and the total lipid and protein concentration of pollen collected from solitary bees. Finally, I visually explored relationships among different site types (i.e., urban restoration sites, urban farms, city parks, rural restoration sites, and working rural farms), on levels of individual lipid class concentrations for all years of data collection.

Materials and Methods

Field sites and Experimental Set-up

Field sites were established across a variety of urban and rural landscapes in King County, Washington. In 2017, a total of 14 sites were selected for preliminary placement of solitary bees to determine which site characteristics were conducive to nesting success based on whether the bees nested in the placed boxes or failed to nest. Of the 14 sites, nesting success was recorded from 12 sites. In 2018, these 12 sites were selected, and an additional 10 sites were chosen for 2019-2020. Sites were chosen based on their proximity to 1) flowering species and 2) site characteristics conducive to solitary bee success, including reasonable protection from strong winds and adequate southeast exposure to sunlight (Rust, 1990). A brief description of sites is presented in Table 1 from Chapter 1. Field sites were established across a variety of urban and rural landscapes including community farms and gardens, public parks, restoration sites, and working farms.

At each site, two bee houses, constructed from 7.5 cm diameter PVC pipe, were placed facing southeast and filled with 40, 8mm diameter paper nesting tubes. For each site, 100 locally purchased *O. lignaria* overwintering pupae (Crown Bees, Woodinville, WA and Watts Solitary Bees, Bothell, WA) were placed in emergence boxes once outdoor temperatures had reached a minimum of 10°C during peak foraging hours, which typically occurred in late March to early April of each year in western Washington. Once adults had emerged and females had left to forage and nest in the boxes over their 6-week lifecycle, which I considered the first foraging period, any remaining pupae and all nesting materials were removed. The first foraging period was designed to be implemented during the phenologically-typical foraging period for *O. lignaria* in western Washington based on prior work (Bosch & Kemp, 2000). The 6 weeks encompassed the life span the adult bees (Bosch et al., 2000). New nesting materials were then placed at all sites with 100 additional *O. lignaria* pupae and allowed to complete a second foraging period over 6 weeks. This second foraging period was designed to simulate asynchrony between *O. lignaria* and its phenologically-typical host plants.

Following completion of the second foraging period, all nesting tubes from a single site and foraging period were randomly placed into two groups approximate equal numbers: one group for nutritional analysis to quantify protein and lipid levels for each site, and one group to quantify larval developmental and adult emergence success. In 2018, completed nesting tubes from each site were used to count the number of eggs, and allocated to molecular analysis to ascertain plant DNA. In 2019 and 2020, nesting tubes selected for nutritional analysis were dissected, pollen provisions were removed and individually stored at -20°C prior to analysis. Nesting tubes selected for developmental and emergence success were placed in Percival Scientific (Perry, Iowa) biological incubators under conditions that simulated the most recent 10-year weekly average temperatures for King County, WA (Weather Underground, 2021), and the photoperiod for the county. For each week, photoperiod was adjusted, and temperatures in the incubators were set to fluctuate between the average high and average low for the relevant week, and were adjusted from mid-May to March-to-April of the following spring.

Larval Development and Emergence Success

Nesting tubes selected for quantifying developmental and emergence success were removed from biological incubators in late fall (late October to mid-November) once *O. lignaria* had reached the late larval or pupal stage. Nesting tubes were dissected, and I counted individual segments inside nesting tubes with whole pollen provisions, or 1st instars that failed to develop to the late larval or pupal stage, as a measure of oviposition. I also counted the number of offspring that had completed development from egg to 5th-6th instar or pupal stage as successful larval development. Once counted, pupae were washed in lukewarm water to remove frass and other debris. Pupae were dried and placed in containers identified by site and foraging period in the biological incubators until early spring the following year. When the temperatures from the biological incubators reached the minimum temperatures required for adult emergence (~10°C), adult bees were allowed to emerge over 6 weeks. The total number of emerged adults were counted, as well as the total number of pupae that failed to emerge. This process was repeated for all years of data collection.

Pollen removal for Plant DNA extraction

Pollen samples consisted of individual pollen provisions from a unique nesting segment from each site as previously described in Chapter 1. Briefly, four to five individual pollen provisions taken at random from the middle of nesting tubes per site were homogenized using a micropestle. A total of six 25mg samples were taken from each homogenized sample for DNA extraction. Following extraction, extracted DNA samples were vortexed and 100µl of each extraction were combined for a single site. A schematic of the sub-sampling process is shown in Chapter 1 (Ch. 1, Fig. 1). In summary, a total of six DNA extractions were performed for each individual site and foraging period in 2018 totaling 264 extractions, and 528 DNA extractions in 2019 and 2020.

Each sample was lysed with a single 5mm stainless steel bead and 180mL ATL buffer (Qiagen, Germantown, Maryland) on a Qiagen Tissuelyzer for 3 min. at 30hz to fully break apart pollen cell walls. I added 20µl of proteinase K, mixed thoroughly, and placed on a heat block at 57°C overnight. The following morning, I added 4µl of RNase A to each sample. I then used a modified protocol from McFrederick and Rehan (2016) and DNeasy Mini Plant Kits (Qiagen, Germantown, Maryland) for DNA extraction of plant DNA. Final elution totaled 100µl.

To ascertain plant richness at each site, I sequenced pollen DNA to identify plants visited by foraging bees in a previous study (Chapter 1). For sequencing, I chose the *rbcL* region for its success in identification of plants to genus (De Vere et al., 2017; Hawkins et al., 2015) and robust reference library (Bell et al., 2017). PCR testing was performed on 10 randomly selected samples with primers described in the previous study (Chapter 1) prior to sequencing submission to ensure DNA was of high quality and primers were successful with these samples. Primers and adapters used were taken from de Vere et al. (2012). Samples were submitted to the Integrated Microbiome Resource Center (IMR) at Dalhousie University (Halifax, Nova Scotia) for library preparation and sequencing on Illumina MiSeq 2x300bp technology.

Plant DNA sequence analysis

All samples were submitted concurrently for sequencing, and analyzed using the following bioinformatic pipeline in 2020. Sequenced samples in paired-end FASTQ format were merged using PEAR (Zhang et al., 2014). Quality control and removal of adaptor contamination was performed using Trimmomatic (Bolger et al., 2014) and clustering of reads was performed at 97% identity using CD-HIT (W. Li & Godzik, 2006). Clustered reads were aligned against an *rbcL* database developed by Bell et al. (2017) using BWA MEM for alignment (H. Li & Durbin, 2009). Alignments were parsed with SAMtools (H. Li et al., 2009), and converted to BLAST b6 format. Custom Python scripting was used to combine individual results files into a merged table for statistical analysis at the genus level.

The sequence reads for plant genera in each sample were converted to proportions for simple downstream analysis. DNA metabarcoding approaches are not an established method for quantification of plant taxa of DNA due to the high potential for bias in amplification of different plant taxa and variations in plastid copy number, as well as bias in DNA extraction, PCR, and sequencing (Hawkins et al., 2015; Kraaijeveld et al., 2015). I used proportion of DNA sequences in each sample as a method of analysis but chose to take a more conservative approach by only using presence to calculate plant richness. As a denoising step, all sequences totaling less than 0.1% of all sequences (total sequences per year) were removed prior to analysis. Subsequently, all sequences totaling less than 0.1% of individual sample sites (all sequences per sample) were also removed prior to analysis.

Protein and Lipid Analysis

All pollen samples were submitted to the University of Washington Northwest Metabolomics Research Center, Seattle, Washington for protein and lipid analysis. Protein quantification was performed using the BCA protein assay (ThermoFisher Scientific, Waltham, MA). Four to five randomly selected pollen provisions from the middle of two to three randomly selected nesting tubes were taken for each unique combination of site, foraging period, and year. Randomly selected pollen provisions from each unique combination were homogenized using a micropestle, from which a 10mg pollen sample was taken to quantify protein concentration (μg/mg).

Lipid analysis was performed using liquid chromatography-mass spectrometry (LC-MS), an analytical chemistry technique capable of quantifying over 1000 lipids into 13 individual classes. The lipid classes are listed in Table 1. Because there was no prior research on the effect of individual lipid classes on solitary bee development, I considered the role of each of the 13 lipid classes on *O. lignaria* development in subsequent analyses. Three randomly selected pollen provisions from the middle of two to three randomly selected nesting tubes for each unique combination of site, foraging period, and year were taken. Pollen provisions from each unique combination were homogenized using a micropestle, from which a 10mg pollen sample was taken to quantify the concentration (μM/L) for each of the 13 lipid classes. LC-MS was performed on a Lipidyzer platform (Sciex, Framingham, MA), an established platform for identifying lipid profiles from tissue samples (Cao et al., 2020). Total lipid concentration for each unique combination of site, foraging period, and year was calculated by taking the sum of the 13 lipid class concentrations.

Statistical analyses

For *O. lignaria* developmental metrics, I considered the number of eggs oviposited, developmental success of eggs to the late instar or pupal stage, and adult emergence success at each site, foraging period, and year as response variables. All three response variables were transformed to meet the assumptions of normality. The number of eggs was transformed using $\log_{10}$. Developmental success to the late instar/pupal stage was considered as the proportion of eggs that successfully developed to the late instar/pupal stage. Adult emergence was calculated as the proportion of *O. lignaria* that emerged from overwintering pupae in the following spring. For the proportions, a value of 0.001 was added to each (due to the presence of 0 proportions), and a linearizing transformation was applied to the proportions, ln(proportion/(1-proportion)). I removed data on larval development success and adult emergence when these values were calculated from single digit counts of eggs to avoid bias in assessing development success. Therefore, proportional data on larval development success and adult emergence from sites with <10 eggs were excluded from analyses. Lipid and protein concentrations were transformed using $\log_{10}$ to meet the assumptions of normality when used as response variable. I also used transformed values of lipid and protein concentrations when they were used as predictor variables due to differences in the range of their respective values.

