Apidologie (2024) 55:6

https://doi.org/10.1007/s13592-023-01045-5

Original article

Accessibility and resource quality drive flower visitation patterns among native perennial species

Anne F. Murray[1], Karl A. McKim[1], Amani Khalil[1], Xinlu Chen[2], Feng Chen[2], and Laura Russo[1]

[1] Department of Ecology and Evolutionary Biology, University of Tennessee, Knoxville, TN 37996, USA
[2] Department of Plant Sciences, University of Tennessee, Knoxville, TN 37996, USA

Received 27 July 2023 – Revised 21 October 2023 – Accepted 10 November 2023

Abstract – Pollinators navigate a complex and dynamic nutritional landscape while foraging for floral resources. Bees are a group of flower-visiting insects that rely on pollen as their sole protein source, and thus, bees have strong incentives to seek pollen with high protein content. Indeed, research has shown that bees may prefer to visit flowers with high-protein pollen, but the mechanisms by which bees are able to detect plants with this high-protein pollen are unknown. One hypothesis is that plants with high-protein pollen advertise this resource quality through volatile emissions. We established 17 native perennial plant species from three plant families (Fabaceae, Asteraceae, and Lamiaceae) in a large field experiment to explore the relationship between nutritional quality, inflorescence volatile emissions, and pollinator visitation. We sampled twenty garden plots composed of these native plant species for 2 years. Our results showed that floral morphology significantly affected pollinator visitation; floral morphology that restricted the accessibility of floral resources reduced the overall foraging female bee visitation rate. In contrast, the visitation rate of foraging female bumble bees was higher on plants with floral morphology that restricted access. Moreover, we showed that (1) plants with less accessible inflorescences had significantly higher pollen protein content and (2) lower volatile emissions, while (3) there was a significant interaction between accessibility and pollen protein for foraging female honey bee visitation; honey bees preferred accessible flowers with lower pollen protein. We found no evidence in support of the hypothesis that floral volatiles advertise pollen protein content. Overall, floral accessibility related significantly to both floral volatile emissions and pollen protein content, determining both the identity of floral visitors and affecting the frequency with which they visited.

chemical ecology / floral volatiles / nutritional rewards / native bees / plant–insect interactions pollinators / pollen / floral morphology

1. INTRODUCTION

Pollinators maintain diverse landscapes and productive agriculture (Kremen et al. 2002). Bees are key pollinators and seek nutritional resources including pollen, their primary source of proteins and lipids, and nectar, a source of carbohydrates and, to a lesser extent, amino acids (Council 2007; Vaudo et al. 2016). Bees generally prefer high-quality nutritional resources and visit inflorescences with high pollen protein content at higher rates (Russo and Danforth 2017; Russo et al. 2019; Vaudo et al. 2020; Krishna and Keasar 2018); however, the nutritional requirements and preferences of many native bee species remain unclear. Regardless, nutritional resources support the metabolic activities of bees and are also critical to nest provisioning; larvae that are fed higher protein diets tend to

Corresponding author: L. Russo, lrusso@utk.edu
Manuscript editor: David Tarpy

Published online: 02 January 2024

have better health and developmental outcomes (Roulston and Cane 2000; Vaudo et al. 2018). The quality of these resources varies and depends on the plant family and whether species are native (Russo and Danforth 2017; Vaudo et al. 2020; Roulston and Cane 2000; Venjakob et al. 2022). Because pollen and nectar are the sole nutritional sources for all bees, differentiating between the relative quality of available floral resources is crucial to successful foraging.

