Temporal dynamics of plant and fungal communities based on pollen sampled from honey bee hives in Southern Ontario, Canada

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Abstract

Multitrophic interactions are inherent to the ecological networks of terrestrial ecosystems and can exhibit dynamic temporal changes within a season. In floral communities, pollen and nectar act as hubs for various microorganisms, including fungi that can alter plant-pollinator interactions. In mixed pollen samples collected by *Apis mellifera* L., the associations between plants and fungi foraged by bees may be complex and not yet fully characterized. Exploring the temporal succession of the multitrophic interaction is an area that requires further investigation. Forty-two pollen samples were retrieved from 13 hives dispersed in urban and peri-urban locations in Southern Ontario Canada where the honey bee is not native. Using metabarcoding of the ITS region, we identified a total of 77 plants and 46 fungi. Among the foraged plants visited, the top ten were all non-native or invasive taxa for Southern Ontario, with *Trifolium repens* L. and *Sonchus arvensis* L. as most common taxa. For fungal taxa, the main yeasts and moulds were identified as *Starmerella* and *Mucor* taxa. Plant richness was found to have a significant association with fungal richness. Moreover, plant and fungal taxa richness and Shannon diversity increased with time from spring to late summer. Only plant taxa composition varied over the active foraging season suggesting a more homogenous fungal taxa community. Diverse flowers can further play a role in the spread of fungal organisms having a variety of ecological functions and trophic levels. The study of their interactions with flowers, pollinators, and humans, are deserving of more investigation.

Keywords: *Apoidea; DNA metabarcoding; pollination ecology; urban pattern*

1. Introduction

Animal vectorization of pollen is essential for maintaining floral communities in temperate and tropical regions (Ollerton et al., 2011). Among animals, bees (Hymenoptera: Anthophila) are the most efficient pollen transporters due to morphological and behavioral adaptations, e.g., the presence of scopae (carrying the pollen), and a specialized pollen and nectar diet. Among all bee species, the western honey bee, *Apis mellifera* L. (Hymenoptera: Apidae) is one of the most common and globally widespread. This social bee species has been domesticated and managed mainly for agricultural pollination, honey production, as well as other hive products (Pirk et al., 2017). Honey bee workers are essential for providing food for the colony throughout the year, and, as generalists, visit many different plant species in search of pollen and nectar (Hung et al., 2018). If food is abundant, honey bees prefer certain flower species over others. In contrast, if the abundance or quality of local flowers is insufficient, foragers must compensate by visiting more plant species over larger spatial distances (Garbuzov et al., 2015). Moreover, the specific foraging strategy of honey bees has been used as a bioindicator to monitor the quality of the environment e.g., heavy-metal concentrations in honey (Zaric et al., 2018) or the presence of metallothioneins and pesticides in pollen (Badiou-Bénéteau et al., 2013). Due to the number of flowers visited per bee and per hive, honey bees can also be deployed to monitor pathogens, and used as a biocontrol delivery agent of fungicides to flowers, e.g., against the grey mold *Botrytis cinerea* Pers. that damages strawberries (Hokkanen et al., 2015).

The flowering plants visited by the honey bee workers contain a unique microbiome, including some fungi, oomycetes, bacteria, and viruses, which have evolved various dispersal strategies enabling attachment to visiting pollinators (Manirajan, 2018). Honey bee workers carry microorganisms not only from flower to flower but also from flowers to the colony via collected nectar and pollen (Figueroa et al., 2020). Consumption of more diverse floral resources can improve the immunity of honey bees (Di Pasquale et al., 2013). For example, when *Lactobacillus* bacteria were included in the honey bee diet, honey bee health improved reducing bacterial dispersal (Pietropaoli et al., 2022). Floral nectar is indeed known to include a high abundance and diversity of bacterial and fungal (e.g., yeast) communities that can withstand high sugar levels (Aizenberg-Gershtein et al., 2013). After anthesis, flowers are rapidly colonized and dominated by specific yeasts arriving by air or via animal-vectored pathways (Klaps et al., 2020). For instance, a part of fungal communities are driven by regurgitation of collected nectar to moisten and glue the pollen grains to shape corbicular pollen and after the transport to the colony as bee bread (Gilliam, 1997). Moreover, the foraging behaviour of honeybees is also influenced by the volatile organic compounds (VOCs) of the flower that may result from the modification of nectar chemistry by flower-inhabiting fungi. For example, presence of the yeast *Metschnikowia reukaufii* Pitt & Miller (Metschnikowiaceae) leads to the production of distinctive VOCs that increase the attraction of *A. mellifera* to the flower (Rering et al., 2018). Fungi may, therefore, change plant-pollinator interactions and ultimately increase plant fitness by increasing pollination and improving seed production (Yang et al., 2019).

92 Moreover, the presence of fungal microorganisms in pollen and nectar is essential for the
93 proper development, health, and survival of larvae and adult bees (Dharampal et al.,
94 2019; Parish et al., 2020). In contrast, other microorganisms are known to threaten
95 fitness. The parasitic microsporidian *Nosema apis*, for example, reduces the longevity of
96 the colony and bee brood success in *A. mellifera* (Webster et al., 2004).
97 Many perennial questions related to plants and fungi in bee macro-ecology are
98 constrained by technical difficulties in identifying and differentiating organisms from
99 matrices such as pollen (Bell et al., 2022). The advent of high-throughput sequencing has
100 significantly enhanced the detection and quantification of plant and fungal species by
101 DNA metabarcoding of nuclear ribosomal DNA Internal Transcribed Spacer (ITS) region
102 (Richardson et al., 2015; White et al., 1990), improving both the number of samples that
103 can be analyzed and the level of taxonomic resolution achieved (Bell et al., 2022). DNA
104 metabarcoding is a powerful tool to assess the community structure of organisms that can
105 be measured using two approaches: alpha diversity (i.e., within communities) and beta
106 diversity (i.e., between communities). This approach is particularly well suited to
107 uncovering temporal patterns in plant and fungal species richness within bee-collected
108 pollen, offering new insights into the plant-bee-fungal interactions.
109 Previous research revealed that honeybee pollen baskets collected at hive
110 entrances showed changes in the plant community visited during foraging (Danner et al.,
111 2017; Noël et al., 2023). Such variation likely reflects temporal changes in floral diversity
112 and composition and may drive shifts in the flower-associated fungal community.

