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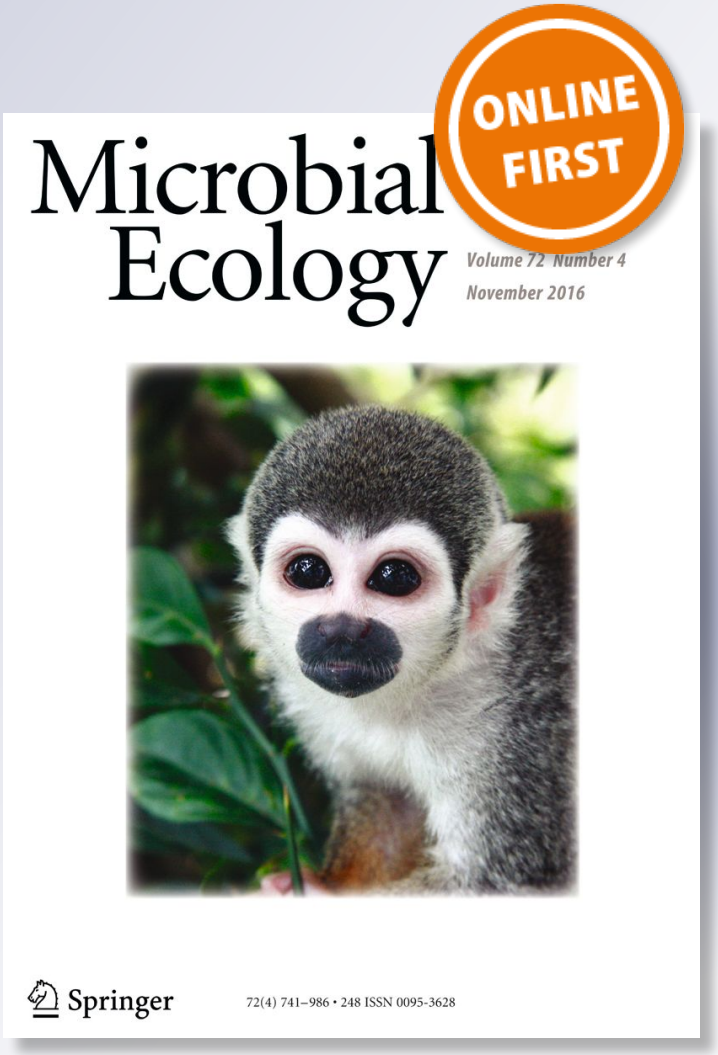
Quinn S. McFrederick & Sandra M. Rehan

Microbial Ecology

ISSN 0095-3628

Microb Ecol
DOI 10.1007/s00248-018-1232-y



Springer

Microbial Ecology
https://doi.org/10.1007/s00248-018-1232-y

INVERTEBRATE MICROBIOLOGY

Wild Bee Pollen Usage and Microbial Communities Co-vary Across Landscapes

Quinn S. McFrederick[1] · Sandra M. Rehan[2]

Received: 3 June 2018 / Accepted: 12 July 2018

Abstract

Bees forage for pollen and nectar at flowers but simultaneously acquire pathogenic, commensal, and likely beneficial microbes from these same flowers. Characterizing pollen usage of wild bees is therefore crucial to their conservation yet remains a challenging task. To understand pollen usage across landscapes and how this affects microbial communities found in the pollen provisions collected from flowers, we studied the generalist small carpenter bee *Ceratina australensis*. We collected *C. australensis* nests from three different climatic zones across eastern and southern Australia. To characterize the plant, fungal, and bacterial composition of these pollen provisions, we used a metabarcoding and next-generation sequencing approach. We found that the species richness of plant types, fungi, and bacteria was highest in a subtropical zone compared to a temperate or a grassland zone. The composition of these communities also differentiated by zone, particularly in pollen composition and fungal communities. Moreover, pollen composition strongly correlated with fungal community composition, suggesting that variation in pollen usage across landscapes results in variation in microbial communities. While how these pollen usage and microbial community patterns affect bee health merits additional work, these data further our understanding of how flowering plant community composition affects not only the pollen usage of a generalist bee but also its associated microbial communities.

Keywords Microbiome · Metabarcoding · Pollen · Yeast · Polylecty

Introduction

While there is great interest in protecting wild pollinator populations, elucidating bee pollen usage remains time consuming and difficult [1, 2]. Yet understanding bee foraging is essential for understanding bee health [3]. Laboratory experiments have shown that variation in diet directly affects bee development and immunity [4–6]. Flowers, however, also act as transmission sites for bee pathogens, parasites, and commensal (and possibly beneficial) microbes [7–9]. Foraging therefore appears to affect bee health via several different mechanisms.