Bees are central place foragers that provide their hives or nests with pollen and nectar. Yet it is not well understood how bees assess floral quality and determine which plants or specific inflorescences to visit. Foraging bees respond to visual cues such as floral pigments and morphology (Barragán-Fonseca et al. 2020; Wilson et al. 2017; Gerlach and Schill 1991), and floral morphology varies widely; individuals from Fabaceae have complex features such as keels, while many species in Lamiaceae have inflorescence lips that can limit or exclude pollinators from obtaining floral rewards (Krishna and Keasar 2018). Floral morphology affects pollinator abundance and visitation rate (Thompson 2001). While floral morphology is thought to act as a long-distance cue for visitation (Krishna and Keasar 2018), complexity in floral morphology can also act as a filter. For example, morphologically complex flowers restrict visitors to certain groups of specialists to promote a higher floral fidelity and plant fitness (Krishna and Keasar 2018). Morphological attributes such as corolla tube length and width act as a filter to limit visitation to pollinating insects of a certain size (Stang et al. 2007). A recent review by Krishna and Keasar showed that increases in floral complexity reduced the overall number of pollinator visitors in most systems. To add to the complexity of foraging, pollinators encounter volatile compounds released by plants (Gerlach and Schill 1991). Plants emit volatiles from each of their different tissue types (Dobson et al. 1990; Son et al. 1996), and this collective scent plays a large role in bee attraction and influences visitation preferences (Byers et al. 2014; Kantsa et al. 2019). Many floral volatiles act as attractants, and some of the most well-known and common are the terpenoids β-ocimene and linalool (Dobson 2006). Volatile profiles vary across angiosperm families and are loosely associated with certain orders of bee visitors (Dudareva and Pichersky 2006). For example, a recent study showed general associations between Apidae and Andrenidae bee presence and certain chemical classes such as phenylpropanoid/benzenoids in Mediterranean environments (Kantsa et al. 2019). These volatiles can affect bee behavior and induce them to land and forage (Burkle and Runyon 2019; Dobson 1994; Raguso 2004; Larue et al. 2016).

Further, floral resource quality can be communicated through volatile emissions. For example, the pollen itself often generates a unique odor attractive to bee visitors (Dobson and Bergström 2000). "Honest signals" involve volatile compounds that accurately reflect the nutritional quality of the available floral resources (Knauer and Schiestl 2015; Schiestl 2015). A well-known example is nocturnal hawkmoths following the floral scent of their host plant *Nicotiana* and receiving a nectar reward (Rusch et al. 2016). Similarly, in black mustard (*Brassica nigra*), the emission of phenylacetaldehyde was considered an honest signal because it correlated with nectar availability to visiting *Bombus impatiens*. There are also documented examples where the volatile signal is decoupled from nectar production. For example, in *Polemonium viscosum*, 2-phenylethanol does not correlate with nectar production and can reduce bumble bee visitation (Galen et al. 2011). These signals are species specific rather than universal, and it is unclear if nutritional quality is consistently communicated through floral volatiles to visiting bees (Raguso 2008).

Moreover, though bees exhibit preferences for plants with high-protein pollen, it is unknown whether the protein content of the pollen is communicated through floral volatiles or whether bees require chemotactile cues to determine nutritional quality (Ruedenauer et al. 2023). Though nectar is considered the traditional floral reward for pollinating insects, and thus may be advertised by plants, there is evidence that bees are able to distinguish, and preferentially visit, plants with high-protein

pollen (Russo et al. 2019). To investigate whether volatile emissions signal pollen quality to visiting pollinators, and whether this interacts with the complexity of the floral morphology, we evaluated the pollen protein content of 17 plant species from three flowering families. (1) Asteraceae pollen is thought to have low protein and high lipid content with highly accessible flowers, (2) Fabaceae pollen is thought to have high protein and low lipid levels, but less accessible flowers, and (3) Lamiaceae inflorescences tend to produce small amounts of lower-quality pollen (Vaudo et al. 2020) and provide mixed access. We evaluate the bee community as a whole, as well as two groups of bees with well-studied foraging preferences: bumble bees (*Bombus* spp.) tend to prefer a specific protein-to-lipid ratio, while honey bees (*Apis mellifera*) tend to forage broadly (Vaudo et al. 2015, 2016; Leonhardt and Blüthgen 2012). These two groups of bees also differ in their preferences and ability to taste nutritional aspects of pollen and nectar (Leonhardt and Blüthgen 2012; Ruedenauer et al. 2017, 2021).

Here, we use a large field experiment to study bee visitation to flowering plant species native to southeastern North America. We conducted a common garden experiment replicated across five landscapes over two field seasons to assess bee visitation, pollen protein, and floral volatiles. Our goals were to (1) quantify pollen protein content, (2) analyze inflorescence volatile emissions, (3) test whether volatile emissions signal pollen protein content, (4) determine whether bee visitation relates to volatile emissions and/or pollen protein, and (5) evaluate whether floral morphology affected bee visitation or volatile emission. Our hypothesis was that plant species with higher-quality pollen (i.e., higher protein content) would produce higher concentrations of floral volatiles (an honest signal), leading to higher bee visitation rates.