A linear mixed-effects model was used to examine the main effects of foraging period, protein concentration, and total lipid concentration, and interactions between foraging period and protein concentration, foraging period and total lipid concentration, and protein concentration and total lipid concentration, on each *O. lignaria* developmental metric. I also used a linear mixed-effects model to examine the main effects of plant richness and foraging period, and their interaction, on total lipid concentration and protein concentration. Finally, I used a linear mixed-effects model to examine the effect of each individual lipid class concentration on each *O. lignaria* developmental metric. Site was included as a random effect in all linear mixed-effect models. Some sites were grouped due to their close proximity (< 600 m apart, the flight distance of *O. lignaria*). This resulted in 5 groups for the 12 sites used in 2018, and 16 groups for the 22 sites used in 2019 and 2020. Initially, year was included as a random effect, but because its inclusion or absence did not alter the results, year was excluded as a random effect for parsimony. Analyses were conducted using lme4 (Bates et al., 2015) and lmerTest (Kuznetsova et al., 2019) in R (R Core Team 2020).

Results

The main effects of total lipid concentration, protein concentration, and their interaction were nearly significant in predicting *O. lignaria* oviposition. Total lipid concentration was nearly significant in predicting lower oviposition (*P*=0.07), while protein concentration and the interaction between lipid and protein concentrations were nearly significant in predicting increased oviposition (*P*=0.06) (Table 2). Protein concentration was not significant in predicting larval developmental success or adult emergence. However, the interaction between lipid concentration and foraging period was significant in predicting larval developmental success and adult emergence success (Table 2). Lipid concentrations were high in both foraging periods, and were associated with a higher rates of larval developmental success (Fig. 1A) and greater adult emergence success (Fig. 1B). Foraging period was also a significant predictor of adult emergence success (Table 2), with greater adult emergence observed in the first foraging period.

Plant richness was not a significant predictor of total lipid concentration or protein concentration (Table 3). Foraging period was not a significant predictor of protein concentration; however, foraging period did predict lipid concentration, with higher lipid concentrations observed in the second foraging period (Table 3, Fig 2). The results indicate that foraging bees accumulated higher lipid concentration in pollen during the second foraging period. The interaction between plant richness and foraging period was not significant. The mean (±SD) plant richness was 12.06 (±7.80) and 11.64 (±7.28) in the first and second foraging period, respectively.

A few lipid class concentrations were significantly associated with *O. lignaria* developmental success. Higher concentrations of hexosylceramide (HCER) were associated with lower oviposition (Table 4). No other lipid class concentrations were significantly associated with oviposition. Higher concentrations of HCER and cholesterol ester (CE) were significantly associated with increased larval developmental success (Table 4). No other lipid class concentrations were significantly associated with larval developmental success or adult emergence.

Variation in the concentration of each of the 13 measured lipid classes is shown using heatmaps in Figure 3. Overall, I observed high levels of CER and TAG in 2019, and high levels of DAG and TAG in 2018 (Fig. 3A). Pollen collected during the second foraging period tended to have higher lipid levels, specifically in CER, DCER, HCER, and PE. Pollen collected during the first foraging period had relatively consistent levels of lipids across all lipid classes, but with lower levels of LCER (Fig. 3B). When considering site classifications, each site classification had higher levels of some lipids than others, except in the case of urban farms, which tended to have the lowest overall lipid levels for all classes (Fig. 3C). Restoration sites had the highest levels of HCER, while working farms had the highest levels of FFA and high DCER and HCER; city parks showed high levels of PC, PE, and SM, while rural parks had higher levels of LPC and LPE lipid classes. Collectively, all sites classified as rural tended to have higher levels of DCER, FFA, and HCER lipid classes than sites classified as urban (Fig. 3D).

Discussion

The primary pathway for bees to obtain protein and lipids required for growth is to acquire these nutrients from pollen and nectar, an evolutionary development separating bees from wasps (Cane, 2016; Roulston & Cane, 2002). Female bees require large amounts of energy for foraging and oviposition, energy which is derived from pollen and nectar. Female *O. lignaria* can oviposit up to 30 eggs per day (Phillips and Klostermeyer, 1978), which requires large amounts of time to be spent foraging for resources. Upon hatching, neonates consume the pollen available to them while developing to the pupal stage. The process of larval molting was once thought to be cued primarily by a critical weight, but recent research has demonstrated that starvation cues are also an important signal for metamorphosis in *O. lignaria* (Helm et al., 2017). For these reasons, the nutritional content of the pollen collected by foraging mothers and fed to developing offspring is extremely important in the success of solitary bees. Plants vary in the nutrient content they offer to bees, potentially affecting the number of plants foraging bees must visit to satisfy their nutrient requirements (Vaudo et al., 2015; Yeamans et al., 2014).

In terms of oviposition success, the nearly significant linkages of protein, lipids, and their interaction seemed to be at odds with their known roles in successful bee development. While foraging mothers consume small amounts of pollen during foraging (Cane et al. 2017), it is unknown whether female bees can determine the quality of pollen, or the role consumed pollen may play in oviposition. In this study, *O. lignaria* developmental success from the egg to the late instar or pupal stage was not associated with increased protein concentration in pollen (Table 2). Total lipids appeared to be important to development, but were linked to foraging period (Table 2, Fig. 1A). Given the importance of these nutrients in bee development, it is assumed that more nutritious pollen would result in increased developmental success. However, bees may be able to accumulate the necessary concentrations of proteins and lipids from available plants, regardless of the plants they are visiting during the period in which they are active. *Osmia lignaria* forage in diverse landscapes, particularly in urban and suburban areas that are both fragmented due to human development and comprised of a large diversity of plant species due to ornamental plantings, restoration projects, and urban parks. Bees must select pollen from those flowers that best support offspring development and from a larger availability of floral resources. In this study, the interaction between lipid concentration and foraging period was significant in predicting increased larval developmental success and subsequent adult emergence success. Later foraging periods hosted a higher number of flowering plants and higher accumulation of lipids by foraging bees, even though plant richness did not differ between foraging periods. Late-blooming floral resources potentially provide more opportunities for bees to collect pollen with higher lipid concentration, which provide the bees offspring with the lipid levels necessary for successful development. The results suggest that *O. lignaria* are able to successfully find floral resources that support offspring development across both foraging periods, and that these resources might not be a limiting factor in urban or rural environments in western Washington.

Among 13 individual lipid class concentrations, only two were associated with increased successful larval development (Table 4). Studies on the roles that individual lipid classes play in solitary bee development are absent from the literature, though previous studies have been conducted in other insect species. For example, free fatty acids were reported to be essential as a source of energy and precursors for secondary metabolites, such as pheromones and maintaining homeostasis, in the development of the flesh-fly, *Sarcophaga (Liopygia) argyrostoma* Robineau-Desvoidy (Diptera: Sarcophagidae) (Kaczmarek et al., 2020; Manning, 2001). In honey bees, fatty acid profiles are associated with inhibiting bacterial microbes in brood combs (Manning, 2015). As part of nesting behavior, *O. lignaria* females have been known to drag their abdomen along nesting segments, depositing chemicals to aid in nest recognition; such chemicals may arise from cuticular lipids in nesting females (Guédot et al., 2006; Pitts-Singer et al., 2012). To my knowledge, the precise role of hexosylceramide or cholesterol ester in *O. lignaria* development is not known; however, esters have been associated with *Colletes* spp. (Hymenoptera: Colletidae), a ground nesting solitary bee, in construction and coating to waterproof brood cells (Hefetz et al., 1979). In this study, increases in hexosylceramide (HCER) was associated with reduced oviposition (Table 4); however, it is difficult to ascertain the biological significance of this finding given the lack of knowledge on the roles individual lipid classes play in bee development. HCER was significant in predicting increased larval development success (Table 4), suggesting this lipid is connected to development inside the nest. Additional studies are needed to better understand the role of lipid classes in the development of solitary bees.

Presence of various lipid classes varied by year, foraging period, among urban and rural sites, and site classification (Fig. 3). The presence of specific lipid classes was lowest in 2019 and highest in 2018 and 2020 (Fig. 3A). Higher lipid class levels were present in the second foraging period across all years, and stayed relatively constant in the first foraging period. Interestingly, the second foraging period was considered as the ‘asynchronous’ foraging period for *O. lignaria*, even though nutritional quality increased during this period without an increase in the number of plant genera identified in pollen. The 'synchronous' foraging period could be due to commercial breeding and selection in *O. lignaria* to be timed with agricultural uses in early spring, such as for fruit tree pollination (Bosch et al., 2006; Torchio, 1976). However, later emergence may be more beneficial to *O. lignaria*, as well as other solitary bees, given the potential for higher nutritional quality of pollen. Across different site classifications, rural sites and working farms generally had the highest numbers of lipid classes, with urban farms hosting the lowest number of lipid levels (Fig. 3C). The lipid class levels in urban farms are interesting considering the use of *O. lignaria* to supplement community farm pollination in western Washington even though these sites do not seem to be the most beneficial for *O. lignaria* success. High lipid class concentrations in restoration sites also signal a potentially positive impact for other native pollinators with lipid concentration requirements. Lastly, even though rural sites have fewer plant species than urban areas, considering the number of ornamental plantings in urban sites, rural sites tended to contain plants with higher lipid resources (Fig. 3D).

Protein provides the building blocks of energy for insect development. Lipids provide storage for these resources, ensuring insects can survive periods of dormancy including diapause. Further investigation of the role of individual lipid classes in developmental success of solitary bees and the levels at which they are required for reproductive success is important in understanding how landscape changes, including the presence or absence of specific floral species, will affect solitary bees and better inform conservation efforts. Considering the lack of direct connections between nutritional content and *O. lignaria* developmental success found in this study, the highly diverse plantings established as part of pollinator conservation efforts in restoration sites, city parks, or hedgerows may already be providing the necessary resources for solitary bees. Focused conservation efforts could benefit from planting more floral resources with high levels of cholesterol ester and hexosylceramide in their pollen, but further analysis of the exact role of these lipid classes in solitary bee development are necessary. Finally, the relationship between foraging period and lipid concentration suggests that plants flowering in early summer provide more nutrients to pollinators than those in the early spring. Focusing on the planting of highly nutritious pollen-producing plants during early spring would benefit spring-emerging pollinators.