The vast majority of bees use nectar and pollen for their food, and forage availability is thought to be a major driver of bee populations [10, 11]. Foraging decisions of generalist pollinators are driven by various factors including availability, competition, and landscape characteristics [3, 12]. Bumble bees and honey bees detect and make foraging decisions based on the nutritional content of floral resources [13–16]. The sweat bee *Lasioglossum zephyrum* has smaller offspring when using pollen with low protein content and does not adjust the size of the provision according to protein content [4]. In contrast, maternal manipulation of offspring size via pollen provision quality and quantity occurs in the socially polymorphic bee *Megalopta genalis* and the subsocial bee *Ceratina calcarata* and determines whether a daughter bee becomes a foundress or a worker [17–20]. Bee pollen usage is therefore driven by a complex mix of factors that vary by species but can be consequential for the fitness of the bee or colony.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00248-018-1232-y) contains supplementary material, which is available to authorized users.

✉ Quinn S. McFrederick
quinnmc@ucr.edu

Sandra M. Rehan
sandra.rehan@gmail.com

[1] Department of Entomology, University of California, Riverside, 900 University Avenue, Riverside, CA 92521, USA

[2] Department of Biological Sciences, University of New Hampshire, 38 Academic Way, Durham, NH 03824, USA

Published online: 01 August 2018

While bee foraging is influenced by these diverse factors, an additional consequence of foraging on shared flowers is exposure to pathogens and parasites. The microsporidian *Nosema ceranae* and the trypanosome *Crithidia bombi* are both transmitted among and between bee species at flowers [8, 21]. A broad range of pollinators harbors similar parasites including *Ascosphaera* fungi, microsporidians, and deformed wing virus, and floral transmission may be a likely source of these pathogens [22]. While floral transmission of parasites and pathogens is still a nascent field, it is becoming clear that bees obtain more than just pollen and nectar from flowers.

In addition to parasites and pathogens, flowers also house commensal and possibly beneficial bacteria that are shared among bee taxa [23]. Flowers, halictid bees, and megachilid bees all harbor closely related lactobacilli, indicating that flowers serve as hubs of transmission for these bacteria [7]. Plant-associated fungi, which are likely obtained via the pollen, occur in the guts of larval megachilid bees [24]. Within a population of the small carpenter bee, *Ceratina calcarata*, specific types of pollen correlate with specific bacteria, suggesting that the plants from which the pollen was obtained serve as hotspots of transmission or ecological filters for those bacteria [25]. If these florally transmitted bacteria are beneficial to the host, it is likely facultative, as they show more uneven distributions among wild bees compared to the socially transmitted bumble bee and honey bee associated bacteria [7, 26]. While our knowledge of these flower- and bee-associated bacteria remains sparse, we now know that in addition to nutrition and pathogens, bees obtain commensal and possibly beneficial microbes from flowers.

One of the missing pieces in the study of the mutualism between flowers and bees is an understanding of how pollen usage across disparate habitats affects microbial communities and pathogen exposure within a bee species. To address this gap, we studied the small carpenter bee *Ceratina australensis*, which is a habitat and floral generalist [27], across several disparate habitats in Australia. We asked several questions: (A) How does pollen usage vary across disparate habitats? (B) Do bacterial and fungal communities found in the pollen and nectar that the bee collects and feeds to her offspring also vary by habitat? (C) Does pollen provision composition correlate with microbial community composition? and (D) Does pathogen prevalence differ between landscapes? To answer these questions, we used metabarcoding of pollen provisions to determine what pollen types, bacteria, and fungi were present across landscapes. To identify pathogen presence, we screened for bacterial, fungal, trypanosome, and microsporidian pathogens in the pollen provisions.

Materials and Methods

Specimen Collection

We collected 52 *Ceratina australensis* nests across three climactic regions in eastern Australia (Fig. 1) in January 2015. The Queensland site (28.24° S, 152.09° E) is subtropical, the Victoria site (34.15° S, 142.16° E) is grassland, and the South Australia site (34.94° S, 138.50° E) is temperate. Each nest was collected at dawn or dusk from dead broken stems and chilled until processed. Nests were split lengthwise and contents transferred to sterile cryovials with forceps that were flame sterilized between samples. Samples were flash frozen in liquid nitrogen and then transferred to a − 80 °C freezer for storage until DNA extraction.

Molecular Analyses

For molecular analyses, we first extracted DNA from each of 91 pollen provisions using previously described protocols [25, 28]. Briefly, to lyse pollen, Gram-stain positive bacteria, and other resistant spores and cells, we bead beat each sample in 180 μL Qiagen buffer ATL with a single 3.2 mm steel bead and ~ 50 μL of 0.1 mm glass beads at 30 Hz for 6 min. We then added 20 μL of proteinase K and incubated the samples overnight at 56 °C. To complete the extractions, we then followed the manufacturer's instructions for the Blood and Tissue DNeasy 96 kit (Qiagen, Valencia, CA). We included two blank controls in the extractions that were subjected to all downstream steps.