2. MATERIALS AND METHODS

2.1. Plant material and field sites

In the springs of 2019 and 2020, we established five field sites at four research and education centers owned by the University of Tennessee. Four sites, one at the UT Gardens (Knoxville, TN) (UTG), one at the UT Arboretum (FTREC, Oak Ridge, TN) (UTA), and two sites at the Plateau Research and Education Center (PREC, Crossville, TN) (UTP) were established in 2019. A fifth site was added in the spring of 2020 at the UT Organic Crops Unit (ETREC, Knoxville, TN) (UTO), a hybrid organic/conventional farm (Table S1). We planted four research plots (3 m×2.4 m) at five sites; each plot contained four individuals each of six species of native perennials grown in rows. Individual plants were planted 0.3 m apart for a consistent floral density. Three plots at each site consisted of plants from a single family (Asteraceae, Fabaceae, Lamiaceae), while the fourth was a mixed-family plot comprising two plant species from each family. Plots were located at least 50 m apart, and plot identity was randomized at each site after plot locations were determined. The arrangement and density of plants in each plot were identical at all sites.

Three weeks prior to planting, plots were treated with glyphosate to remove grass and weeds at recommended label rates. We then added soil amendments and planted plugs exclusively sourced from a native plant nursery (Overhill Gardens, Vonore, TN). Plots received 18.9 L of water per week in the absence of natural rainfall and were weeded weekly, and plants were trimmed if they started overtaking other plant species in the plots. At the beginning of each season, plants from the greenhouse were used to replace any field mortality. We added mulch to all plots in 2020 to improve water retention and weed suppression. Although the experiment included 18 native perennials, in total, 17 of these were sampled for pollinator visitation, floral volatile emissions, and pollen protein during their bloom period in 2020 and 2021 (Table I). The last species, *Lycopus virginicus*, produced insufficient pollen to be included in this study.

2.2. Pollen Collection

Pollen samples were collected from all the field sites during each species' respective bloom period. Fresh pollen was sampled in

Table I Plant species included in the study, grouped by plant family. The accessibility of the floral morphology is denoted by either "high" or "low"

Family		
Fabaceae	Lamiaceae	Asteraceae
Thermopsis villosa, Fernald & B.G. Schub., low	*Conradina verticillata*, Jennison*, low	*Coreopsis lanceolata*, L.*, high
Baptisia albescens, Small*, low	*Pycnanthemum muticum*, Pers.*, high	*Helianthus occidentalis var-dowellianus*, Riddell*, high
Lespedeza hirta, Hornem., low	*Lycopus virginicus*, L., high	*Eurybia saxicastelli*, Newsom, high
Baptisia tinctoria, L., low	*Physostegia leptophylla*, Small, low	*Stokesia laevis*, Greene, high
Senna marilandica, L.*, low	*Blephilia subnuda*, Simmers & Kral*, low	*Helianthus hirta*, Raf.*, high
Amorpha herbacea, Walter, high	*Collinsonia canadensis*, L., low	*Verbesina occidentalis*, L., high

*Indicates species that volatile emissions were sampled semi-quantitatively due to limited availability of plant material

the morning from at least three flowering individuals ($n = 3$) per species over two growing seasons (2020 and 2021). Due to the variation in morphology, pollen collection methods differed among the plant species. For *C. verticillata*, *B. subnuda*, *P. muticum*, *P. leptophylla*, *C. canadensis*, *B. albescens*, *B. tinctoria*, *L. hirta*, *T. villosa*, and *A. herbacea*, the entire anther was removed with scissors and placed into 1.5-mL microcentrifuge tubes (Eppendorf). Freshly dehisced pollen was manually scraped into collection tubes from the anthers of *C. lanceolata*, *H. occidentalis*, *E. saxicastelli*, *S. laevis*, *H. hirta*, and *V. occidentalis*. The pollen of *S. marilandica* was collected by applying a sonicating toothbrush directly to the poricidal anthers, releasing pollen into a scintillation vial. All collected pollen samples were immediately set on ice, transported to the laboratory, and stored at −20 ºC until analysis.