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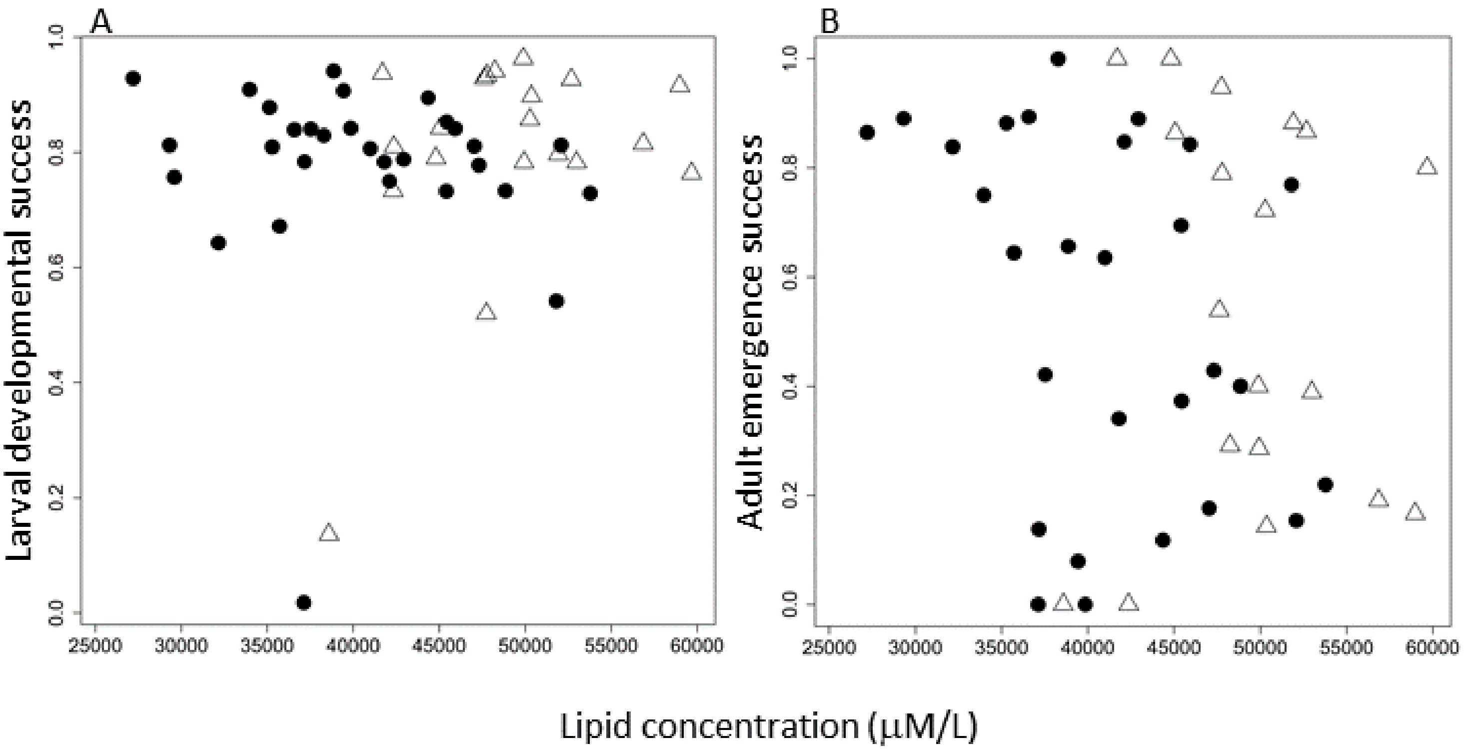

Figure 1. Lipid concentration by larval developmental success (A) and adult emergence success (B). Symbols represent a single site between 2019-2020 during the first foraging period (closed circles) and second foraging period (open triangles).

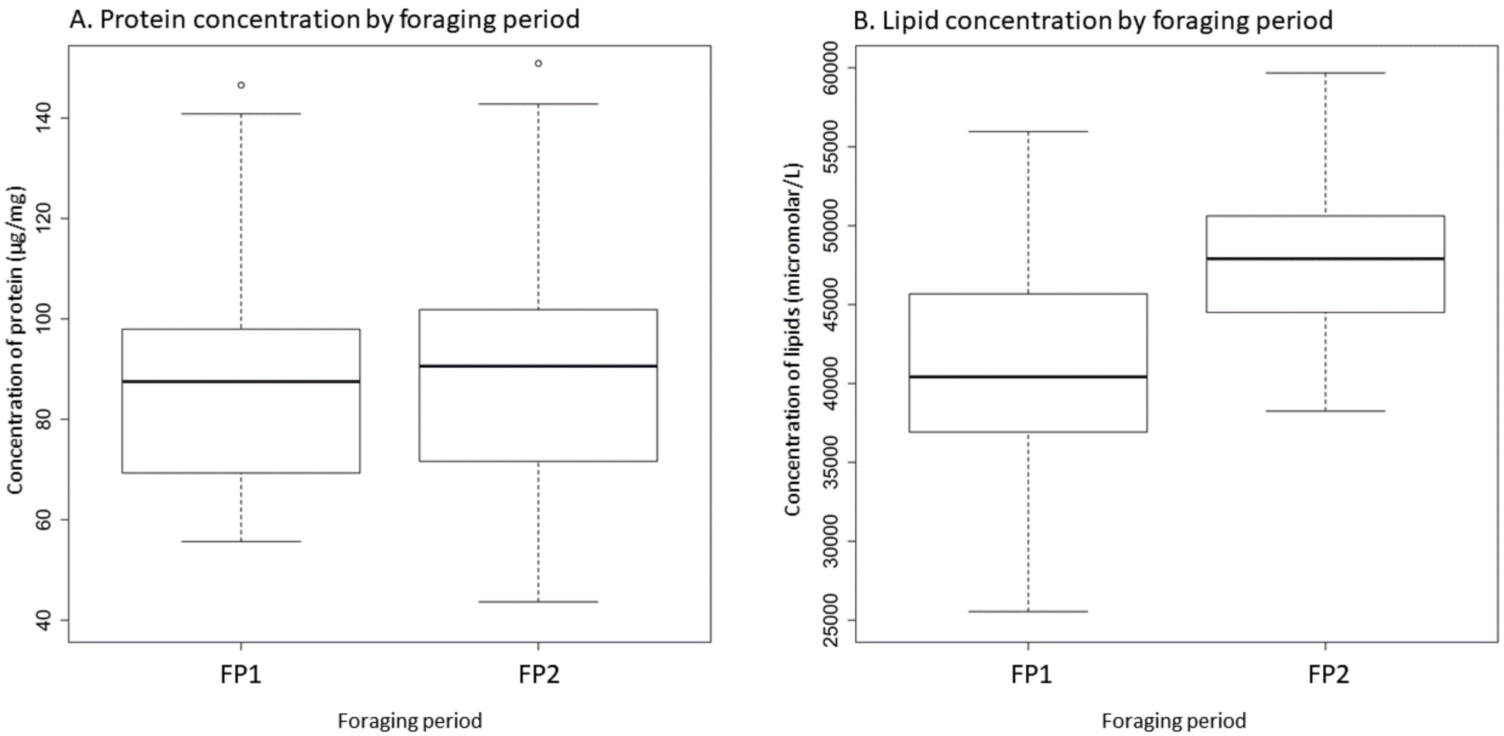

Figure 2. Concentration of protein (A) and lipids (B) in pollen during the first (FP1) and second (FP2) foraging periods, 2018-2020.

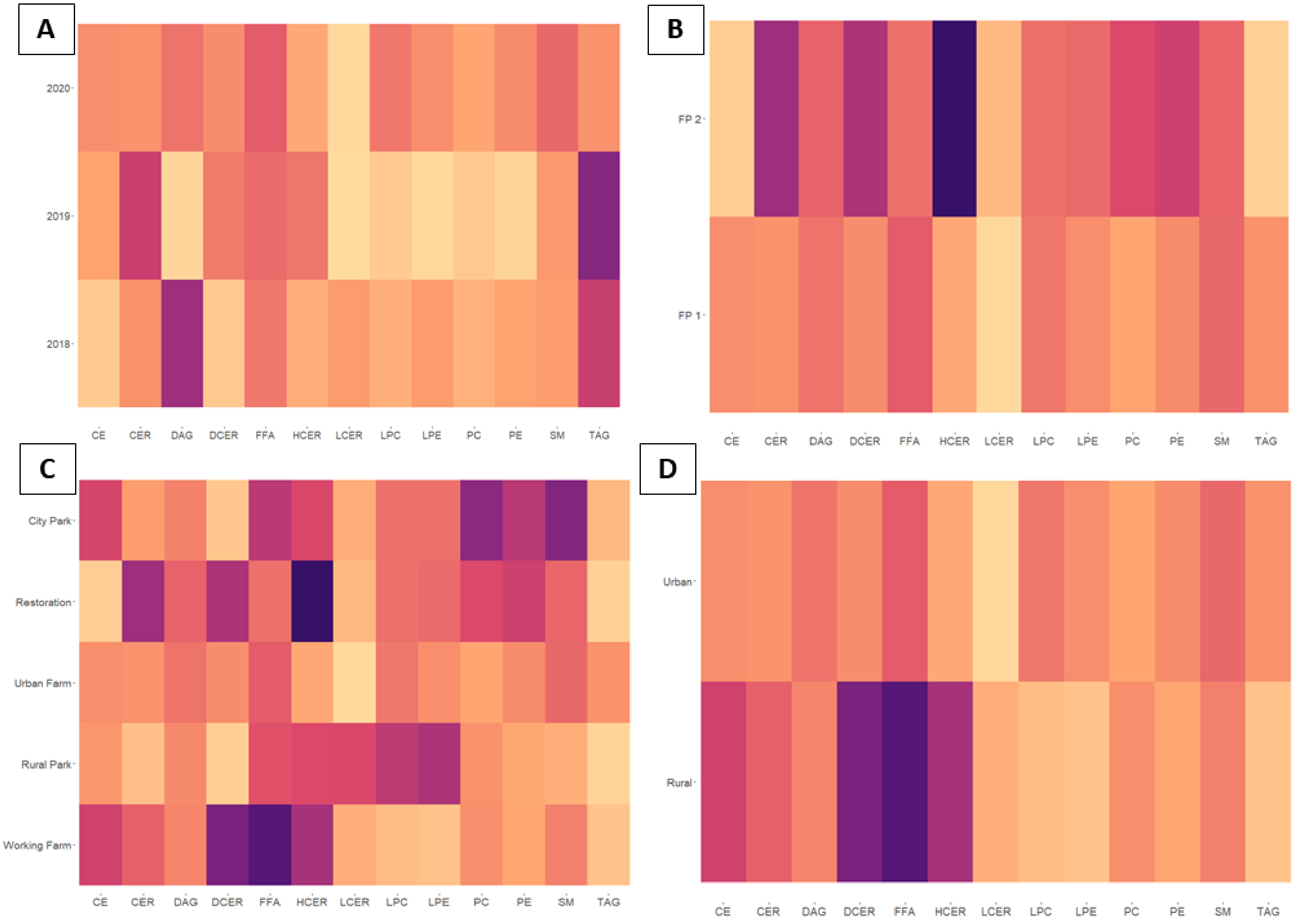

Figure 3. Heatmaps of the presence of each of the 13 lipid classes (cf. Table 1) by (A) years studied, 2018-2020; (B) first and second foraging periods; (C) site classification into five categories, including city parks located within the city limits of Seattle, WA, sites actively undergoing intensive restoration, urban farms including community gardens, educational gardens, and public farming plots; parks located outside the city limits of Seattle, WA in King County, WA; sites classified as urban, located in developed areas, and rural, outside developed areas; sites located on rural agricultural farms; and (D) site classification into two categories, urban developed area in the city limits of Seattle, WA, and rural areas outside city limits. Lipid class data were normalized by row and column for visual comparisons. Darker colors indicate higher occurrence of a lipid class concentration; lighter coloration indicates lower occurrence.