We used the resulting DNA extractions for several analyses. First, we prepared separate metabarcoding amplicon libraries for bacteria (the 16S rRNA gene [16S]), fungi (the internal transcribed spacer [ITS]), and plants (RuBisCO large subunit [rbcl]). Nectar does not contain DNA, and our rbcl primers may not detect plants that the bees visited for nectar only, but we note that a similar barcoding approach has been applied to honey, which contains incidental pollen [29]. For 16S and rbcl, we used previously described primers [25] while we used ITS1f and ITS2 [30] to design new primers that incorporate inline 8-mer barcodes and the Illumina sequencing primers into the resulting amplicon. All primer sequences used in this study are reported in Table S1. We used previously described PCR protocols for preparation of all three libraries [25]. After purification of the resulting PCR product using the PureLink Pro 96 kit (Invitrogen, Carlsbad, CA), we performed a second round of PCR using 1 μL of purified PCR product as the template and the PCR2F and PCR2R primers to complete the Illumina sequencing construct [25, 31]. To obtain roughly equal molar libraries, we used SequalPrep normalization plates (Invitrogen, Carlsbad, CA) and then performed a final

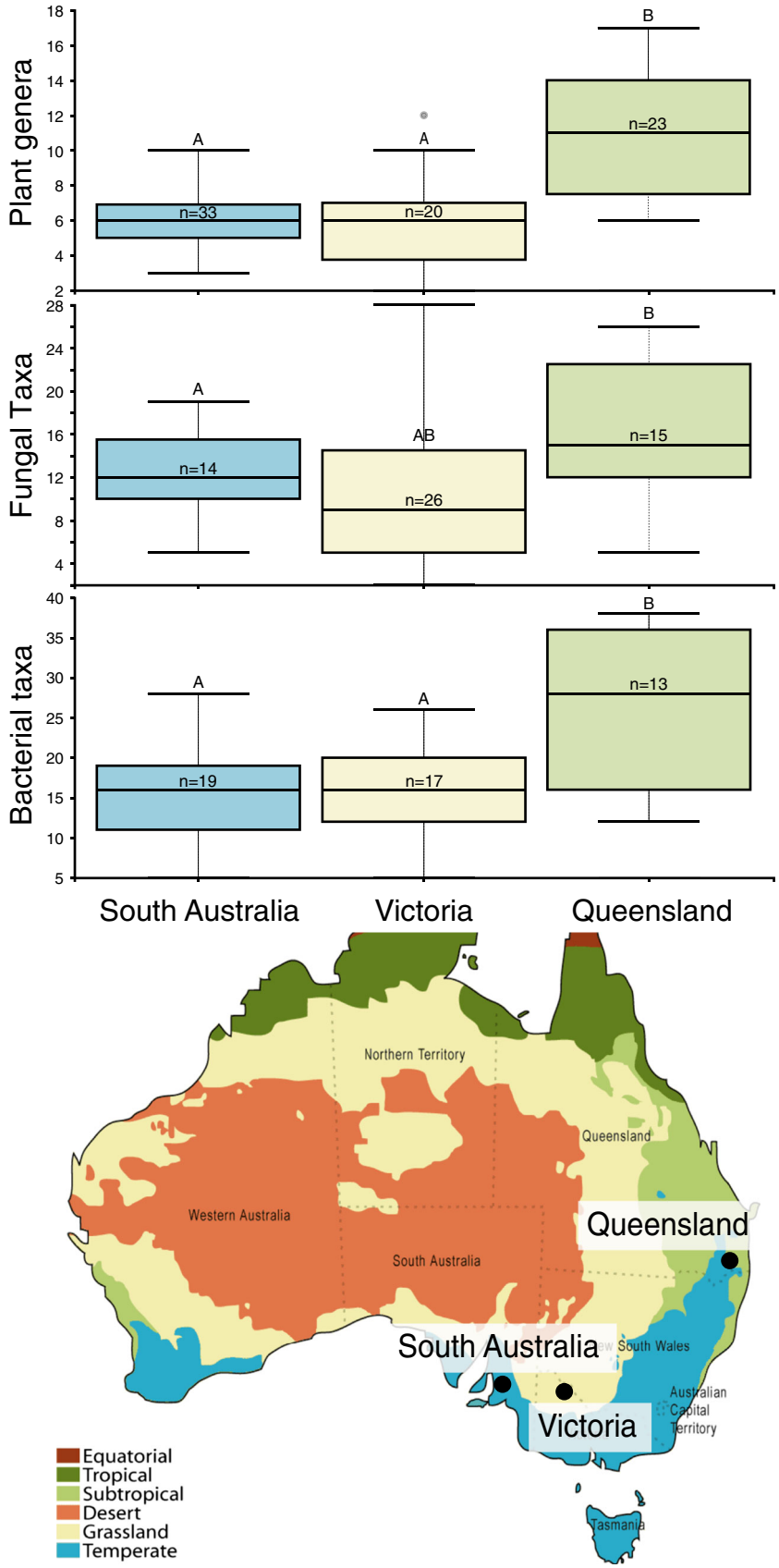


Fig. 1 Alpha diversity of pollen provisions across sites and location of sampling points across Australia. The subtropical site (Queensland) exhibited greater diversity across the three kingdoms compared to the grassland (Victoria) or temperate (South Australia) sites