Dynamic interactions between plants and fungi could, in turn, influence plant-pollinator interactions and the health and fitness of pollinators through a multitrophic interaction . The combination of eusociality and the highly polylectic behaviour of honey bees is, therefore, a great opportunity to discover new associations between fungal and plant communities during the active foraging season. In this context, our study assessed the plant and fungal species richness and composition by DNA metabarcoding from corbiculate pollen loads of honey bees returning to the hive in a non-native environment along a temporal gradient.

2. Materials and methods

2.1. Pollen collection

In 2019, permission to sample pollen from honey bee hives at 13 sites in the city of Toronto and the surrounding region in Southern Ontario, Canada was granted (Figure 1). This region is the economic centre of Canada and is made up of sprawling urban and peri-urban settlements surrounded by agricultural croplands and a large protected natural area gathered under the name, The Greenbelt (www.greenbelt.ca). Each site consisted of 1-10 hives that were managed by local beekeepers. One hive per site was selected for pollen sampling and chosen based on the strength of the colony activity at the end of winter to ensure pollen sampling was not detrimental to colony survival or future generations of honey bees. We assumed that the selected hives had similar colony strength. Of the selected hives sampled from May to September, a plastic pollen trap with a removable trellis was permanently installed at the entrance (Figure S1). At each selected site, pollen samples were collected between 2 to 5 times over the 5 months of sampling to reach a total of 44 samples (Table S1). Pollen sampling occurred on sunny, non-windy days in the fourth week of each month, when it was possible, by inserting the trellis for approximately half a day. The trellis is designed to scrape the corbiculate pollen basket from the hind legs of honey bees entering the hive, causing the pollen to fall into a collection basin. Pollen samples were collected and stored in glass jars at -20°C. To prepare samples for DNA metabarcoding, each defrosted sample was thoroughly mixed by lightly kneading all corbiculate pollen per sample with a mortar and pestle. A single

sub-sample (average mass ± standard deviation/sample = 0.705 ± 0.180g, N = 44) was taken from the total mass of each collected sample.

2.2. DNA extraction, amplification & sequencing

DNA was extracted from each pollen sample by adding lysis solution from the Nippon Gene kit ISOPLANT (Nippon Gene Co., LTD, Tokyo, Japan) and then grinding the mixture at 1500 rpm for 2 minutes using a 'Shake Master Neo' (bms, Shinjuku, Tokyo, Japan). Once ground, the mixture was left to stand at 65°C for 10 minutes. The sample was then centrifuged at 12,000 x g for 1 minute and the supernatant removed. A purification solution and chloroform from the Nippon Gene kit ISOPLANT were added to the DNA solution, which was shaken before being returned to the centrifuge at the same speed for 15 minutes. Once DNA was extracted and cleaned, amplicon libraries were prepared using a two-step tailed polymerase chain reaction (PCR) protocol used by Noël et al. (2023) using primer pair 18S ITS1-u1/5.8S ITS1-u2, amplifying the ITS1 region (Cheng et al., 2016). The ITS1 region serves as a genetic marker for identifying plants and fungi in complex samples, such as honeybee pollen (Blaalid et al., 2013; Wang et al., 2015). The first PCR amplification was coupled with MiSeq-specific adapters and Illumina index sequences. The second PCR amplification was conducted using index primers. The generated library was sequenced using MiSeq Illumina technology (Illumina, San Diego, CA, USA) through a 2 × 300 paired-end run and data compressed as a FASTQ file. Among

163 the 44 samples of the study, two were removed during this process (Table S1) because the
164 sequencing depth did not reach 1,000 reads (Sponsler et al., 2020).
165
166 ***2.3. Bioinformatics***
167 The data were analysed with QIIME2 (Bolyen et al., 2019) by transforming raw sequence
168 libraries into Amplicon Sequence Variants (ASVs). In detail, the raw sequence data were
169 imported using the Casava 1.8. (paired-end) demultiplexed instructions, after which the
170 DADA2 plugin was applied to trim, denoise, and merge paired-end reads of the
171 demultiplexed sequences to improve the quality profiles of the reads. Of the reads, the
172 primers linked to the ITS1 region of each sequence were trimmed. Reads were also
173 truncated at 250 bp for forward and reverse reads based on the information linked to the
174 demultiplexed sequences. The results obtained after processing by the DADA2 process is a
175 community matrix indicating which ASV is present in each sample and hit counts. For the
176 same genetic marker, ASVs were identified by querying two reference databases (i.e.,
177 fungi and plants) with 97% confidence of similarity, usually considered as the species-level
178 threshold (Stackebrandt et al., 1994). This taxonomic classification uses machine-learning-
179 based classification methods with the *classify-sklearn* function in QIIME2. Plant taxonomic
180 classification was performed using a customized reference database called 'Toronto'. The
181 Toronto plant database was based on a list of 1,723 Angiosperm plants (filtered from
182 1,937 vascular plants) present in the city of Toronto (Cadotte, 2021). This exhaustive list of
183 species is most representative for the taxonomic diversity present in this region based on