Table 1. Lipid class and code identifiers quantified using liquid chromatography-mass spectrometry (LC-MS)

Code	**Lipid Class**
CE	Cholesterol Ester
CER	Ceramide
DAG	Diacylglycerol
DCER	Dihydroceramide
FFA	Free Fatty Acid
HCER	Hexosylceramide
LCER	Lactosylceramide
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
SM	Sphingomyelin
TAG	Triacylglycerol

Table 2. Effects of lipid concentration (mM/L), protein concentration (mg/mg), foraging period, and interactions on *O. lignaria* oviposition, larval developmental success, and adult emergence success. Values in bold indicate significance ($P \leq 0.05$).

Predictor variable	**Oviposition**	**Larval developmental success**	**Adult emergence**
Foraging period (FP)	*t*=0.25; df=48; *P*=0.80	*t*=-1.81; df=43; *P*=0.08	***t*=-2.62; df=37; *P*=0.01**
Lipid (L)	*t*=-1.89; df=44; *P*=0.07	*t*=-0.07; df=43; *P*=0.94	*t*=-0.29; df=37; *P*=0.77
Protein (P)	*t*=-1.94; df=44; *P*=0.06	*t*=-0.07; df=43; *P*=0.95	*t*=-0.24; df=37; *P*=0.81
L × FP	*t*=-0.18; df=49; *P*=0.86	***t*=2.03; df=43; *P*=0.05**	***t*=2.83; df=37; *P*=0.01**
P × FP	*t*=-0.44; df=46; *P*=0.66	*t*=-0.86; df=43; *P*=0.40	*t*=-1.15; df=37; *P*=0.26
L × P	*t*=1.93; df=44; *P*=0.06	*t*=0.06; df=43; *P*=0.95	*t*=0.24; df=37; *P*=0.81

Table 3. Effect of plant richness and foraging period, and their interaction, on total lipid concentration and protein concentration. Values in bold indicate significance ($P \leq 0.05$).

Predictor variable	**Lipid concentration**	**Protein concentration**
Plant richness (PR)	t=-0.41; df=50; P=0.68	t=-0.26; df=57; P=0.80
Foraging period (FP)	**t=2.51; df=52; P=0.02**	t=0.58; df=57; P=0.56
PR × FP	t=0.56; df=51; P=0.58	t=0.53; df=57; P=0.60

Table 4. Effect of individual lipid classes (cf. Table 1) on *O. lignaria* oviposition, larval developmental success, and adult emergence. Values in bold indicate significance (*P* ≤0.05).

Predictor variable	**Oviposition**	**Larval developmental success**	**Adult emergence**
FFA	*t*=-1.50; df=54; *P*=0.14	*t*=0.72; df=48; *P*=0.48	*t*=-0.39; df=42; *P*=0.70
HCER	***t*=-2.84; df=44; *P*=0.01**	***t*=1.87; df=41; *P*=0.05**	*t*=1.56; df=36; *P*=0.13
CE	*t*=-1.41; df=50; *P*=0.17	***t*=2.31; df=48; *P*=0.03**	*t*=0.85; df=42; *P*=0.40
CER	*t*=-0.32; df=58; *P*=0.75	*t*=-0.09; df=47; *P*=0.93	*t*=0.27; df=41; *P*=0.79
DAG	*t*=-1.86; df=58; *P*=0.07	*t*=-0.05; df=48; *P*=0.96	*t*=-1.23; df=42; *P*=0.23
LCER	*t*=-0.44; df=46; *P*=0.66	*t*=-0.58; df=36; *P*=0.57	*t*=0.61; df=40; *P*=0.55
DCER	*t*=-0.05; df=24; *P*=0.96	*t*=1.50; df=21; *P*=0.16	*t*=-0.82; df=18; *P*=0.42
LPC	*t*=-0.55; df=55; *P*=0.58	*t*=-0.94; df=48; *P*=0.35	*t*=0.25; df=42; *P*=0.81
LPE	*t*=0.42; df=52; *P*=0.68	*t*=-0.82; df=48; *P*=0.41	*t*=0.15; df=42; *P*=0.88
PC	*t*=-0.46; df=54; *P*=0.65	*t*=0.14; df=48; *P*=0.89	*t*=0.44; df=42; *P*=0.67
PE	*t*=0.16; df=57; *P*=0.87	*t*=-0.37; df=48; *P*=0.71	*t*=-0.01; df=42; *P*=0.99
SM	*t*=-0.98; df=55; *P*=0.33	*t*=-0.09; df=48; *P*=0.93	*t*=-0.28; df=42; *P*=0.78
TAG	*t*=1.39; df=47; *P*=0.17	*t*=0.78; df=48; *P*=0.44	*t*=0.81 ; df=42; *P*=0.42

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Chapter 3: Symbiont associations with native solitary bees: an exploratory analysis of fungi and bacteria in bee-collected pollen provisions

Abstract

Symbionts such as bacteria and fungi are an important part of the ecosystem and exist on and in almost every living (and non-living) component of the environment. Symbionts are linked to the physiological development of many insects, and in some cases thought to be a defense system against parasites and diseases. Current symbiont associations in solitary bees are not well known, especially in areas of the Pacific Northwest region. I collected pollen provisions from the native spring-foraging solitary bee, *Osmia lignaria* Say, across two years and two foraging periods per year at 22 sites in and around Seattle, Washington. Using Next-Generation Sequencing to identify bacterial classes and fungal genera within the provisions, I found novel connections between bee foraging activity and bacteria and fungi, including the presence of potentially harmful fungi. Relatively few overall bacterial sequences in DNA analyses were detected, and bacterial richness accumulated by bees increased during the later foraging period. Fungal sequencing identified *Ascosphaera* spp. in solitary bee nests, many species of which have known adverse effects on honey bees. Fungal richness decreased with increasing plant richness in bee-collected pollen provisions; an unexpected result given the conducive environment for fungi in a temperature region. This study provides new information on symbionts present in urban and rural areas of the Pacific Northwest, and their potential connections to foraging pollinators.

Introduction

In recent decades, the role of microorganisms in affecting the health of numerous species, from ants to humans, has become an engaging area of study due to the increased availability of molecular tools to ascertain these relationships (Jackson et al., 2002). In humans, microbiome bacteria are a topic of extensive research and economic value, with scientific discoveries linking the presence or absence of certain bacterial clades with the presence of diseases such as cancer, psoriasis, and cirrhosis (Chen et al., 2011; Gao et al., 2008; Plottel & Blaser, 2011). In insect species, microbial symbionts have been identified as playing key roles in development and survival, and are linked to population success (Engel & Moran, 2013; Moran, 2006). For example, aphids transmit the bacteria *Buchnera aphidicola* vertically from mother to offspring, which provides necessary amino acids to developing nymphs (Douglas, 1998). Termites harbor a diverse community of microbes that break down cellulose, fix nitrogen, and degrade uric acid in the hindgut (Mikaelyan et al., 2015; Santana et al., 2015). In mosquito species a microbial symbiont affects reproduction and survival, allowing microbial manipulation to mediate the transmission of diseases such as malaria to humans (Thongsripong et al., 2018).

The connection between symbiotic microbes and pollinator health has also received much attention, especially in the European honey bee, *Apis mellifera* L., in response to Colony Collapse Disorder (Cox-Foster et al., 2007). In bees, foraging females collect pollen and nectar during the flowering season to bring back to their offspring. Symbionts such as bacteria and fungi are naturally occurring on flowering plants, particularly on the surface of flowers (Junker et al., 2011). These microorganisms could be naturally occurring on flowers, or deposited by pollinators and other insect species that visit the flower (Belisle et al., 2012; Thomson, 1992). Because social and solitary bees visit a plethora of flowering plant species during their foraging periods, they create a complex network of visitations and consequently facilitate the movement of microorganisms among flowers (Crowley-Gall et al., 2021).

Bacteria associated with the health of social and eusocial bee species has been found to be consistently linked across seasons and geographic areas, which could suggest that there is a core microbiome distinct across time and space (Martinson et al., 2011). Moreover, the presence of bacterial diversity has been linked to the health and survival in several bee species. For example, beneficial gut bacteria in bumble bees have been shown to reduce the infection rate and presence of the gut parasite *Crithidia bombi* (Koch & Schmid-Hempel, 2011). Stress tolerance, disease resistance, and stronger immunity to agrochemicals and parasites have been associated with the presence of certain microbiota in honey bees (Daisley, Chmiel, et al., 2020). Lactic acid bacteria collected from flowers have been identified as playing a mutualistic role in honey bee nutrition and health, dominating the crop of bees and providing fermentation for food resources (Olofsson & Vásquez, 2008; Vásquez et al., 2012).