2.3. Pollen protein analysis

To quantitate the protein levels in collected pollen, the Bradford protein assay was employed following a modified protocol from (Vaudo et al. 2016). Collected pollen was separated into 1-mg portions and placed in a drying oven (Quincy) for 24 h at 36EightC. After drying, 1.5 mL of 0.1 M NaOH (Fluka) was added to each pollen sample. To fracture the pollen grains, a Microson Ultrasonicator (Misonix Incorporated) probe was submerged into the solution for 90 s, and samples were subsequently stored for 24 h at 5 ºC. Immediately prior to testing, the pollen solutions were centrifuged at $2000 \times g$ for 30 s. The Bio-Rad Protein Assay Kit (Bio-Rad Laboratories) with a bovine γ-globulin protein standard was then prepared following the manufacturer's protocol. The processed samples and the standards were prepared in triplicate and pipetted into a sterile 95-well plate (VWR Avantor). Absorbance readings were taken at 595 nm on a SynergryHi microplate reader using Gen 5.0 software (Biotek). A 5-point calibration curve ($r > 0.97$) was generated to calculate protein levels.

2.4. Evaluating floral access

To evaluate the role of floral morphology to pollinating insects, plants were divided into high- and low-access categories. All of the Asteraceae, *P. muticum* (which had an aster-like inflorescence), and *A. herbacea* (which had exposed anthers) were considered high access. The remaining Fabaceae and Lamiaceae species were considered low access because they had a floral morphology that restricted access to flower-visiting insects, such as keel petals, poricidal anthers, or long narrow corollas (Table I).

2.5. Pollinator visitation

Pollinator visitation data were collected in 2020 and 2021 following a standardized sampling methodology. Surveys were conducted weekly on clear days (April–October) between 9 AM and 5 PM from the first bloom to the final inflorescence for each plant species. Upon arrival at a plot, we recorded the temperature and counted the number of inflorescences per species in bloom. For each collection event, we recorded the time as well as the presence or absence of cloud cover. Each plant species with at least one open flower was sampled using a modified handheld vacuum (Russo et al. 2019). For 5 min, we collected any insects that landed on an inflorescence and contacted the reproductive parts of the plant species being observed. When pollinators landed, we did not make the distinction between nectar and pollen collection. Fresh collection chambers were used for each plant species sampled, and full chambers were labeled and immediately stored on ice. Specimens were stored at −20 ºC until the end of the season, pinned, labeled, and databased. For the purposes of this study, we focused just on bee visitors. Bees were sorted to species level using keys from Discover Life (Discoverlife.org) (Marshall and Marshall 2006), then verified by Sam Droege of the US Geological Survey (USGS), and are vouchered at the University of Tennessee.

2.6. Volatile collections

All volatile samples were taken from inflorescences collected from the UTG plots described above during 2020 and 2021. Nine plant species generated sufficient concentrations of volatile compounds for collection, while the remaining 8 species did not generate detectable concentrations of volatiles from the inflorescence material available. Plant inflorescences were collected 1–2 days after opening, and at least three individual plants were sampled per species ($n = 3$), except for *Baptisia albescens* ($n = 2$) and *Stokesia laevis* ($n = 1$). Whole inflorescences were selected for volatile sampling. For species with clustered inflorescences or flower heads (all Asteraceae, *P. muticum*, *B. subnuda*), whole inflorescences were sampled, while for species with individual flowers (remaining Lamiaceae and Fabaceae), the flower and peduncle were sampled. The inflorescence or flower heads were weighed, and the values were recorded. Samples were removed from field plots with shears, placed in water, and immediately transported to the laboratory for sampling. The transportation window between removing the inflorescence from the field and the sample collection was 20 min. All materials for volatile collections were made between 10 and 11 AM to standardize the time of day in volatile release. In the laboratory, inflorescences were placed inside a closed loop circulating system (ARS) chamber (1 L), and charcoal-purified air was circulated at a rate of 600 mL/min for 5 h. Volatile emissions were collected using Porapak-Q™ air traps (volatilecollectiontrap.com) which were rinsed with 3 mL GC–MS grade methylene chloride prior to use (MilliporeSigma, MA). At the end of the sampling period, air traps were eluted with 200 mL of methylene chloride containing 0.003% 1-octanol (MilliporeSigma, MA) as an internal standard. Volatile samples were then stored at −80 ºC until analysis using a gas chromatography-mass spectrometer (GC–MS). The plant material was dried for 36 h at 65 ºC, and the final dry weight was recorded.