purification on the pooled libraries. To check the quality of these libraries, we used the Bioanalyzer 2100 (Agilent, Santa Clara, CA). These protocols allowed us to simultaneously sequence all libraries on the Illumina MiSeq using the Illumina sequencing primers and V3 2 X 300 reagents.

For detection of possible parasites, we screened our fungal and bacterial barcoding taxonomy for known bee pathogens such as *Ascosphaera*, *Melissococcus,* and *Serratia.* We additionally performed PCR screens using DNA extractions that produced amplicons in the above metabarcoding reactions. To detect trypanosomatid parasites (such as the bumble bee pathogen *Crithidia bombi* or the honey bee pathogen *Lotmaria passim*), we performed PCR screens using the SEF/SER primers, which detect a broad range of trypanosomatids [32]. For each 10 μL reaction, we used one unit of New England Biolab's standard taq buffer, 200 μM dNTPs, 0.2 μM forward and reverse primer, 0.25 U Taq polymerase (New England Biolabs Ipswich, MA), and 2 μL DNA template. We used an annealing temperature of 57 °C for 35 PCR cycles. We additionally screened for *Nosema ceranae* and *Nosema apis* using species specific primers [33] and the conditions described above with the exception of a 58 °C annealing temperature. We visualized all products on 1.5% agarose gels. To test for differences in infection rates across sites, we ran Pearson's chi-squared test in R.

Bioinformatics and Statistical Analyses

We analyzed the metabarcoding data with QIIME2 [34]. We visualized quality scores and trimmed the reads when quality scores dropped below 35. We then used DADA2 [35] to bin reads into exact sequence variants (ESVs). We classified the reads in several ways, depending on the barcode. For 16S rRNA data, we trained the SILVA 128 database [36] to the section of the 16S rRNA gene that is amplified with our primers. For ITS, we used the UNITE database [37]. We used the scikit-learn classifier to assign taxonomy to 16S rRNA gene and ITS reads. For rbcl, we used a database that was recently compiled for metabarcoding studies of pollen [38] and the RDP classifier [39]. For all three loci, we performed additional BLASTn [40] searches against NCBI databases: 16S microbial for the 16S data and nt for the ITS and rbcl data. We then compared the top BLAST hit to the classifier taxonomy to quality check the assignments. We used these assignments to remove chloroplast and mitochondrial contaminants from the dataset. We additionally removed ESVs that were present in the blank controls and therefore likely represent reagent or human-sourced contaminants. To quality check the rbcl taxonomic assignments, we verified the presence of the plant genus at the collection locality using the Atlas of Living Australia [41].

For alpha and beta diversity analyses, we used these three quality-filtered and contaminant-controlled datasets. For the 16S rRNA gene, we found that quality and contaminant control removed the majority of reads from most samples. For example, 16S rRNA gene reads assigned to chloroplast and mitochondria accounted for 28% of all reads, while a *Pseudomonas* contaminant that was abundant across all samples (including the two blank controls) accounted for an additional 58%. To be able to include a reasonable number of samples (53%) in downstream analyses, we rarified to 153 reads per sample. Surprisingly, rarefaction curves mostly leveled off at this depth, which in combination with the dearth of bacterial reads indicates that bacteria were likely not abundant in these pollen provisions. For ITS, the blank control only had one ESV, which was assigned to *Monographella nivalis*, and occurred in only one sample. We removed this ESV from further analyses. We ran ITS analyses at two rarefaction depths: 1249 and 530. As both community composition and diversity analyses were similar at both sampling depths, we used 530 reads per sample in order to retain 70% of the samples in downstream analysis. The rbcl reads were overall more abundant compared to 16S rRNA or ITS reads. Rarifying at 3015 reads per sample allowed us to retain 87% of our samples in downstream analyses.

To detect differences in species richness by site, we performed Kruskal-Wallis overall and pairwise tests in QIIME2. To avoid type-1 errors, we used the Benjamini-Hochberg correction [42]. To determine which ESVs or plant genera were differentially abundant by site, we used ANCOM in QIIME2 [43].