Unlike social and eusocial bee species, solitary bees lack the horizontal transfer of symbionts within a social colony and essentially, must accumulate microbiota from their environment. Soil-dwelling bees are known to be exposed to bacteria and fungi in soil (Janzen, 1977), and several species provide brood care to their offspring similar to social bees, transferring microbiota through mouth contact (Kaltenpoth et al., 2010). Some cavity nesting species line their nests with secretions of bacteria that are then acquired by developing larvae through pollen consumption, and subsequently accumulated in pupae through larvae spinning (Kaltenpoth et al., 2010). The size and composition of bacterial communities can be linked to the developmental stage of solitary bees, indicating changes in microbial community gut structure as bee larvae develop into adults (Mcfrederick et al., 2014). Microbes also play a role in comprising the diet of solitary bees, not just as an accidental hitchhiker transported from flower to bee bread, but as a source of nutrients for offspring. In a study of the microbes associated with the solitary bee *Osmia cornuta* (Latreille), several microbial species were identified in pollen and nectar provisions of developing larvae (Lozo et al., 2015). Diet manipulation experiments have shown microbial presence to be a vital source of amino acids and fatty acids for developing larvae (Dharampal et al., 2019). However, the depth of information regarding the connection between specific bacteria and solitary bee health is limited, and further research is necessary to ascertain the role of important bacterial clades in the health and fitness of solitary bee species.

Bees also acquire fungi during flower visitations and accumulate fungal communities in pollen and nectar provisions fed to larvae. Fungi, which are preferential to warm, wet conditions, benefit from the environments of the hive or solitary nest, and many fungi grow on pollen (Kačániová et al., 2011). Fungi have been observed in the nests of honey bees, bumble bees, and solitary bee species (Dharampal et al., 2020; Disayathanoowat et al., 2020; Menezes et al., 2015). In addition to the potential benefits of mutualistic fungi to developing larvae, some fungal species are known to be pathogenic to bees. For example, *Ascosphaera apis* causes chalkbrood in honey bee colonies, which can cause stress and exacerbate mortality (Evison, 2015). Stonebrood is a fungal disease caused by several species of *Aspergillus*, and can detrimentally affect honey bees colonies, and can even be transferred to humans (Gilliam et al., 2007). Honey bee diseases are also linked to fungi, such as the microsporidia *Nosema apis* and *Nosema ceranae*, which are parasites that live in the digestive tract of honey bees and reduces the ability of bees to obtain nutrients and decreases their lifespan (Rubanov et al., 2019). Solitary bees raised in groups, such as the alfalfa leafcutter bee *Megachile rotundata* (Fabricius), are also known to carry and suffer the adverse effects of fungal diseases; populations of *M. rotundata* are known to be destroyed by an outbreak of chalkbrood caused by *Ascosphaera aggregata* (Pitts-Singer & Cane, 2011). Although fungi are considered to be widespread in pollen and nectar provisions, and among immatures in both solitary and social bees, fungi are not considered a main source of mortality in wild bees (Minckley & Danforth, 2019).

Past research on the potential benefits of bacteria and fungi in solitary wild bees is limited as most prior work on bee-symbiont interactions have focused on honey bees given their economic importance (Engel et al., 2014; Kwong et al., 2014; Leonard et al., 2020). Past work has reported on the benefit of certain mycelium fungi to bees in stressed situations such as exposure to pesticides and other chemicals (Stamets et al., 2018). Fungi are often a footnote in studies identifying bacteria in bee larvae or pollen provisions, and rarely linked to foraging behavior, geographic location, or survival. In this study, I used a field-based approach to collect pollen from the solitary cavity-nesting bee *Osmia lignaria* Say, and Next Generation Sequencing to identify the presence of bacteria and fungi in provisions of developing larvae, to ascertain relationships between plant richness and bacterial and fungal presence, and ascertain the effect of bacterial and fungal presence and richness on *O. lignaria* developmental success.

Materials and Methods

Field sites and Experimental set up

Sites were set up across a range of urban and rural landscapes in King County, Washington over two years, 2019-2020. Using information from a pilot study that sought to determine site characteristics conducive to *O. lignaria* nesting success (Chapter 1), I established 22 sites for use in both years. At each site, I deployed two bee houses constructed from 7.5 cm diameter PVC pipe, each containing 40 paper tubes, each being 8mm in diameter. Bee houses faced southeast to capture adequate sunlight for bee nesting success. Paper tubes were purchased from a local supplier (Crown Bees, Woodinville, WA; Watts Solitary Bees, Bothell, WA) and were handled with gloves. One hundred locally purchased *O. lignaria* overwintering pupae (Watts Solitary Bees, Bothell, WA) were placed at each site in emergence boxes attached to bee houses over two foraging periods in each year. The foraging periods used for this study are described in Chapters 1 and 2. Briefly, the first foraging period corresponded to the natural emergence of adult *O. lignaria* in western Washington (~10 °C), which typically occurs in late March to early April. Bees were allowed to forage for six weeks once temperatures had reached a minimum of 10 °C, after which remaining pupae and nesting reeds were removed. I then placed new nesting tubes and 100 additional *O. lignaria* pupae in new emergence boxes. Bees were then allowed to forage for an additional six weeks, after which nesting tubes were removed (~late July). Nesting tubes from each site were randomly placed into one of two groups in approximate equal numbers. One group was used to measure larval development and adult emergence, and one group was used for genetic sequencing to identify bacteria and fungi in pollen provisions. For nesting tubes used to measure the presence of bacterial and fungi, the tubes were dissected using sterile tools, and pollen was placed into sterile storage vials and maintained at -20°C until processed. Nesting tubes used to quantify developmental and emergence success were placed in Percival Scientific (Perry, Iowa) biological incubators under conditions that simulated average temperatures in King County, WA based on the recent 10 years (Chapter 1). For each week, temperatures in the incubators were set to fluctuate between the average high and average low for the relevant week, and set to reflect the photoperiod for the week. The biological incubators were adjusted from mid-May to March-to-April of the following spring.

Larval Development and Emergence Success

Nesting tubes selected for quantifying developmental and emergence success were removed from biological incubators in late fall, at which point most offspring had reached the pupal stage (~late October to early November). Nesting tubes were opened to count the number of larvae that successfully developed to the late instar (5th-6th) or pupal stage. Pupae were washed in lukewarm water to remove frass and other debris. Pupae were then allowed to air-dry, and placed in the biological incubators under conditions described above. The following spring, bees were allowed to emerge over 6 weeks. The total number of successfully emerged bees was counted along with number of pupae that failed to emerge. Successful adult emergence for each site, foraging period, and year was defined as the number of pupae from the previous year that successfully emerged in the following spring.

Pollen removal and DNA extraction for plant DNA

To ascertain plant richness at each site, I sequenced pollen DNA to identify plants visited by foraging bees; full details are presented in Chapter 1. Briefly, pollen samples were lysed with 5mm stainless steel beads and 180mL ATL buffer (Qiagen, Germantown, Maryland) on a Qiagen Tissuelyzer for 3 min. at 30hz. I added 20µl of proteinase K, mixed thoroughly, and placed on a heat block at 57°C overnight, then added 4µl of RNase A. I then used a modified protocol from McFrederick and Rehan (2016) and DNeasy Blood and Tissue kit (Qiagen, Germantown, Maryland) for DNA extraction. Final elution totaled 100µl. Following DNA extraction, sub-samples from a single site were combined prior to sequencing submission. In sequencing, the *rbcL* region as used given its success in identification of plants to genus (De Vere et al., 2017; Hawkins et al., 2015) and robust reference library (Bell et al., 2017). PCR testing was performed on 10 randomly selected samples and primers prior to sequencing submission to ensure DNA was of high quality. Primers and adapters used were taken from De Vere et al. (2012). Samples were submitted to the Integrated Microbiome Resource Center (IMR) at Dalhousie University (Halifax, Nova Scotia) for library preparation and sequencing on Illumina MiSeq 2x300bp technology.

DNA extraction and sequencing to detect bacteria and fungi

For bacterial and fungal sequencing, I randomly selected four to five pollen provisions from the center of nesting tubes. I also randomly selected four pollen provisions from the front of the nesting tube, which faced outward in the nest, and four pollen provisions from the back of the nesting tube, which were the inward most provisions from the entry point. Because females fill nest tubes from back to front, using pollen from the back, center, and front sections of the nesting tubes helped to ensure an adequate representation of bacterial and fungi throughout the foraging period. Each group of pollen provisions (i.e., from the front, middle, and back of nesting tubes) from each unique combination of site, foraging period, and year were homogenized using a micropestle, from which two sub-samples were taken for DNA analysis.

To thoroughly lyse pollen cells following sub-sampling, I added 180mL of ATL buffer (Qiagen, Valencia, CA) and a 5mm stainless steel bead and 25mg of each pollen sample, including a blank sample in each pretreatment and subsequent extraction. Samples were lysed for 3 minutes at 30hz using a Qiagen Tissuelyzer, then 20µl of proteinase K was added and vortexed to mix thoroughly. Samples were placed in a heat block at 57°C overnight. The next morning, 4µl of RNase A was added to each sample, and I followed the manufacturer's instructions for the remainder of the DNeasy Blood and Tissue Kit (Qiagen), excluding the second spin column step. For the final elution, a total of 100µl AE buffer was pipetted directly onto the spin column membrane and incubated at room temperature for 15 minutes. In total, 264 microbial DNA extractions were performed each year, encompassing both foraging periods. Front, middle, and back provisions taken from the nesting tubes were pooled separately, and sequenced separately for each site.

Following DNA extraction, samples were tested for DNA quality using PCR and gel electrophoresis. PCR protocol followed McFrederick and Rehan (2016), and gel electrophoresis confirmed DNA quality was excellent to continue with sequencing. Samples were submitted to the Integrated Microbiome Resource center (IMR) at Dalhousie University in Halifax, Nova Scotia. All metabarcoding amplicon libraries for bacteria (16S rRNA gene [16S]) and fungi (the internal transcribed spacer [ITS2]) were prepared by IMR. Sequencing of all libraries was performed by IMR on the Illumina MiSeq using Illumina sequencing primers and V3 2x300 bp reagents.