184 available taxonomic and biogeographic information. We lumped *Lotus tenuis* Waldst. &
185 Kit. ex Willd. (Fabaceae) into *Lotus corniculatus* L. (Fabaceae) as it belongs to a species
186 complex (Grant and Small, 2011). Plant species recorded were determined to be native or
187 non-native using the field guide in Del Tredici (2020) and Cadotte (2021). Of the non-
188 native species, we classified invasive species based on the species list provided by the
189 Early Detection & Distribution Mapping System (EDDMapS) Ontario
190 https://www.eddmaps.org/ontario/species/). For fungi, the reference dataset was
191 retrieved from the UNITE website on February 4th 2021 (Abarenkov et al. 2020) using the
192 RESCRIPt pipeline (Robeson et al., 2021). Each reference database was trained with the
193 naïve-Bayes classifier implemented in QIIME2 and the reference database that
194 taxonomically assigned most ASVs was selected. The taxonomic results were then filtered
195 to remove non-target mitochondrial and chloroplast sequences(Jimenez et al., 2021). The
196 generated community matrix was further filtered to remove ASVs with a frequency of less
197 than 10 hits within and across samples.
198
199 ***2.6. Community and statistical analysis***
200 All data wrangling and statistical analyses were performed using RStudio software (R
201 version 4.0.1; R Core Team, 2020). The number of hits per ASV was considered as
202 quantitative data of the plant and fungal community matrices (Deagle et al. 2019).
203 We calculated both the alpha and beta diversity metric using the *phyloseq* and
204 *microbiome* packages (Lahti and Shetty, n.d.; McMurdie and Holmes, 2013). Alpha

diversity is based on the observed/estimated species richness and indexes within modality, here the sample site. Beta diversity is the differentiation of species composition between independent samples. For alpha diversity metrics, we considered the richness (i.e., the number of distinct ASVs observed) and the Shannon's diversity index per sample. The Shannon's diversity index measures biodiversity (using species richness and relative abundance) as the logged probability that two randomly selected individuals from the same sampling unit belong to different species (Shannon, 1948).

Fungal alpha diversity metrics (i.e., ASV richness and Shannon's index) were first modelled as a function of plant alpha diversity metrics. Given the unbalanced experimental design, mixed modelling was used to explain fungal alpha indexes based on associated plant alpha diversity indexes. The fungal ASV richness and Shannon's index were modelled using Generalized Linear Mixed-Model (GLMM) fitted with Poisson error distribution and Linear Mixed-Model (LMM) fitted with Gaussian error distribution, respectively. Second, all alpha diversity metrics considered in this study were tested using month as a numeric fixed effect and using GLMMs with negative binomial error structure for species richness and LMMs for Shannon's index. In all models, the sampled hive was specified as a random effect. All mixed models were fitted using the package *lme4* (Bates et al., 2015) and *glmmTMB* (Brooks et al., 2017). For all mixed-effects models, assumptions about the residual distributions (i.e. over- and under- dispersion, deviance, heteroscedasticity, uniformity) were checked with the *DHARMa* package (Hartig, 2021).

To measure the dissimilarity between sampled hives (including identified taxa as variables), i.e., beta diversity analysis, the Bray-Curtis dissimilarity index was used after a Hellinger's transformation of the quantitative data. The distance matrix obtained from this index was considered as the response variable in a distance-based redundancy analysis (dbRDA) using the *dbrda* function from the *vegan* package (Oksanen et al., 2012). Sampling month was used as a numeric variable. When significant, a permutation test for dbRDA model was applied using the *anova.cca* function. Pairwise comparisons between months with Holm's correction for multiple testing were performed using *pairwise.perm.manova* function from RVAideMemoire package (Hervé, 2020). To visualise variation in species composition as a function of temporal factor, alternative plotting function (i.e., *ordiplot*), with a scaling (scaling = 2) to show relationships between explanatory variables and species, was used to show the constrained ordination for plants and fungi community compositions. All the graphical representations were generated using the *ggplot2* package (Wickham, 2009).

3. Results

The raw data of pollen load DNA consisted of 2,457,263 reads. After trimming and filtering, the data consisted of 1,664,808 reads and 1,111 ASVs of plants and fungi distributed across 42 pollen samples. A total of 77 plant and 46 fungi taxa were identified from the sampled hives that represent 50.68% of ASVs identified to the species level. At the family and genus level, the ASVs assignation process reached 88.48% and 85.42%, respectively.

3.1. Plant species composition

Among the 77 plant species (Table S2), honey bee workers mainly collected pollen from species that are non-native, herbaceous, and perennial (Table 1). Three non-native species were observed in most samples: white clover *Trifolium repens* (71.4%), field sow-thistle *Sonchus arvensis* (35.7%), and red clover *Trifolium pratense* (28.6%) (Table S2). *Trifolium repens* was the most foraged taxon throughout the sampling period from June to September, while other plant taxa were only foraged once or twice (Figure 2A). *Trifolium repens* was also foraged quasi-evenly across sampling sites (Figure 2B). The three most represented plant families in terms of number of distinct species were: Asteraceae (31 species), Fabaceae (8 species) and Brassicaceae (7 species). Other uncommon, non-native plant species identified included prickly lettuce *Lactuca serriola* (2.4%) and chicory *Cichorium intybus* (7.1%), as well as invasive species, including European buckthorn *Rhamnus cathartica* (2.4%) and garlic mustard *Alliaria petiolata*

(2.4%). We further identified the highly allergenic species giant ragweed, *Ambrosia trifida* (2.4%). Several non-native cultivated species were also identified in a few samples, such as melon *Cucumis melo* L. (2.4%), cucumber *Cucumis sativus* (2.4%), and garlic chives *Allium tuberosum* (2.4%).

3.2. Fungal species composition

Among the 46 fungi taxa (Table S3), the three most commonly observed taxa were: *Starmerella bombicola* (73.8%), *Mucor circinelloides* (66.7%), and *Mucor falcatus* (57.1%). The three most represented fungi families, in terms of number of distinct taxa were: Saccharomycetaceae (6 species), Saccharomycetales – *Incertae sedis* (5 species) and Mucoraceae (5 species). *Starmerella bombicola* and *M. circinelloides* occurred throughout the entire sampling period (Figure 2C). *Mucor falcatus* and *Starmerella jinningensis* occurred only in early summer (Figure 2C). *Starmerella bombicola*, *M. circinelloides* and *M. falcatus* were present in almost all sampling sites (Figure 2D).