We conducted beta-diversity analyses in the R-package vegan [44]. We imported the ESV feature tables (equivalent to OTU tables) for each barcode separately. We first performed non-metric multidimensional scaling (NMDS) with the meta-MDS and then used the resulting Bray-Curtis distance matrices for Adonis analysis. For explanatory variables, we included collection site, nest, and brood cell position within nest. To test multivariate homogeneity of group dispersions, we used the betadisper command in vegan. To examine correlations between pollen types and bacterial communities or fungal communities, we additionally used the Bray-Curtis dissimilarity matrices to conduct Mantel tests. To test for spatial autocorrelation in each dataset, we conducted Mantel tests between geographic distance matrices and Bray-Curtis dissimilarity matrices. We used 1000 permutations for each Mantel test.

Results

After quality-filtering and removal of contaminants and off-target sequences, we obtained a total of 540,533 sequences and 223 ESVs across 87 samples for rbcl, 194,246 sequences across and 393 ESVs across 79 samples for ITS, and 29,947 sequences and 789 ESVs across 91 samples for the 16S rRNA

gene. After manual curation of the rbcl data, we binned the ESVs into 75 genera and conducted further analyses using these genera. Sequence data are publicly available under NCBI/EMBL/DDBJ accession numbers SAMN08911168-SAMN08911424.

We screened the 87 samples that were positive for rbcl (the one marker that showed no contamination in the blank controls) for trypanosome and *Nosema* bee pathogens (Table S2). We detected only one trypanosome positive in a pollen provision from Queensland. *Nosema* was more prevalent, occurring in 8% (7/87) of the pollen provisions. We detected *Nosema* at all three sites: 8% in Queensland (2/23), 3% in South Australia (1/34), and 13% in Victoria (4/30) ($\chi^2 = 2.34$, df = 2, $P = 0.31$). We additionally detected low numbers of reads of the bacterial honey bee pathogen *Melissococcus plutonius* (in two different nests from Victoria) and *Serratia* (in one nest from South Australia; Table S3).

Alpha diversity differed by site, with Queensland having significantly greater diversity in all three Kingdoms compared to Victoria (pair-wise Kruskal-Wallis: rbcl $H_{2,42} = 18.5$, $P_{adj.} < 0.001$; ITS $H_{2,40} = 6.7$, $P_{adj.} = 0.029$, 16S $H_{2,29} = 6.2$, $P_{adj.} = 0.019$; Fig. 1), and significantly greater plant and bacterial diversity compared to South Australia (pair-wise Kruskal-Wallis: rbcl $H_{2,55} = 25.5$, $P_{adj.} < 0.001$; ITS $H_{2,28} = 2.7$, $P_{adj.} = 0.15$, 16S $H_{2,31} = 7.0$, $P_{adj.} = 0.019$; Fig. 1). Species richness across all three kingdoms, however, did not differ between Victoria and South Australia ($P \geq 0.20$; Fig. 1). The top five plant genera (*Anthemis*, *Scaevola*, *Alfalfa*, *Glycyrrhiza*, and *Cichorium*) came from three families: Asteraceae, Fabaceae, and Goodeniaceae (Table S4). Most of the abundant fungi belong to families that include saprobes and plant pathogens such as Lophiostomataceae, Didymellaceae, and Sclerotiniaceae (Table S5).

Beta diversity also differed by site. Pollen communities clustered by site in the NMDS ordination, and Adonis analysis confirmed these differences ($F_{2,73} = 17.6$, $P < 0.001$; Fig. 2a). Fungal communities exhibited the same patterns, again with significant differences between sites ($F_{2,52} = 9.2$, $P < 0.001$; Fig. 2b). Two pollen provisions from the same nest collected in Victoria were dominated by otherwise rare fungi, and we removed those samples from the NMDS ordination (Fig. 2b) for clarity. We also ran Adonis analysis with those two samples removed and found that the same patterns held ($F_{2,50} = 9.9$, $P < 0.001$). In contrast to pollen and fungi, bacteria showed less clustering by site. Adonis analysis, however, detected significant differences by site ($F_{2,46} = 1.8$, $P < 0.001$). As the 95% confidence intervals of the sites not only exhibited overlap but also indicated that there could be differences in spread (Fig. 2c), we tested for multivariate homogeneity of group dispersions. While approaching significance ($F_{2,46} = 3.2$, $P = 0.051$), the test indicated that differences in spread among groups did not fully explain the significant Adonis result. Beta diversity also differed by nest for all three