2.7. Gas chromatography-mass spectrometry (GC–MS)

All of the eluted samples were analyzed on a 17A Shimadzu gas chromatograph affixed to a Shimadzu QP5050A quadrupole mass selective detector. Compounds were separated on a Rxi-5Sil MS column (30 m × 0.25 mm i.d. × 0.25 µm thickness) (Restek). The carrier gas was helium at a flow rate of 1 mL/ min. The samples (5 µL) were manually loaded in the injection port (splitless injection) at a temperature of 250 ºC. The temperature program starting at 60 ºC held for 6 min and then ramped at 5 ºC/min to 300EightC for a final hold of 10 min. The MS was operated in electron ionization (EI) mode

at an energy of 70 eV and at a scan range of *m*/*z* 43–350. The identity of compounds was determined by calculating retention indices (RI) (Adams 2007) and comparing mass spectra (MS) to those in the NIST MS library and to authentic reference standards when available. The emissions were calculated by normalizing the area of each compound peak, relative to the area of the internal standard, and then dividing that value by the final sample dry weight (gram). We report the volatile emissions as relative units since authentic standards were not available to quantify each individual compound.

2.8. Data analysis

To assess bee preference, we focused on (A) foraging female honey bees (*A. mellifera*) only, (B) foraging female bumble bees (*Bombus* spp.) only, and (C) foraging females of all other bee species (Table S2). The visitation rate was calculated for each of these groups by dividing the number of visiting bees by the size of the floral display (number of inflorescences multiplied by floral surface area) during each sample. We measured floral surface area by using digital calipers to measure the diameter of 20 randomly selected inflorescences of each species and taking the average surface area of these 20 inflorescences. R (R core Team 2020) was used to run a non-metric multidimensional scaling (NMDS) analysis and general linear mixed-effects model (GLMM) analyses. We also tested for correlations between bee abundance and species richness (count) and bee abundance and pollen protein using Pearson's correlation coefficients. Floral morphology (high and low access) was also evaluated as a fixed effect for pollinator visitation.

We used linear models to test the effect of plant family and floral accessibility on pollen protein, visitation rate, and floral volatiles. We used GLMMs to test the effects of pollen protein, floral volatiles, and floral accessibility on the visitation rate of foraging female bumble bees, foraging female honey bees, and all other foraging female bees. We used presence-only visitation data for each group because the data were zero-inflated and required a log transformation for model fit. Volatile compound data were also log-transformed for linear model analyses. We tested for significant interactions between floral accessibility and both pollen protein content and volatile emissions and removed the interaction terms when they were not significant. We used plot identity as the random effect. We also ran a GLMM to test whether there was a linear relationship between floral volatile production and pollen protein content. For the dataset including both quantitated volatiles and pollen protein, we had 19 observations across four plots because not all plant species produced quantifiable floral volatile emissions. For the pollen protein data, we had 149 observations across 20 plots.

3. RESULTS

3.1. Nutritional resources, volatiles, and pollinator visitation vary by plant family

We spent a total of 164 h sampling and collecting 11,176 visiting pollinators, the majority of which were bees (8,203) (74%). The primary pollen collectors in our study were female bees (referred to as foragers hereafter), and we evaluate their visitation patterns. The most common visitors to the field plots were bees from the families Halictidae and Apidae. Overall, bee collections represented 5 families, 31 genera, and 107 species in total. Bee abundance and species richness were also strongly correlated ($r = 0.78$, $P < 0.001$).