3.3. Plant and fungal community structure

Our results indicated that greater alpha diversity of plants is associated with higher alpha diversity of fungi, as shown by an increase in species richness (z-value = 4.30; p-value < 0.001; Figure 3A) and Shannon's diversity index (t-value = 2.26; p-value = 0.026; Figure 3B). Sampling month had a significant positive influence on plant species richness (z-value = 3.42; p-value < 0.001; Figure 4A), plant Shannon’s index (t-value = 4.21; p-value < 0.001;

Figure 4C), fungal species richness (z-value = 3.43; p-value < 0.001; Figure 4B), and fungal Shannon's index (t-value = 2.11; p-value = 0.035; Figure 4D). This means that the progress of the season significantly increased all considered alpha diversity metrics.

For beta diversity analysis, plant and fungal permutation tests from dbRDA models revealed a significant temporal shift in the composition of foraged plant (df = 4; F = 3.37; P-value < 0.001) and fungal species (df = 4; F = 1.47, P-value = 0.046) over the course of the foraging season. In the plant dbRDA model, the month factor accounted for 26.69% of the total variance, whereas, in the fungal dbRDA model, it explained 13.69% of the total variance. Subsequent post-hoc analyses revealed that the plant community in May was distinct from those in other months and, a significant shift in plant composition was also observed between the plant communities in July and August (Holm's adjusted p-value < 0.05; Figure 5A). The second PERMANOVA further failed to detect a significant influence of temporal progression on fungal composition (Holm's adjusted p-value > 0.05; Figure 5B).

4. Discussion

Our study investigated the community structure of plants and fungi based on corbiculate pollen obtained from foraging honey bees returning to their hives located across the city of Toronto and the surrounding region in Southern Ontario. After describing our dataset, we assessed the interactions between plants and fungi in relation to alpha and beta diversity metrics. Our results revealed biodiversity patterns structuring communities resulting from the foraged pollen. We found that honey bees preferred to forage on 59 ornamental non-native plant species (75% of all identified plants; Table S2) of which 11 are considered invasive species. Some plant species visited by honey bees pose a risk to biodiversity, livestock, and humans. This is the case for the invasive European buckthorn, *Rhamnus cathartica* L., a species subject to import and monitoring regulations imposed by the Canadian government. Another invasive species, garlic mustard, *Alliaria petiolata* (M. Bieb.), is aggressively spreading in forests in Ontario (Welk et al., 2002). Giant ragweed, *Ambrosia trifida* L., is highly allergenic during the flowering period (due to pollen) both at respiratory and epidermal level (plaques, itching) (Rasmussen et al., 2017). Some widespread species such as the non-native viper's-bugloss and the native white snakeroot can cause intoxication in livestock (Davis et al., 2015). These findings highlight how DNA metabarcoding of bee-collected pollen can reveal the widespread presence of ecologically and economically invasive plant species across urban and peri-urban landscapes, underscoring the role of honey bees as effective "bio-sampler" of environmental plant diversity (Sponsler et al., 2020).

316 The mycobiota identified from the surveyed corbiculate pollen revealed
317 similarities in species and genera observed in other studies, such as *Starmerella* spp. and
318 *Mucor* spp. (De Jesus Inacio et al., 2021). We further identified a previously unreported
319 fungal genus that could use pollen to be vectored by honey bees, such as *Lachancea* spp.
320 (Kogan et al., 2023). *Starmerella* spp. were the most common yeast taxa in our survey
321 interacting with foraged pollen. The most prevalent taxon was a yeast, *S. bombicola,*
322 isolated first from Canadian bumble bee honey (Spencer et al., 1970). This fructophilous
323 yeast is well studied for the production of secondary metabolites and to a lesser extent
324 for interactions with flowers and pollinators. A recent study demonstrated that *S.*
325 *bombicola* improved food intake by the honey bee larvae when mixed with a standard
326 pollen diet (Canché-Collí et al., 2021). All *Mucor* spp. in this study have been documented
327 to be able to infect humans depending on the biological context (Table S3) but also exist
328 as moulds in floral pollen, corbiculate pollen or bee bread. For example, the second most
329 prevalent fungal species in this study, *M. circinelloides* that was discovered in 31 (out of
330 42) samples (Table S3), is known to cause disease and infection in humans, including
331 mucormycosis and gastrointestinal disorders (Wagner et al., 2019). For the honey bee,
332 other *Mucor* spp. are beneficial: in bee bread, the presence of *Mucor* spp. inhibits the
333 growth of chalkbrood disease, *Ascosphaera apis* (Maasen ex Claussen) L.S. Olive & Spiltoir,
334 by producing antimycotic compounds (Gilliam et al., 1988).
335 We showed that fungal richness and Shannon's index evenness were positively
336 influenced by floral richness and evenness in pollen loads by intrinsic relationships

between both groups (Klaps et al., 2020). Furthermore, while the plant communities visited by honey bees changed over the foraging season with variation in the range of visited flowers, the fungal community seems homogenous. This means that the fungal communities are more similarly distributed across the foraging season, perhaps due to a gap in the taxonomic knowledge on pollen/nectar fungi (Zhou and May, 2023), but also because we showed high dominance patterns in foraged plants, e.g., *Trifolium repens* or *Trifolium pratense*.

The positive temporal trend in plant species richness may be the result of a decrease in food resources by mid-summer, forcing honey bees to forage on a greater number of plant individuals and species (Noël et al., 2023). This trend declines between mid- and late summer, as well as the beginning of fall following the end of the flowering season for many native and relatively common species. The months with the highest plant species richness were July and August, which could be explained by the higher number of samples obtained from the hives during these months. Concerning the foraged plant composition, it is firmly established in the literature that the composition of pollen collected by honey bees undergoes multiple shifts throughout the seasons (Danner et al., 2017; Noël et al., 2023; Sponsler et al., 2020), which could influence fungal community structure within the corbiculate pollen samples.