Fungal sequenced samples in paired-end FASTQ format were processed using QIIME2 (Bolyen et al., 2019). QIIME2 was used to import the sequences in paired-end mode, demultiplex, perform QC using Deblur (Amir et al., 2017), and align against the taxonomic reference. Bacterial sequences were aligned to the GreenGenes 16S reference database, last updated April 2019, pulled from QIIME2's repository (DeSantis et al., 2006). Individual files were merged by QIIME2 and visualized using QIIME2's viewer utility for taxonomic bar plots. Taxonomy alignment data was exported from QIIME2 for additional analysis through R scripting. Quality control on sequences included removal of all bacterial samples below the 0.1% threshold, a previously accepted standard for bacterial data QC (Poretsky et al., 2014). The same denoising step was used on fungal sequences, removing all samples totaling <0.1% of all sequences prior to statistical analysis. The bioinformatic pipeline used in this study identified bacteria to genus, but due to numerous sequences without successful identification to genera, I chose to use bacteria data identified to the taxonomic level with the greatest level of identification success. Bacterial data were classified to class, and presence/absence of each class was used as a representative of bacterial richness. Fungal data were classified at the level of genus and presence/absence of each genus was used to represent fungi richness.

Statistics

Developmental success to the late instar or pupal stage, and adult emergence success, were considered as response variables. Developmental success to late instar/pupae was calculated as the proportion of eggs that successfully developed. Adult emergence was calculated as the proportion of adults that emerged from the late instar/pupal stage following overwintering. Both response variables were transformed to meet the assumptions of normality. A value of 0.001 was added to each proportion (due to the presence of 0 proportions), and a linearizing transformation was applied to the proportions, ln(proportion/(1-proportion)). Proportional data on larval development success and adult emergence from sites with <10 eggs were excluded from analyses as described in Chapter 1; across both 2019 and 2020, this included 2 sites in the first foraging period and 7 sites in the second foraging. When bacterial richness and fungal richness were used as response variables, they were transformed using $\log_{10}$.

I used a linear mixed-effects model to examine the effect of bacterial richness, fungal richness, foraging period, and all two-way interactions on both *O. lignaria* developmental metrics. I also used a linear mixed effects model to examine the main effects of plant richness and foraging period, and their interaction, on bacterial richness and fungal richness. Site was included as a random effect in both models. Certain sites, due to their proximity and the known flight distance of *O. lignaria* (< 600 m apart), were grouped, resulting in 15 groups for 2019 and 2020. Initially, year was included as a random effect, but because its inclusion or absence did not alter the results, year was excluded as a random effect for parsimony. Statistical analyses were conducted using lme4 (Bates et al., 2015) and lmerTest (Kuznetsova et al., 2019) in R (R Core Team 2020).

In addition, I used non-metric multidimensional scaling (NMDS) to visualize fungal DNA sequencing data and bacterial DNA sequencing data. Post-hoc visualization was performed when classifying sites by foraging period; when classifying sites into rural farms, urban farms (e.g., community gardens, urban gardening plots known locally as ‘P-patches,’ on-campus learning farms), restoration sites (e.g., areas where restoration projects were ongoing), and city parks; and when classifying sites broadly as either urban or rural. Fungal and bacterial data were standardized separately using the Jaccard index. I used the vegan package in R (Oksanen et al., 2020) to calculate distance matrices for each year and foraging period.

Results

Following bioinformatics pipeline analysis, 91% of the sequences from bacteria samples matched to chloroplast DNA across all samples and years. A total of 18 classes were identified in both foraging periods, and are summarized in Table 1. Originally, all bacterial sequences were matched to genera using an established bioinformatics pipeline. Of those bacterial sequences successfully matched to genera, I found multiple genera previously associated with non-*Apis* bee provisions, including *Lactobacillus*, *Saccharibacter*, *Fructobacillus*, and *Wolbachia*. However, each was identified to genus at low sequence counts (e.g., *Lactobacillus* sequence counts <0.1%), which suggests that their identification could be attributed to a sequencing error (Caporaso et al., 2010). Although their sequence counts were too low to meet quality control standards, they are listed in Table 2 due to known prior associations with bee health or microbiome. Overall, relatively few bacterial genera matches were successfully determined, and I chose to conduct subsequent analyses at the highest successful level of taxonomy, which was Class. All further analyses of bacterial richness and bee development focused on taxonomic identification at the Class level.

Several fungal genera were detected for each foraging period and year (Table 3 and Table 4). A total of 16 unique fungal genera were found across both foraging periods in 2019, including 14 genera from the first foraging period and 7 from the second foraging period. In 2020, a total of 27 fungal genera were identified from pollen across all sites, totaling 18 from the first foraging period and 14 from the second foraging period.

In analysis of bacterial and fungal richness on *O. lignaria* developmental metrics, no predictor variables were significant in explaining larval developmental success and adult emergence (Table 5). However, visually, sites with higher larval development success also had higher bacterial richness, particularly during the second foraging period (Fig. 1). Fungal richness was nearly significant in predicting decreased larval developmental success (*P*=0.06). No interactions were significant in predicting developmental metrics, though the interaction between fungal richness and bacterial richness was also nearly significant in predicting larval developmental success (*P*=0.08). The relationship between bacterial and fungal richness, and larval developmental success, is shown in Fig. 1.

Increased plant richness was significantly associated with a decrease in fungal richness, but plant richness was not significant in predicting bacterial richness (Table 6, Fig. 2A). Bacterial richness significantly increased during the second foraging period, while foraging period had no effect on fungal richness (Table 6, Fig. 2D). The interaction between plant richness and foraging period did not predict fungal or bacterial richness (Table 6).

In an NMDS exploratory examination of fungal data (dimensionality=2, stress=0.12), sites classified as city parks were slightly clustered, as were rural farms (Fig 3A). Fungi detected in the second foraging period were also slightly clustered, while those detected in the first foraging period were not. When examining fungal data in urban vs. rural sites, there were no apparent clusters (Fig 3B). Bacterial data (dimensionality=2, stress=0.17) among city parks were slightly clustered in the first foraging period, and more clustered in the second foraging period (Fig. 3C). Bacterial data from the second foraging period sites were also clustered, but not during the first foraging period (Fig. 3D).

Discussion

Bacteria and fungi have long been thought to have associations with insect health (Daisley, Pitek, et al., 2020; Graystock et al., 2017; Lozo et al., 2015). *Osmia lignaria* females are known to encounter >200 flowers per foraging bout from multiple plant species, with several foraging bouts per day (Cane, 2011). Mason bees construct their nests using soil, leading them to potentially interact with hundreds if not thousands of bacterial species throughout their life cycle in soil and on the surface of plants. However, a majority of the sequences found from sites were linked to plants (chloroplasts and mitochondria), and remaining bacterial sequences were quite low. Low bacteria identification cannot be linked to DNA extraction, PCR, or sequencing, considering the methods used in this study are well established as accurate qualitative measurements of bacteria presence, especially at higher taxonomic levels (Bartram et al., 2011; Caporaso et al., 2012). One possibility is that samples in this study may have lower bacterial presence than what was previously known in *O. lignaria* (Lozo et al., 2015; Voulgari-Kokota et al., 2018), widening the range of bacteria previously discovered in the nests of *O. lignaria*. In this study, nests of *O. lignaria* were temporary as nesting tubes were returned after 6 weeks given the experimental design. It is possible that this shorter temporal window, as opposed to leaving nesting tubes in the field, might have reduced bacteria development in the nesting tubes. Regardless of low sequence counts, and even when following quality control procedures, I did find a large number of bacterial classes in this study (Table 1), suggesting that *O. lignaria* still acquired a diverse population of bacteria.

Fungi were detected across a majority of study sites, and many were associated with plants, wood, and leaf litter (Table 4), which are commonly encountered during foraging. Despite detecting fungi across all sites and years, fungal richness at individual sites, foraging period, and year was relatively low in comparison to bacterial richness. This is potentially due to competition with other fungi and bacteria. Fungi associated with soil were also consistently detected by site, foraging period, and year, which is not surprising given that *O. lignaria* collects soil and mud during nesting construction (Pinilla-Gallego et al., 2018). Also, the area of western Washington where this study was conducted has a temperate climate with frequent precipitation, especially between November and April, which are environmental conditions conducive for fungi.

Fungal genera known to be causative agents of disease in bees were detected in 2019. One detected fungal genus that is of concern is *Ascosphaera*, and *A. apis* is the causative agent of chalkbrood in bees (Dharampal et al., 2020). There are several species of *Ascosphaera* that cause disease in bees (Dharampal et al., 2020), including honey bees (Evison, 2015), bumble bees, leafcutter bees (i.e., *A. aggregata* in *Megachile* spp., McFrederick et al., 2014), and solitary bees (Wynns et al., 2013). Some of the urban farm and restoration sites in this study were known to contain or within foraging distance of honey bee colonies. However, in sites where I detected *Ascosphaera*, there were no known honey bee colonies present. Many fungi identified from *O. lignaria*-collected pollen in this study had no known associations with not only *O. lignaria*, but also with other bee species (Table 4), which highlights the need for further research to identify bacterial and fungi to species level and examine their relationship to pollinator health. I also detected several genera, in both 2019 and 2020, known to contain species that are pathogens and are considered to be common molds (Table 4). The most common genera found in 2019, in terms of the number of sites where they were detected, were *Fusarium* and *Podosphaera*, which contain species known to be pathogenic to plants (Ma et al., 2013; Takamatsu et al., 2010), and *Hyaloscypha*, which contain species known to be saprotrophic (Huhtinen, 1989).

Relatively few overall bacterial sequences matched to bacterial classes, even when using established protocols and primers demonstrating previous success in environmental and pollen-based samples (Caporaso et al., 2012; Quinn S. McFrederick et al., 2017). Low sequence counts for bacteria using current protocols are rare in the literature. Of those bacterial classes identified, I observed increased bacterial richness during the second foraging period relative to the first foraging period (Fig. 2C). Bacteria found on plant surfaces and in pollen may develop in larger numbers at warmer temperatures, which would more likely occur during late spring and early summer, which corresponds to the second foraging period. Also, bacteria could be developing faster in the nests during the warmer second foraging period. Bacterial richness was not statistically associated with developmental success (Table 5); however, bacterial richness increased during the second foraging period along with larval development success, suggesting a more intricate relationship between the two variables. Wamer temperatures and faster bacterial growth could also mean the increase in potentially beneficial bacteria in the nest, contributing to increased developmental success later in the season.

Fungal richness was nearly significantly associated with lower bee development success (Table 3), which urges additional scientific exploration into this relationship. Fungal richness was also consistent across foraging periods (Fig. 2D). Plant richness had a statistically negative association with fungal richness (Table 6, Fig. 2B), suggesting that increased visitation to more plant genera reduces fungi in the nest. This would be beneficial if it reduces the number of pathogenic fungal species. Further research of the specific bacteria and fungi, and their potential role in bee development and success, is needed to better understand these relationships.