Pollen collections yielded 240 samples from 17 plant species. The protein quantitation assays showed that the pollen protein content ranged from 18 to 243 µg of protein/mg of pollen. The family Fabaceae had the highest average protein concentration of 197.7 µg/mg which was significantly higher than either the Asteraceae (mean = 110.8 µg/mg) or Lamiaceae (mean = 103.1µg/mg), which did not differ significantly from one another (Figure 1a). This pattern was consistent across the four field sites, except at the Gardens (UTG) where there was no significant difference

between the families. On an individual level, *Baptisia albescens* (F) had the highest overall pollen protein content (234.3 µg/mg). *Helianthus hirta* had the highest Asteraceae pollen protein at 143.2 µg/mg, and *Blephilia subnuda* had the highest protein levels for the Lamiaceae at 131.8 µg /mg. The individual species had consistent protein levels across all of the field sites except for *B. albescens* and *Helianthus occidentalis*, which had higher ($P < 0.05$) protein levels at the Plateau (UTP) and significantly lower levels at the Arboretum (UTA) and Gardens (UTG), respectively.

Twenty-five volatile measurements were taken from the nine plant species which had sufficient floral biomass for volatile collections. Plant volatile emissions from nine species were identified (Table S3) and then semi-quantitated. In total, 71 compounds were identified from several chemical classes. On average, species from Lamiaceae and Asteraceae had significantly higher emission levels than those from Fabaceae (Figure. 1b). *Conradina verticillata* had the highest average emissions, and the volatile profile we report is similar to previous studies (Dein and Munafo 2019; Gorman et al. 2022). Overall, there was no relationship between pollen protein concentration and average volatile emissions (effect size = −0.002, $P = 0.78$) (Figure 1c). The most commonly detected compounds were monoterpenes, including α-pinene, myrcene, and bornyl acetate, and the sesquiterpene germacrene D. The compound α-pinene was present in most of the species and was often present in the