Fungal richness and evenness also increased throughout the survey period, reinforcing the concomitant pattern with plants: more flower species were visited by honey bees and more fungi taxa were sampled. This pattern aligns with the classical

ecological principle proposed by Preston (1948), where an increase in sampling efforts leads to a rise in the number of observed species and an expansion of sample diversity. More work is now needed to link plant-fungal diversity, as well as that of other microbes, in bee corbiculate pollen.

Flowers are ephemeral organs of the plant, implying that the mycobiota do not develop on specific substrates during the growing season. Fungi may thus rely and depend on animal vector phenology, including hibernating taxa such as bumblebee queens, overwintering honey bee colonies, or solitary bees emerging from natal nests (e.g., Rothman et al., 2019) that could act as reservoirs for flower-inhabiting microbes (Pozo et al., 2018). Traits of pollinators, such as sociality or diet breath, as well as plant traits, such as floral morphology or nectar composition, could further have pivotal roles in shaping mycobiota community structure. Honey bees may forage over large spatial scales to collect resources for the hive. We homogenized corbiculate pollen samples from a pollen trap at the hive entrance, meaning that we may have also homogenized the fungal communities, thereby dampening the contribution of single honey bee foragers. There may thus be more complex community patterns that can only become evident from corbiculate pollen of single foraged plant species or landscape effects. Evaluating pollen samples of single bees could thus help to identify positive and negative interactions that are important for bee health and management, and elucidate more complex interactions between managed honey bees and wild bees (Russo et al., 2021).

Based on personal observations, we know that many non-native plants are visited by native wild bees. In our study, we found only few examples of honey bees visiting native perennial herbaceous plants for pollen, however, although we did identify a few, including boneset, *Eupatorium perfoliatum* L., and cup plant, *Silphium perfoliatum* L. (Table S2), that are promoted by the City of Toronto for restoration to support wild and native bees (Toronto, 2022). Future research can aim to identify foraged plant species visited disproportionally more (or exclusively) by native and wild bees compared to non-native managed honey bees (Hung et al., 2018). Such an effort could help focus research questions on competition between honey bees and wild bees for native floral resources, and enhance conservation efforts (i.e., which species to include in plant mixes; Müller et al. 2024). New knowledge can thus be acquired by repeating the work done here with individual honey bees or wild bee species with more limited foraging ranges, and more divergence in fungal communities.

5. Conclusion

In this study, honey bees tended to forage mainly on non-native plants that are widespread across the city of Toronto and its surroundings. Although two of the most visited non-native plant species, white and red clover (*Trifolium repens* and *T.* pratens) are generally perceived as positive in urban and agricultural landscapes, it is important to note that some overabundant non-native species are pollinated by non-native honey bees

399 that can proliferate and become invasive. With a growth in urban beekeeping as a hobby,
400 the potential to augment pollination functions to current and future invasive plant species
401 warrants attention. Fungal diversity was further concordant with plant diversity in pollen
402 loads sampled at honey bee hives. Future studies can aim to understand and disentangle
403 complex bee-flower-fungal interactions to elucidate outcomes that are positive (e.g.,
404 nutritional benefits to bees, vectorization of fungi) or negative (e.g., proliferation of
405 phytopathogenic or allergy-inducing taxa).

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

CRediT authorship contribution statement

Alicia Decolle: Data curation, Formal analysis, Investigation, Software, Visualization, Writing – original draft. **Ayako Nagase:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Resources, Writing – review & editing. **J Scott MacIvor:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Resources, Writing – review & editing. **Bertanne Visser:** Writing – review & editing**. Frédéric Francis:** Project administration, Supervision, Writing – review & editing. **Grégoire Noël:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Supervision, Validation, Visualization, Writing – original draft.

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Supplementary materials

Supplementary materials associated with this article can be found, in the online version, at doi: XXX

Data availability

Sequencing data were deposited on BioProject with accession number PRJNA851359 in the NCBI BioProject database **(https://www.ncbi.nlm.nih.gov/bioproject/).** All R code and the datasets are available on https://github.com/gregnoel/Plant_Fungi_Pollen_Metabarcoding2022 .

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642 *Table 1. Plant number and proportion according to native status, stratum status, and plant*

643 *lifecycle among all samples.*

	Number of plant species	Proportion of plant species (%)
Native status		
Non-native	48	61.54
Invasive	11	14.10
Native	18	23.08
Stratum status		
Herbaceous	66	84.62
Woody	11	14.10
Plant lifecycle		
Perennial	49	62.82
Biennial	10	12.82
Annual	18	23.08