Studies of the associated between bacteria and fungi, and solitary bee health, such as the one presented in this chapter, give insight into their role on solitary bee nesting and development. Further studies of symbionts associated with solitary bees could inform conservation efforts. For example, different plants likely host different microbiota. Visitation to more plants would seem to allow bees to interact with a higher diversity of symbionts. While fungal richness played no role in larval development and adult emergence success in this study, certain fungi, such as *Ascosphaera*, are associated with negative effects on bee health (Evans & Schwarz, 2011; Le Conte, Yves, Ellis, Marion, Ritter, 2010; Pereira et al., 2019). Although the results of this study did not link bacterial or fungal symbionts with *O. lignaria* developmental success, future research that works to ascertain the precise roles that specific bacterial and fungal species play in solitary bee health would enhance bee conservation efforts.

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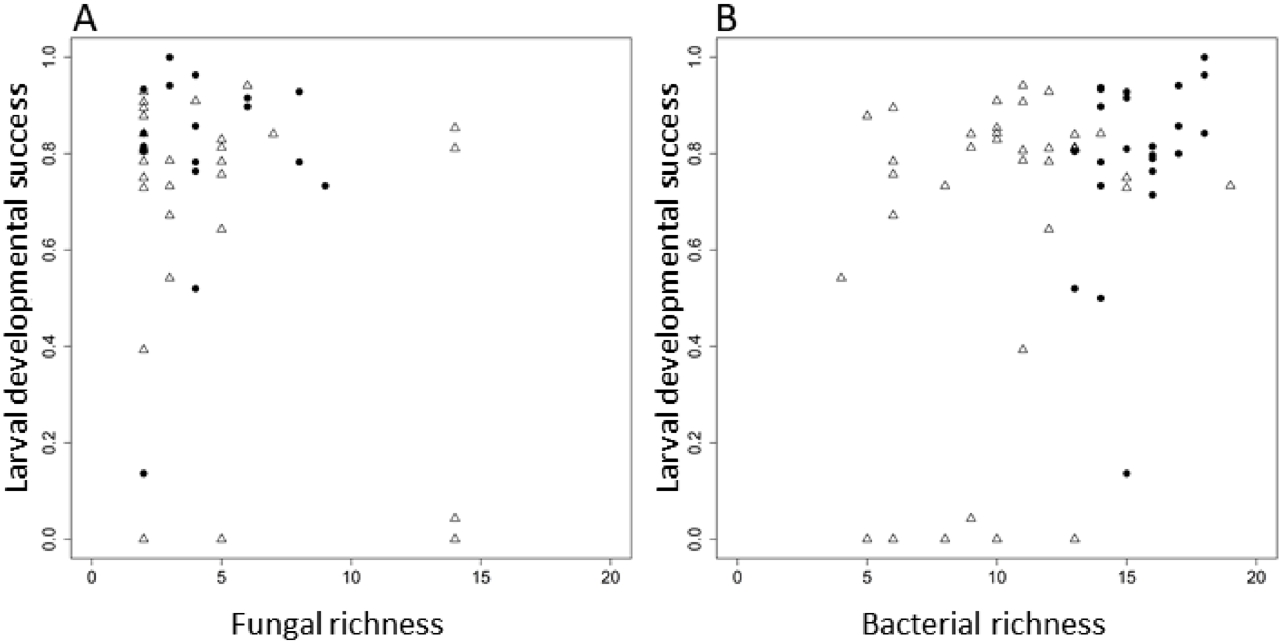

Figure 1. The proportion of larval developmental success (A) by bacterial richness (A) and fungal richness (B) during the first (open triangles) and second (closed circles) foraging period, 2019-2020.

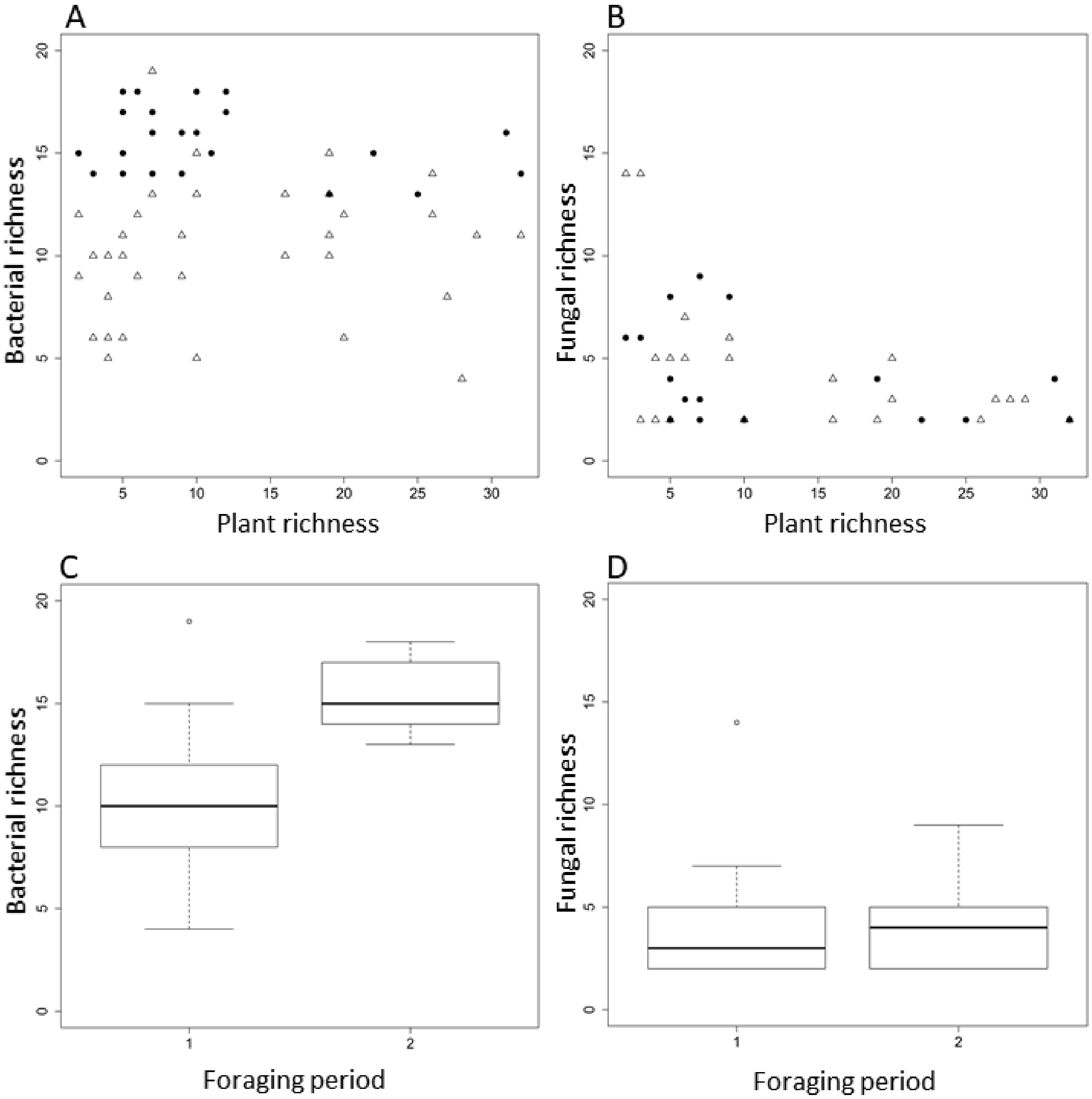

Figure 2. Bacterial richness (A) and fungal richness (B) by plant richness, 2019-2020 during the first (open triangles) and second (closed circles) foraging periods. Bacterial richness (C) and fungal richness (D) by foraging period, 2019-2020.