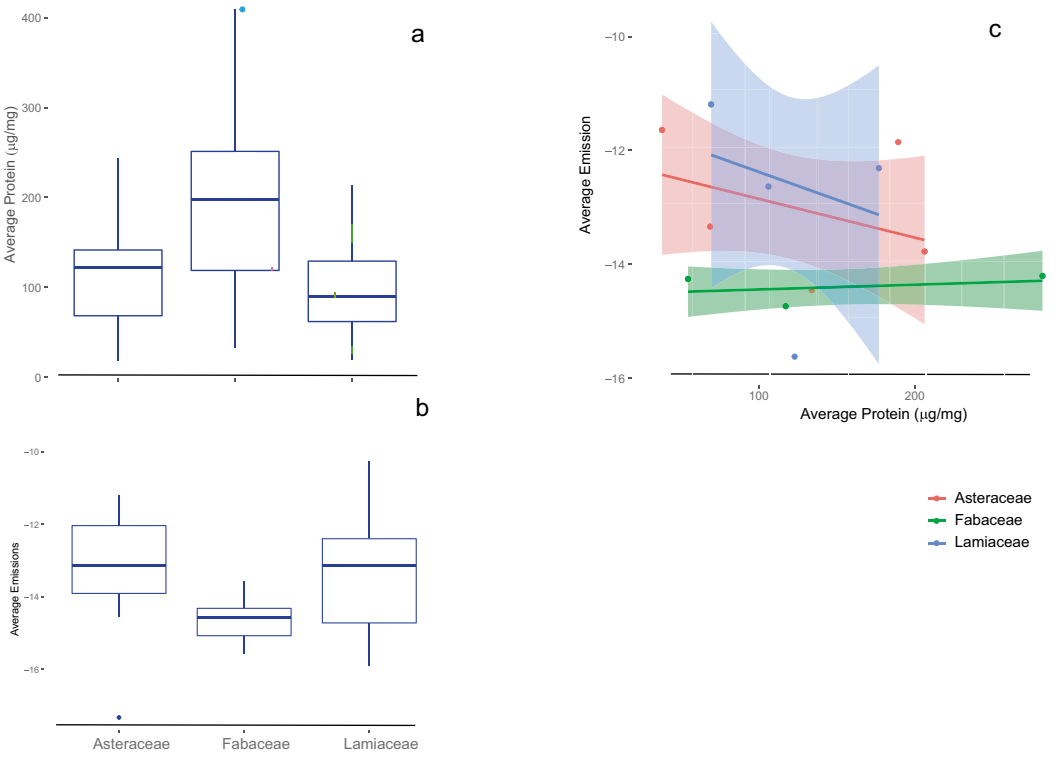

Figure 1 Pollen protein concentrations by plant family (**a**), individuals from Fabaceae had the highest protein concentrations ($P < 0.05$). Volatile emissions by plant family (**b**), individuals from Lamiaceae had significantly higher volatile emissions than the other families ($P < 0.05$). The boxplots represent the median, first, and third quartiles. A GLMM analysis showed no significant relationship between pollen protein concentrations and volatile production in the flowers (**c**)

highest quantities, but this varied. For example, the monoterpene pulegone emitted from *Pycnanthemum muticum* was the highest concentration compound in that species. Overall, volatile profiles differed significantly between plant families (ANOSIM $R = 0.55$, $P < 0.001$), but there was a notable overlap between Asteraceae and Lamiaceae (Figure 2).

Some compounds were unique to a single species, for example, *Coreopsis lanceolata* (A) produced lilac aldehydes, and the *cis*- and *trans*-stereoisomers of Beta-ocimene were detected in *Senna marilandica* (Farré-Armengol et al. 2017). Compounds from the mint biosynthetic pathways were observed, including limonene, isopulegone, pulegone, and menthone, in *P. muticum* and *Blephilia subnuda*. Aldehydes were also detected, such as the fragrant benzaldehyde in *B. subnuda* and phenylacetaldehyde in *C. lanceolata.* The alcohol 1-octen-3-ol was detected in *P. muticum* and *C. verticillata*, and 2-ethyl-1-hexanol was detected in *Senna marilandica.* Green leaf volatiles including E2-hexenyl acetate and E3-hexenyl acetate were tentatively observed in *B. subnuda* and *Stokesia laevis*, respectively. Several sesquiterpenes were detected in individuals from each plant family including those with unknown structures.

3.2. Flower accessibility and pollen protein drive bee visitation patterns among native perennial species

Overall, the plant species with low floral accessibility had higher protein content (effect size = 27.65, $P = 0.0049$), lower visitation rates across all foragers (effect size = −0.27, $P < 0.001$), and significantly lower floral volatile emission (effect size = −1.45, $P = 0.008$) (Table II). The other (i.e., not *Bombus* or *Apis*) foragers visited high-access inflorescences at a higher rate than low-access inflorescences (Figure 3a). We found a significant interaction between accessibility and pollen protein for foraging honey bee visitation rates (effect size = 0.01, $P = 0.03$) (Figure 4c, d). In addition to the interaction, there was a significant negative relationship between pollen protein and female honey bee visitation rate (effect size = −0.001, $P = 0.002$) and a significant decrease in honey bee visitation to low-access flowers (effect

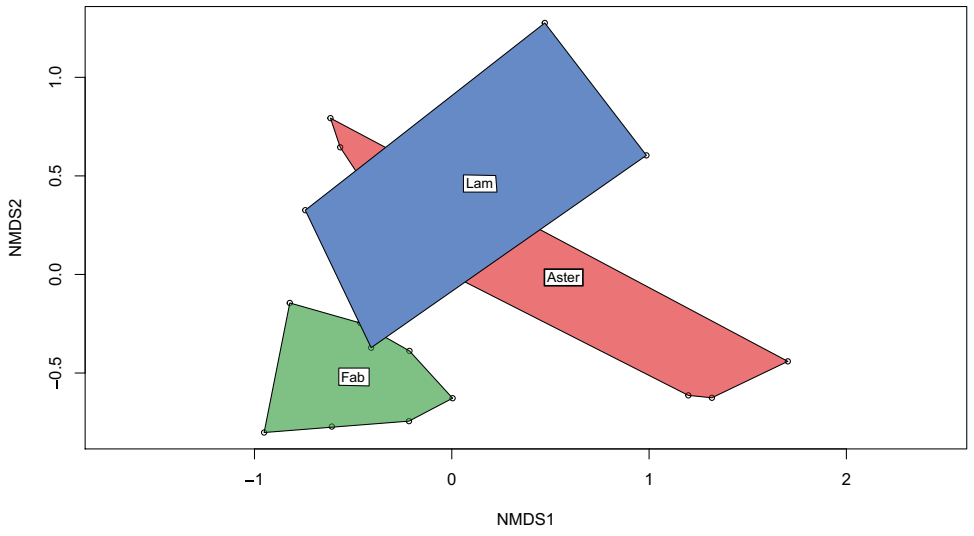

Figure 2 NMDS of volatile compound composition identified in each plant family: Asteraceae (Aster, red), Fabaceae (Fab, green), and Lamiaceae (Lam, blue)