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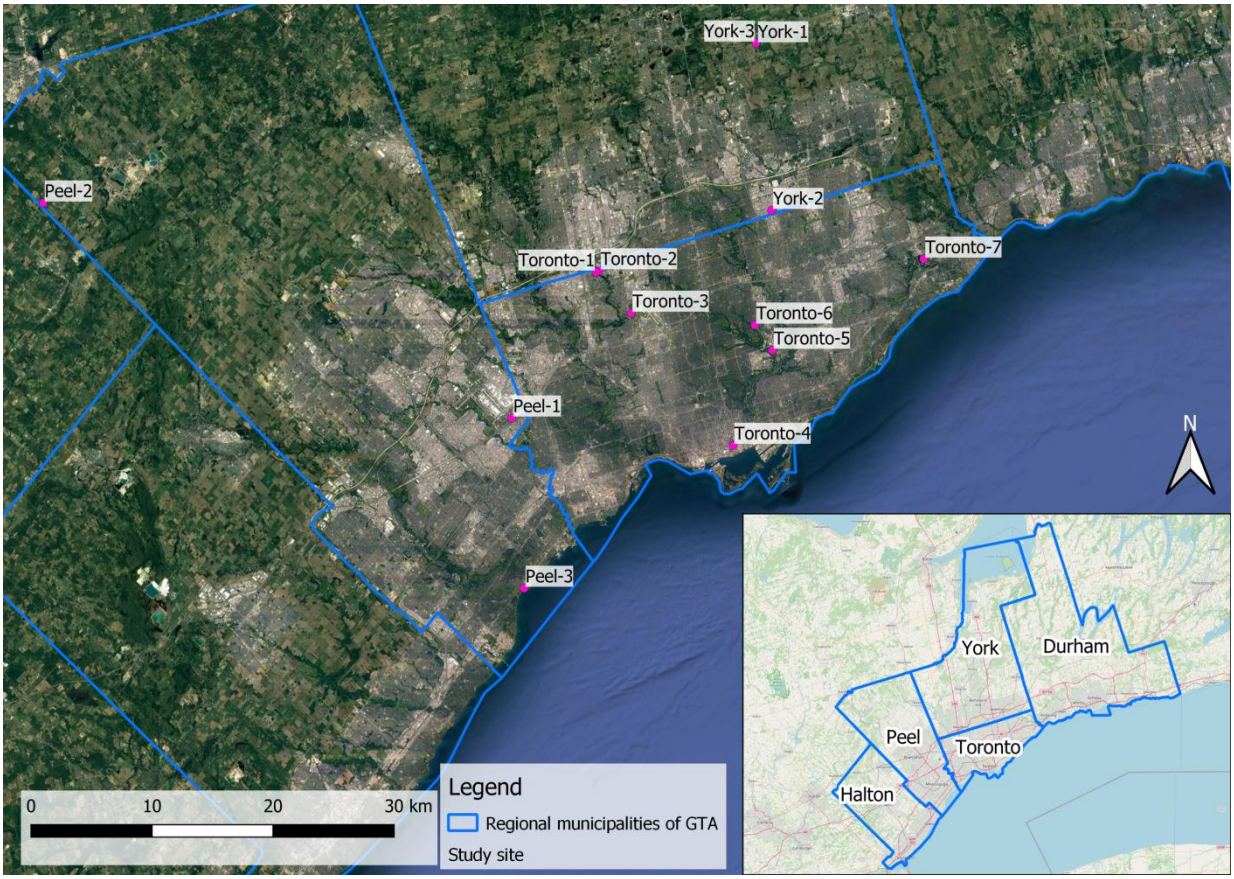


Figure 1. Apiary map. Distribution of the 13 pollen sample collection sites around the Greater Toronto Area, Canada

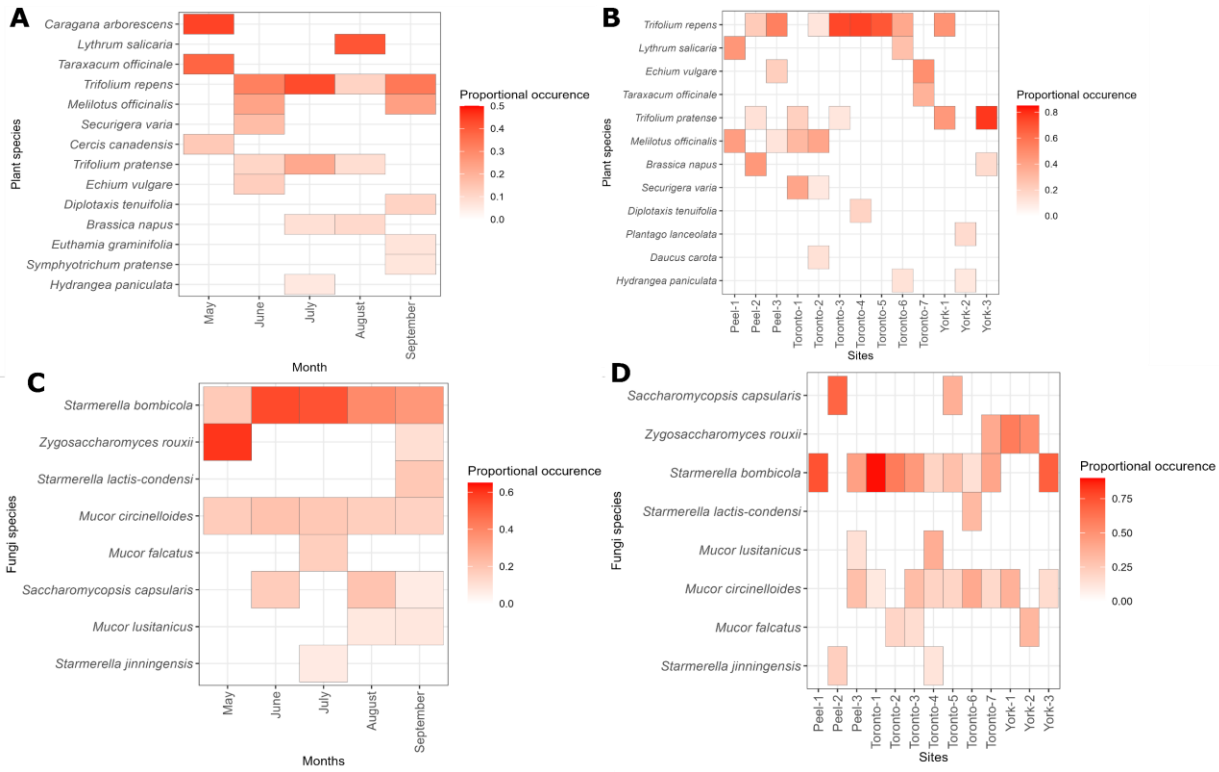


Figure 2. Heatmaps showing the most dominant taxa for plants according to month (A) and site (B), as well as for fungi according to month (C) and site (D). We filtered all taxa occurrences > 5% proportion for plants and fungi according to month (A & C) and all taxon occurrence > 10% proportion for plants and fungi according to collection site (B & D).