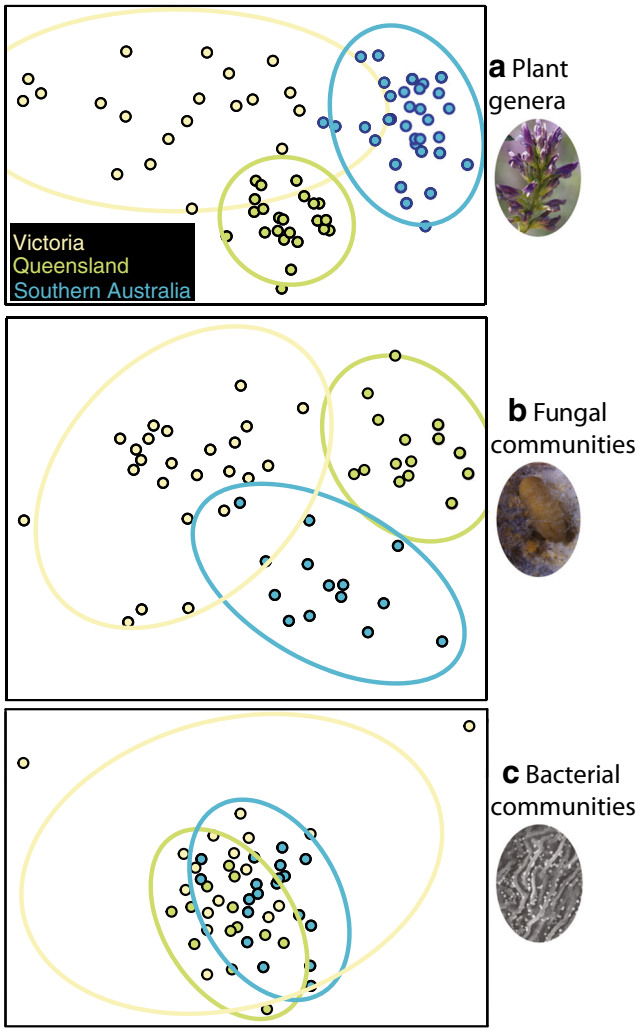


Fig. 2 NMDS ordinations of plant, fungal, and bacterial communities found in the nests of *Ceratina australensis*. Ellipses are 95% confidence areas. **a** Plant genera. **b** Fungal communities. **c** Bacterial communities

barcodes (rbcl $F_{35,37} = 6.0$, $P < 0.001$; ITS $F_{27,24} = 4.5$, $P < 0.001$; 16S $F_{31,14} = 1.4$, $P < 0.001$), indicating significant within-site variation. Brood cell position was not significant for any of the three barcodes.

To test for correlations between pollen and fungi, pollen and bacteria, and fungi and bacteria, we conducted Mantel tests on Bray-Curtis dissimilarity matrices. Pollen and fungi ($N = 46$, Spearman's $\rho = 0.41$, $P = 0.001$) and pollen and bacteria ($N = 44$, Spearman's $\rho = 0.18$, $P = 0.002$) matrices were significantly correlated. Furthermore, fungi and bacteria were also correlated ($N = 34$, Spearman's $\rho = 0.18$, $P = 0.003$). However, whether these correlations are driven by interactions or by geography was not clear, as each community showed significant spatial autocorrelation (pollen $N = 76$, Spearman's $\rho = 0.57$, $P = 0.001$; fungi $N = 55$, Spearman's $\rho = 0.43$, $P = 0.001$; and bacteria $N = 49$, Spearman's $\rho = 0.16$, $P = 0.001$).

Specific pollen types and fungi differed in abundance across sites (Fig. 3). The South Australia site had five pollen