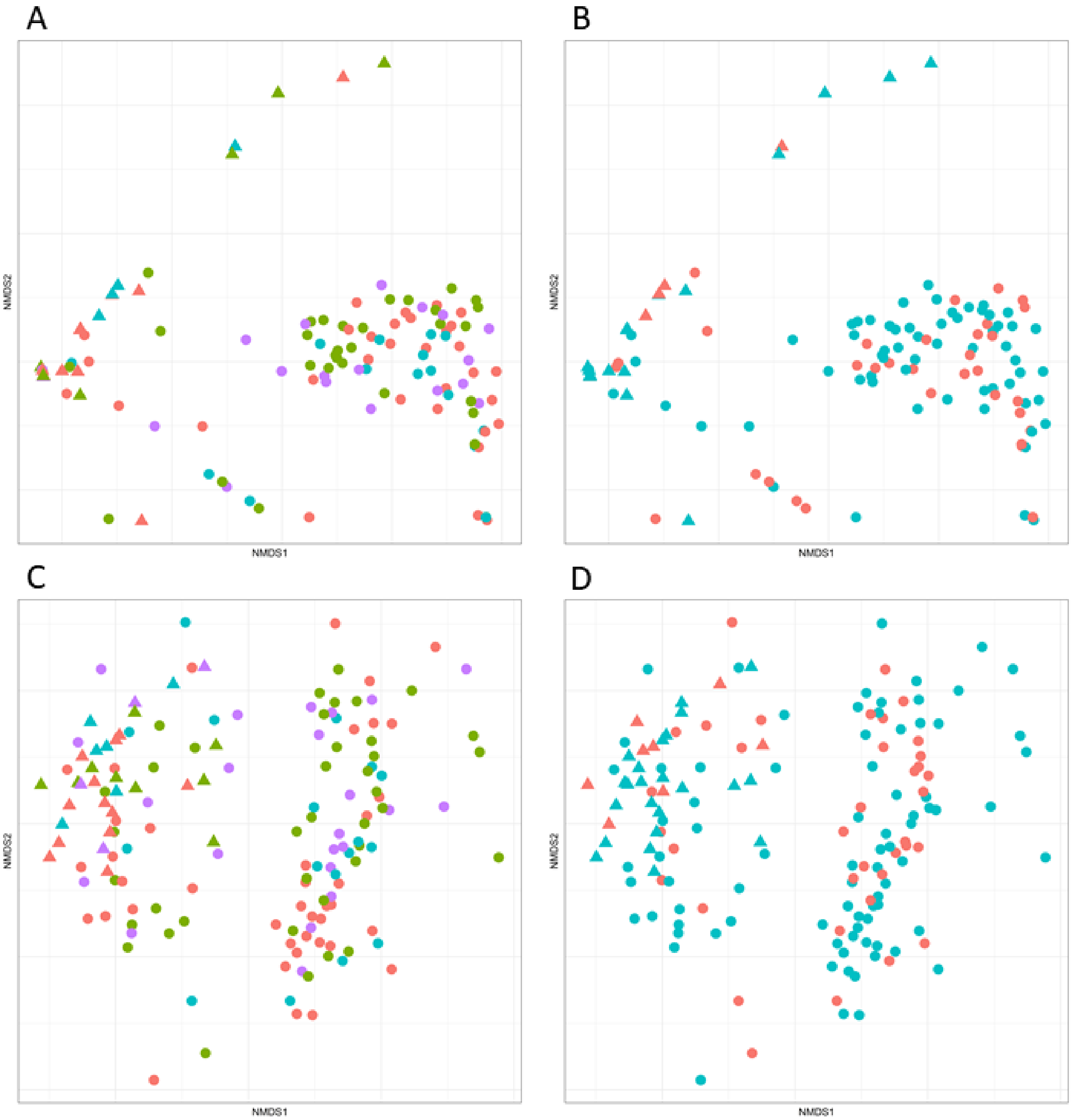

Figure 3. Non-metric dimensional scaling of fungal richness (dimensionality=2, stress=0.12) and bacterial richness (dimensionality=2, stress=0.17) by foraging period and site classification for all sites from 2019-2020. (A) Fungal richness by site classification. Symbols indicate the first (triangles) or second (circles) foraging period, and sites are also classified into four categories by color, including city parks (orange), restoration sites (green), rural farms (blue), and urban farms (purple). (B) Fungal richness by urban (blue) or rural (orange) classification, and during the first (circles) or second (triangles) foraging period. (C) Bacterial richness by site classification. Symbology as in (A). (D) Bacterial richness by urban or rural classification, and during the first or second foraging period. Symbology as in (B).

Table 1. Bacterial classes found in each foraging period in 2019 and 2020 as ascertained from DNA sequencing.

Year	Foraging Period	Classes Present
2019	1	Acidimicrobiia, Acidobacteria-6, Actinobacteria, Alphaproteobacteria, Bacilli, Bacteroidia, Betaproteobacteria, Clostridia, Cytophagia, Deltaproteobacteria, Flavobacteriia, Gammaproteobacteria, Pedosphaerae, Saprospirae, Sphingobacteriia, Thermoleophilia, Verrucomicrobiae
2019	2	Acidimicrobiia, Acidobacteria-6, Actinobacteria, Alphaproteobacteria, Bacilli, Bacteroidia, Betaproteobacteria, Clostridia, Cytophagia, Deltaproteobacteria, Ellin6529, Flavobacteriia, Gammaproteobacteria, Sphingobacteriia, Thermoleophilia, Verrucomicrobiae
2020	1	Acidimicrobiia, Acidobacteria-6, Actinobacteria, Alphaproteobacteria, Bacilli, Bacteroidia, Betaproteobacteria, Clostridia, Cytophagia, Deltaproteobacteria, Ellin6529, Flavobacteriia, Gammaproteobacteria, Pedosphaerae, Saprospirae, Sphingobacteriia, Thermoleophilia, Verrucomicrobiae
2020	2	Acidimicrobiia, Acidobacteria-6, Actinobacteria, Alphaproteobacteria, Bacilli, Bacteroidia, Betaproteobacteria, Clostridia, Cytophagia, Deltaproteobacteria, Ellin6529, Flavobacteriia, Gammaproteobacteria, Pedosphaerae, Saprospirae, Sphingobacteriia, Thermoleophilia, Verrucomicrobiae

Table 2. Detected bacterial genera from bioinformatic pipeline identification. Genera were not included in analyses due to low sequence counts that were below acceptable quality control limits, and are listed only because they have been reported to be associated with bee health or microbiome.

Genera	Foraging period	Total sequence count over foraging period	References
Fructobacillus	2	97	(McFrederick et al., 2014; McFrederick et al., 2012)
Lactobacillus	1	13	(Leonhardt & Kaltenpoth, 2014; Mcfrederick et al., 2014; McFrederick et al., 2012; McFrederick et al., 2013; McFrederick et al. 2017)
Saccharibacter	1	96	(Mcfrederick et al., 2014; McFrederick et al., 2012)
Wolbachia	2	17	(Gerth et al., 2011, 2015; McFrederick et al., 2014; McFrederick et al., 2012)

Table 3. Fungal genera found in each foraging period in 2019 and 2020 as ascertained from DNA sequencing.

Year	**Foraging Period**	**Genera Present**
2019	1	*Acicuseptoria, Ascosphaera, Aureobasidium, Cladosporium, Dichostereum, Erysiphe, Fusarium, Hyaloscypha, Podosphaera, Puccinia, Ramularia, Russula, Septoglomus, Vararia*
2019	2	*Acicuseptoria, Dioszegia, Fusarium, Hyaloscypha, Podosphaera, Puccinia, Subplenodomus,*
2020	1	*Acicuseptoria, Aroramyces, Auriporia, Baltazaria, Cyphellostereum, Cystangium, Dicohstereum, Dictyonema, Fusarium, Hyaloscypha, Laetiporus, Lecanactis, Marasmiellus, Podosphaera, Russula, Scytinostroma, Subplenodomus, Vararia*
2020	2	*Acarospora, Acicuseptoria, Catathelasma, Cora, Fusarium, Hyaloscypha, Leptosphaeria, Neocucurbitaria, Paraleptosphaeria, Phoma, Podosphaera, Puccinia, Sarcodon, Subplenodomus*

Table 4. Fungal genera identified over all sites and years (2019-2020), type of site in which genera were found, and known associations and hosts.

Fungi (genera)	Site type	Known associations
Fusarium	Urban restoration, urban farm, city park, rural restoration, rural farm	Saprotroph, soil; plant pathogen, cereal crops (species specific)
Hyaloscypha	Urban restoration, urban farm, city park, rural restoration, rural farm	Saprotroph, soft and hardwood trees, herbaceous litter
Podosphaera	Urban farm, city park	Pathogen, powdery mildew (many hosts)
Dichostereum	Urban farm, city park	Unknown, tree bark
Vararia	Urban farm, city park	Saprotroph, hardwood bark
Russula	Urban farm, city park	Ectomycorrhizal symbionts
Acicuseptoria	Rural farm, city park	Plant pathogen
Subplenodomus	Rural farm, city park	Saprotroph and parasitic, herbaceous and woody plants
Puccinia	Urban farm, city park	Plant pathogen, rusts
Ascosphaera	City park	Bee pathogen (chalk brood fungus)
Cladosporium	City park	Mold, plant pathogen (species specific)
Septoglomus	Rural restoration	Unknown, genus established 2011
Ramularia	Rural restoration	Ascomycete, plant pathogen
Erysiphe	Rural restoration	Plant pathogen, powdery mildew (species specific)
Aureobasidium	City park, rural restoration, rural farm	Epiphyte, endophyte; soil, water, limestone

Dioszegia	Urban restoration	Saprotroph, yeast
Scytinostroma	City park, rural restoration, rural farm	Plant pathogen
Cyphellostereum	City park, rural restoration, rural farm	Basidiolichen, bark
Paraleptosphaeria	Urban restoration, urban farm, city park, rural farm	Unknown, soil
Baltazaria	City park, rural restoration, rural farm	Recent reclassification of *Puccinia* (2018), plant pathogens
Lecanactis	City park, rural restoration, rural farm	Crustose lichen, bark
Auriporia	City park, rural restoration, rural farm	Saprotroph, wood
Dictyonema	City park, rural restoration, rural farm	Basidiolichen, wood
Cystangium	City park, rural restoration, rural farm	Varied, ecomycorrhizal
Laetiporus	City park, rural restoration, rural farm	Pathogenic, trees (chicken of the woods)
Marasmiellus	City park, rural restoration, rural farm	Saprotroph, can be parasitic and mycorrhizal
Aroramyces	City park, rural restoration, rural farm	Unknown, tropical associations
Leptosphaeria	City park, rural restoration, rural farm	Plant pathogen, parasitic (species specific)
Acarospora	Rural farm	Crustose lichens, rock, soil, can be parasitic to other lichen
Catathelasma	Urban restoration, city park	Mycorrhizal, conifers
Cora	Urban restoration, city park	Basidiolichen
Phoma	Urban restoration, city park	Saprotroph, parasitic, plant pathogen (species specific), soil
Neocucurbitaria	Urban restoration	Unknown
Sarcodon	City park	Ectomycorrhizal

Table 5. Effects of fungal richness, bacterial richness, foraging period, and all two-way interactions on *O. lignaria* larval developmental success and adult emergence success.

Predictor variable	**Larval developmental success**	**Adult emergence**
Fungal richness (FR)	*t*=-1.89; df=41; *P*=0.06	*t*=0.25; df=27; *P*=0.81
Bacterial richness (BR)	*t*=-0.70; df=41; *P*=0.51	*t*=0.03; df=27; *P*=0.97
Foraging period (FP)	*t*=-0.24; df=41; *P*=0.81	*t*=-0.76; df=27; *P*=0.45
FR × BR	*t*=1.77; df=41; *P*=0.08	*t*=-0.49; df=27; *P*=0.63
FR × FP	*t*=-0.62; df=41; *P*=0.54	*t*=1.33; df=27; *P*=0.20
BR × FP	*t*=0.33; df=41; *P*=0.74	*t*=0.49; df=27; *P*=0.63

Table 6. Effect of plant richness and foraging period, and their interaction, on fungal richness and bacterial richness. Values in bold indicate significance ($P \leq 0.05$).

Predictor variable	**Fungal richness**	**Bacterial richness**
Plant richness (PR)	**t=-2.42; df=36; P=0.02**	t=1.38; df=57; P=0.17
Foraging period (FP)	t=-0.87; df=39; P=0.39	**t=3.10; df=57; P<0.01**
PR × FP	t=1.04; df=38; P=0.30	t=-1.10; df=57; P=0.29

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