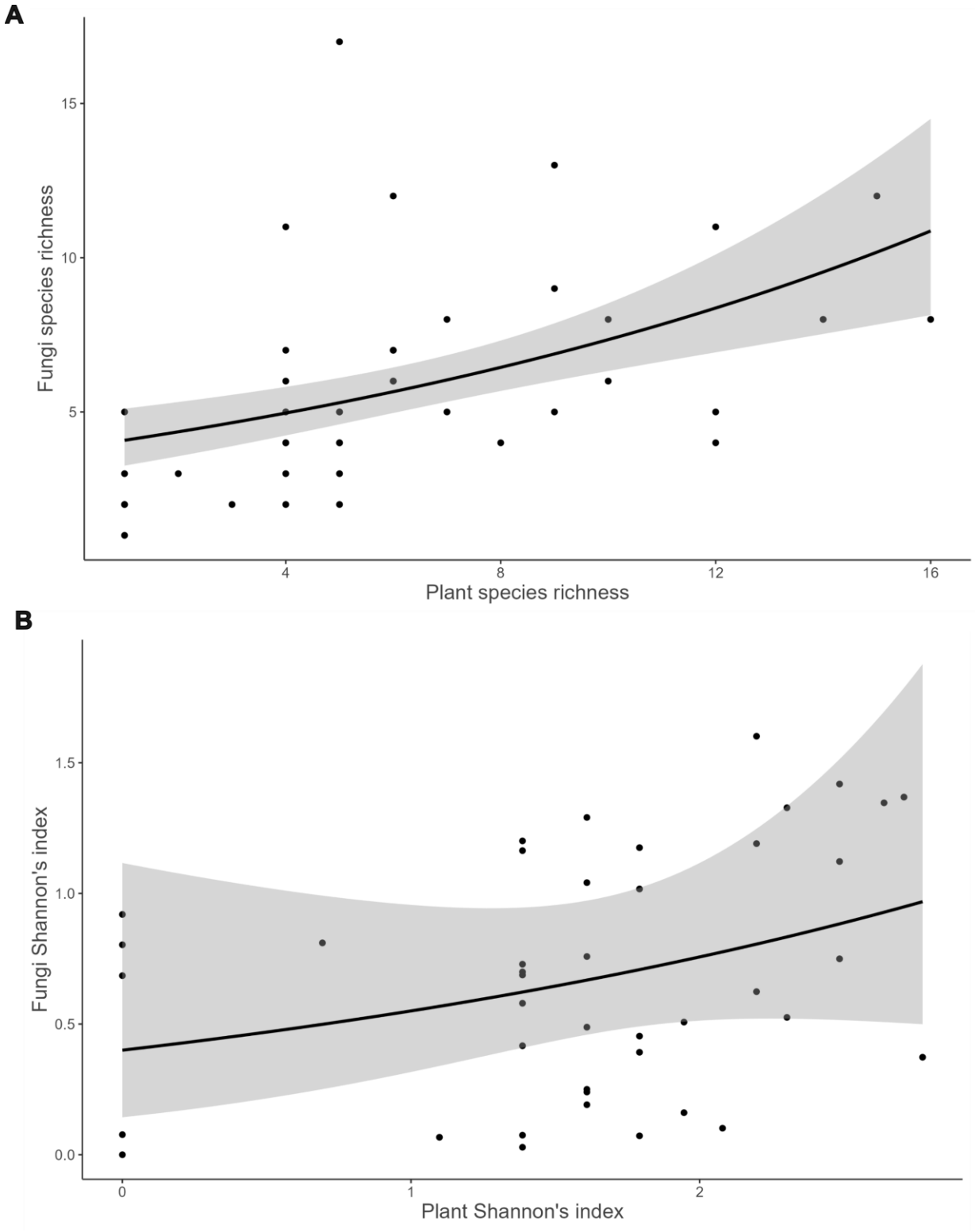


Figure 3: Foraged plant richness and plant Shannon's index effect on fungi richness (A) and

fungi Shannon's index (B), respectively (N = 42). Shaded areas for both graphics correspond to the 95 % confidence interval superimposed on black lines. The black dots of both graphics correspond to pollen samples (n = 42).