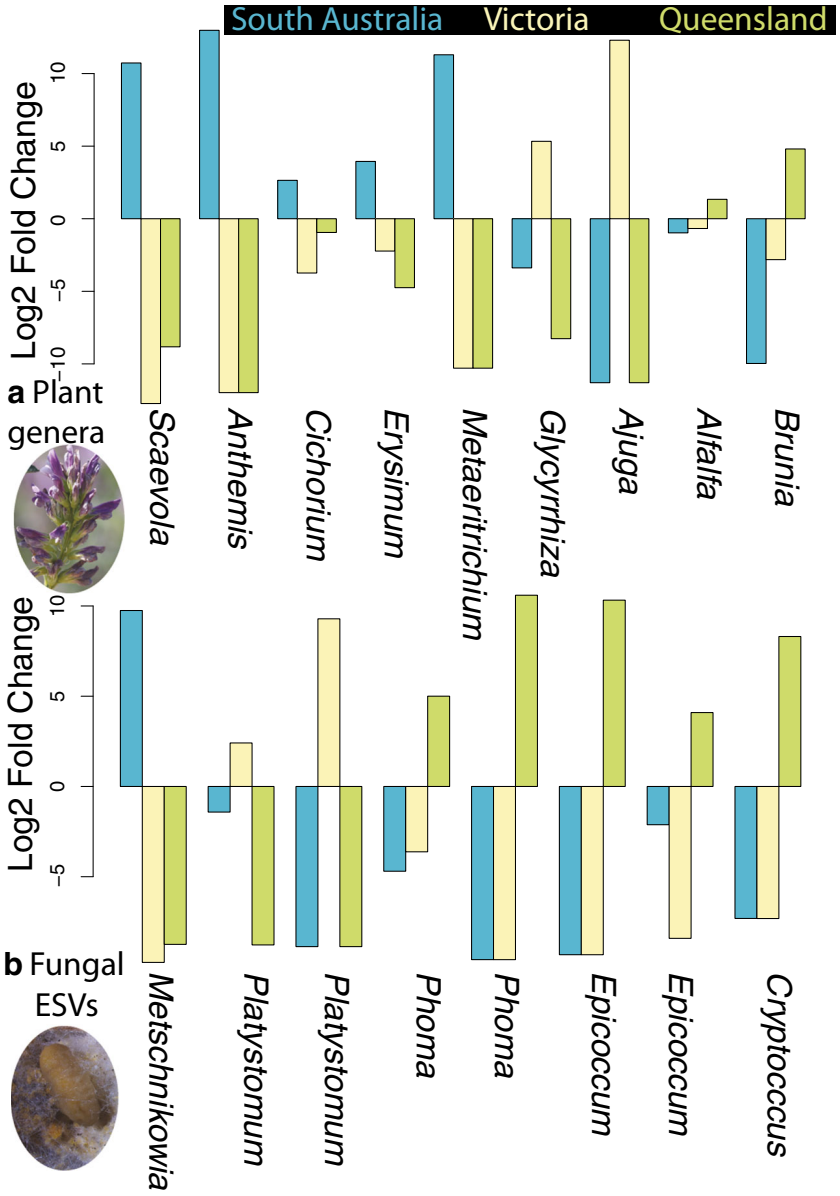


Fig. 3 Plant genera and fungal ESVs that are differentially abundant across habitats. For plant genera, only those that were one of the 10 most abundant genera are shown. For fungi, all differentially abundant ESVs are shown. Queensland had fewer plant genera yet more fungi with greater abundance compared to the other two sites. **a** Plant genera. **b** Fungal ESVs

types from several families that were more abundant compared to Victoria or Queensland. The Victoria and Queensland sites each had two genera that were overrepresented in comparison to the other sites. In contrast, the Queensland site had more fungi (five) that were overrepresented, while the Victoria site had two and the South Australia site had one. Ancom analysis did not detect any bacterial taxa that with significant differential abundance across sites.

Discussion

Ceratina australensis pollen usage differs across habitats. The subtropical climate of Queensland harbors the highest flowering plant species richness of the three study sites (Queensland = 10,592, Victoria = 6339, and South Australia = 5920 flowering plant species; [41]). These species richness patterns are represented in the pollen provisions of this generalist forager, which contain pollen from more plant genera in Queensland compared to Victoria or South Australia (Fig. 1). Moreover, the composition of the pollen provisions differed strongly across sites (Figs. 2 and 3), further indicating that the pollen usage of these bees differs across habitats.

Our data suggest that this variation in pollen usage across habitats leads to variation in microbial communities across habitats. The highest fungal and bacterial species richness in the pollen provisions were found in Queensland, and fungal communities were strongly correlated with pollen composition of the provision. Moreover, the species richness of fungi in the pollen provisions does not follow the overall species richness recorded in the different habitats (Queensland = 1894, Victoria = 2281, and South Australia = 1010 fungal

species; [41]), suggesting that these patterns are not driven by variation in microbial communities alone. Bacterial communities also correlated with pollen communities but were less differentiated across habitats compared to fungal communities. The microbes that we detected in pollen provisions are commonly isolated from flowers [7, 45–47], further suggesting that greater diversity in floral utilization leads to greater microbial diversity in a bee's pollen provisions. Whether this variation in microbial diversity arises from greater pollinator or floral diversity across sites merits further study. As plant and microbial diversity positively co-vary across habitats, however, we are not able to entirely exclude the possibility that microbial communities follow a similar diversity gradient independently of plants or that different pollen chemistries filter microbiomes differently across habitats. Even so, the bulk of evidence supports the hypothesis that floral diversity drives microbial diversity across habitats.

Flowers are known to be hubs of microbial diversity. For example, in our previous research, we found that bees and flowers share microbes [7] and that specific pollen types correlated with specific bacterial types in the provisions of the North American small carpenter bee *C. calcarata* [25]. Others have shown that flower visitors introduce yeast and bacteria into nectar microbial communities [46, 48]. These microbes affect nectar chemistry, volatile emission, pollinator attraction, and plant reproductive fitness [49–52]. The pollination mutualism is clearly entangled in multiple layers of interactions.

The work presented here furthers our understanding of these intricate interactions by highlighting how they vary across landscapes. We show that the diversity of plants that a generalist pollinator visits across landscapes correlates with the diversity of microbes that she introduces into her brood's food. Foraging habitats of pollinators across landscapes consequently shape microbial communities not only in flowers but also in the provisions that were collected from those flowers.

These pollen usage patterns may also result in introduction of bee pathogens into a bee's nest. Although the pathogenicity of these microbes is not known in small carpenter bees, we detected trypanosomatids, microsporidians, and bacterial pathogens in pollen provisions. These pathogens were rare enough that no obvious patterns in their distribution emerged, but further study of their pathogenicity in small carpenter bees is warranted. We did not detect fungal pathogens of bees such as *Ascosphaera* [53], *Aspergillus* [54], or *Beauveria* [55] across any of the study sites.

As with other amplicon studies of plant material with low bacterial biomass [56], the majority of our 16S rRNA gene reads were from chloroplasts and mitochondria. We have previously found variation in the quantity of bacteria found in pollen provisions [7], and our results here provide further evidence that pollen provisions are not necessarily bacterial-rich habitats. Flower pollen can harbor substantial numbers of bacterial cells [57], as can nectar [58], and whether the variation in the number of bacteria in pollen provisions arises from a small number of bacteria in the incoming food or inhibition by fungi or some other agent deserves further study. While the bacterial reads in our study were sparse, the most abundant bacteria in the pollen provisions have been previously reported from pollen provisions in North and Central America [7, 25, 59]. For example, the ten most abundant bacteria included *Saccharibacter floricola* and *Rosenbergiella collisarenosi* (floral nectary inhabitants) and the bee- and flower-associated *Lactobacillus micheneri* [47, 60, 61]. Fungal read counts were much higher than bacteria in most of our samples, indicating that fungi may be more common than bacteria in these provisions. Many of these fungi were plant pathogens and may be inconsequential to bee fitness but highlight the role that bees may play as vectors for plant pathogens [9]. Others, such as yeasts that were abundant in certain provisions, have been posited to be bee parasites, commensals, or mutualists in bee pollen provisions [54]. The flower-associated yeasts *Metschnikowia* and *Starmerella* [62, 63] were among the more abundant fungi present in the pollen provisions but only accounted for 7% of ITS reads. Although the consequences for bee health remain unknown, we found that *Metschnikowia*, for example, was more abundant in South Australia compared to the other two sites. From the plant perspective, *Metschnikowia* in flower nectar can increase pollinator visitation [49, 64] but can reduce plant reproductive fitness [45]. How these plant-pollinator-microbe interactions play out across diverse landscapes also deserves further study.

The generalist foraging and nesting habits of *C. australensis* are thought to have allowed for its remarkable range across a diversity of habitats [27]. Population genetic analyses suggest that this expansion started out of Queensland, through Victoria, and more recently into South Australia [27]. Here we show that this expansion also led to a switch in pollen usage and microbial associations. Our data suggest that as *C. australensis* populations expanded into Victoria and South Australia, the diversity of flowers on which they forage diminished, leading to a decrease in microbial diversity introduced into their nests. Migration rates between populations appear to be low, as the populations show clear structure [27]. The loss of diversity in forage and microbial associates as *C. australensis* migrated into new habitats did not hinder its ability to establish new populations.

Ceratina australensis exhibits social polymorphism where some nests are solitary while others are incipiently social [65]. The frequency of social and solitary nests did not vary across habitats [27], indicating that the variation in forage and microbes that we discovered here is likely unrelated to social behavior in this bee. Female offspring size was lowest in Victoria [27], but because alpha-diversity of forage is similar between South Australia and Victoria, the composition and

quality of forage available in Victoria are likely more important for bee size than diversity per se. Sex ratios also varied across sites, with female-biased populations occurring in Victoria and Queensland, but even sex ratios in South Australia [27]. While we found abundant *Wolbachia* in the pollen provisions of *C. calcarata* in North America [25], we detected only a few *Wolbachia* reads from two provisions from Victoria. *Wolbachia* is therefore probably not responsible for the female-biased sex ratios found in certain *C. australensis* populations.

By using simultaneous metabarcoding of pollen, fungi, and bacteria of each of our samples, we were able to determine how pollen usage and microbial patterns co-vary across landscapes in the small carpenter bee *C. australensis*. While there are clear patterns of diversity across these landscapes, what these patterns mean for bee health requires further study. The contribution of forage quality versus forage alpha-diversity also merits further study. Additionally, the differential abundance of plant pathogens and yeasts across these landscapes suggests that the pollination mutualism could vary in costs and benefits to both plant and bee partners across landscapes. Carefully designed studies investigating how pollinators serve as vectors of beneficial and deleterious microbes among themselves and their plant partners will continue to provide fascinating insights into these symbioses.

Acknowledgements We thank Wyatt Shell and Rebecca Dew (UNH) for assistance with nest collections as well as Laura Leger (UCR) for assistance with pathogen screens.

Funding Information US Department of Agriculture NIFA Hatch funds (CA-R-ENT-5109-H) to QSM and (NC1173) to SMR, National Science Foundation MSB-ECA award #1638728 to QSM and National Science Foundation IOS-Behavior #1456296 to SMR, as well as National Geographic (9659-15) to SMR supported this research.

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