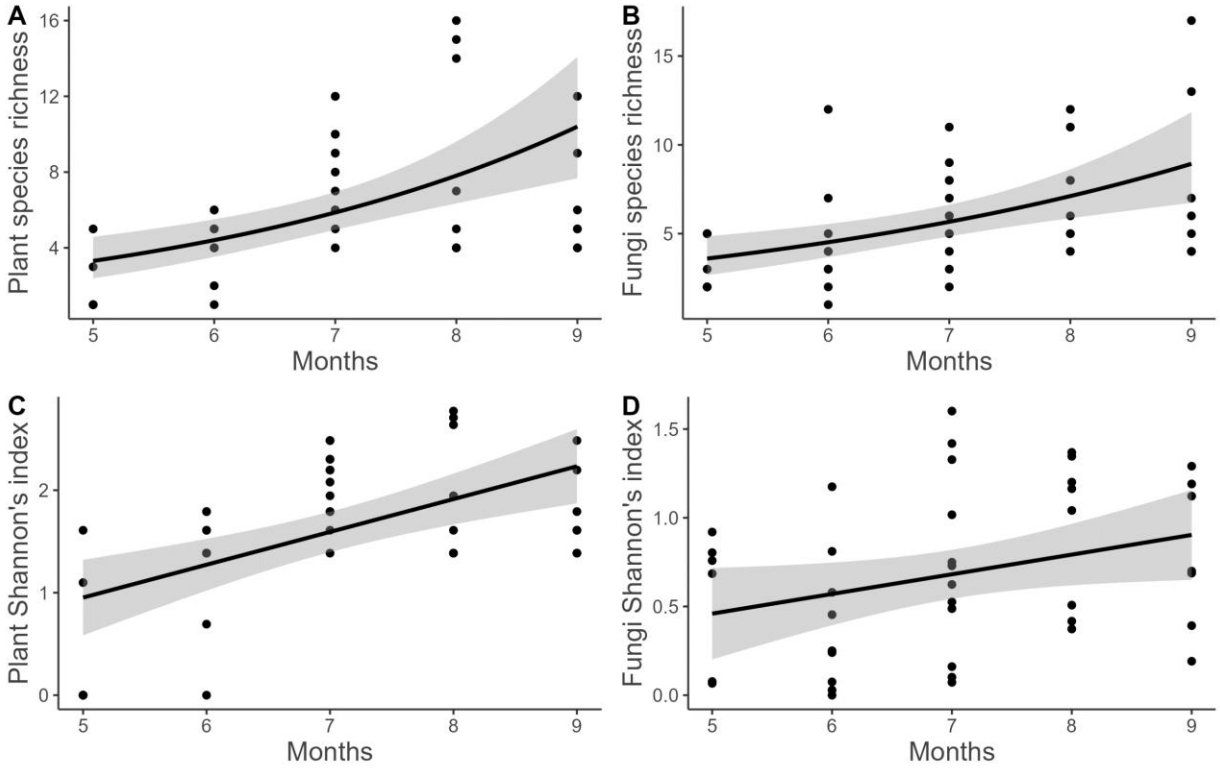
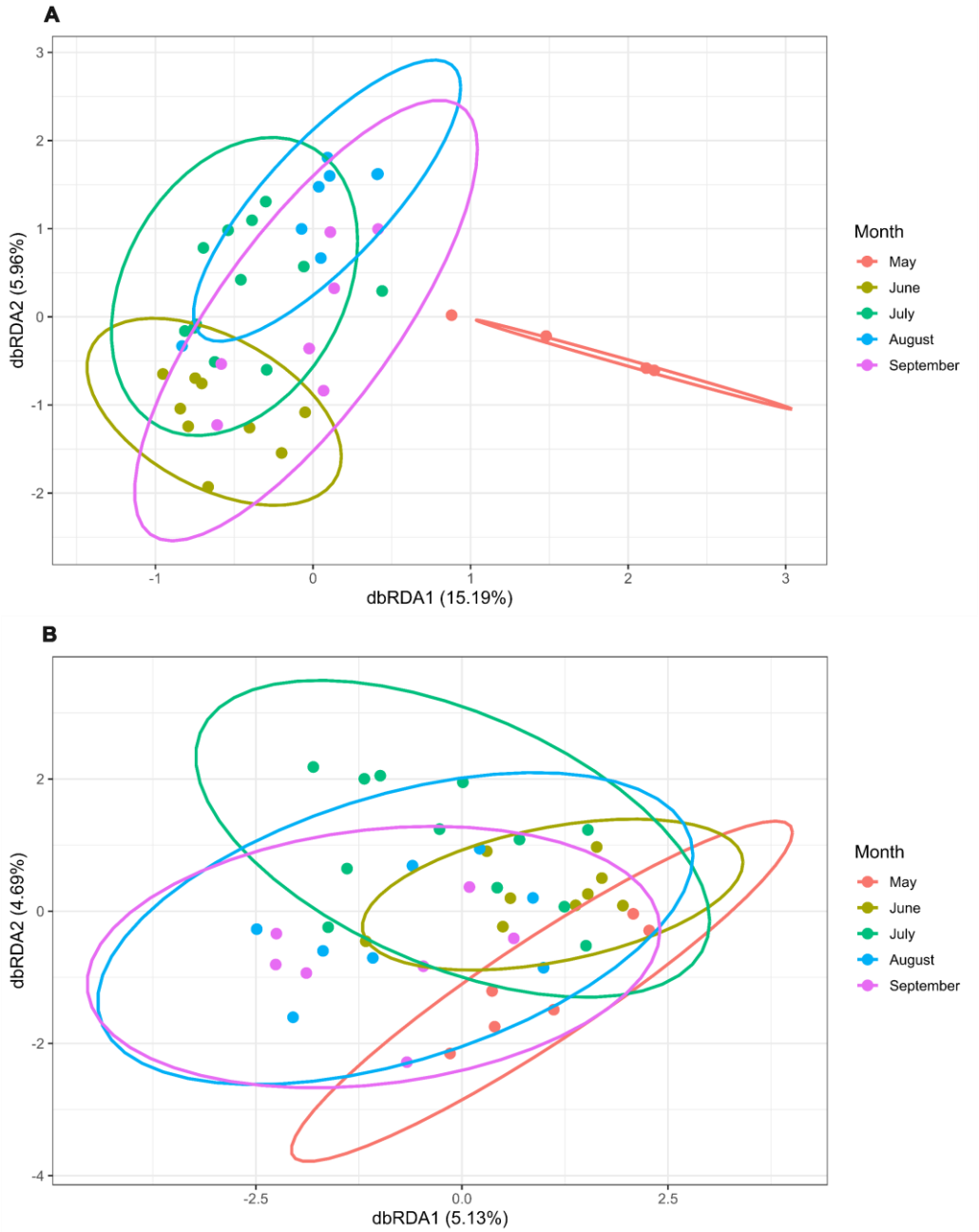


Figure 4: Temporal effect on foraged plant richness (A), fungi richness (B), plant Shannon's index (C), and fungi Shannon's index (D) (N = 42). Shaded areas for both graphics correspond to the 95 % confidence interval superimposed on black lines. The black dots of all graphics correspond to pollen samples.


A
dbRDA2 (5.96%)
dbRDA1 (15.19%)
Month
May
June
July
August
September
B
dbRDA2 (4.69%)
dbRDA1 (5.13%)
Month
May
June
July
August
September

675 *Figure 5: Distance-based redundancy analysis (dbRDA) of plant (A) and fungi (B) communities. Each point corresponds to a*
676 *sample and is colored according to the sampling month that are represented by 95% prediction confidence ellipses.*

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