size = −1.74, $P = 0.0009$, Figure 3b). Foraging bumble bees visited low-access, high-protein species more frequently (Figure 3c), but there was no significant interaction between accessibility and volatile production for foraging bumble bee visitation rates ($P > 0.05$) (Figure 4e, f).

Overall, the three plant families were each visited by distinct bee communities (ANOSIM $R = 0.45$, $P < 0.001$) (Figure 5). The genus *Lasioglossum* had the greatest overall abundance, visited the most plant species, and had the highest number of species (25) of all genera collected in the study. The three most abundant species collected were *Halictus ligatus/poeyi* (1,347), *Apis mellifera* (1,221), and *Bombus impatiens* (929). While the majority of bees visiting the Asteraceae were *Halictus* and *Lasioglossum*, visitation to the Lamiaceae was dominated by *Lasioglossum* and *A. mellifera*. Meanwhile, the genera found visiting the Fabaceae were largely those that could buzz-pollinate (e.g., *Bombus* and *Augochloropsis*) or open keel petals (e.g., *Megachile*), likely as a result of the accessibility as discussed above.

4. DISCUSSION

This study illustrates the importance of floral morphology and accessibility in relation to factors that may drive bee visitation, such as floral volatiles and pollen protein. Plants with less accessible inflorescences had significantly higher protein content and significantly lower overall bee visitation. Overall, we show that, rather than advertising high-protein pollen, plants restrict access to that resource and do not exhibit higher floral volatile production. In contrast, it appears that inflorescences with low-protein pollen have highly accessible flowers. In other words, we show that in these species, floral volatiles do not serve to communicate high-protein pollen to visiting insects. Further, accessibility interacts with the effects of pollen protein on honey bee visitation rates. Therefore, the mechanisms by which bees are able to detect or forage preferentially on plants with high-protein pollen remain unknown.

4.1. Nutritional resources, volatiles, and pollinator visitation vary by plant family

Our study shows that three focal plant families (Asteraceae, Fabaceae, Lamiaceae) exhibited significant differences both in their volatile production and pollen protein content. Notably, individuals from Lamiaceae and Asteraceae had a significantly higher floral volatile production than those from Fabaceae, while the Fabaceae had a significantly higher pollen protein content than either the Asteraceae or Lamiaceae. The higher levels of volatile emissions found in Lamiaceae individuals have also been reported in Mediterranean ecosystems (Kantsa et al. 2019).

The range of volatile compounds emitted by the plant species reflects the diversity and complexity of olfactory sensory cues bees encounter while foraging (Schiestl 2015). The composition of the floral volatiles emitted was most similar within plant families, with overlap between Asteraceae and Lamiaceae. The volatile profile of Asteraceae and Lamiaceae plant species was dominated by monoterpenes, while Fabaceae individuals released mainly sesquiterpenes. The number of compounds collected varied, but generally, Lamiaceae individuals produced the highest number and most diverse structures, while those from Fabaceae emitted fewer compounds on average. The volatile profiles published here to the best of our knowledge have not yet been reported except for the mountain mint (*P. muticum*) and Cumberland rosemary (*C. verticillata*) (Dein and Munafo 2019; Murray et al. 2020). The biological role of the detected volatile compounds varies; for example, the cis-Beta-ocimene has been reported as an attractant to species of *Bombus* (Farré-Armengol et al. 2017) and was the major compound in *S. marilandica* (Fabaceae) which experienced high visitation rates from five bumble bee species. The lilac aldehydes detected in *C. lanceolata* are derivatives of linalool oxides and are reported attractants to nocturnal moths (Ilc et al. 2016).

Studies on honey bees have shown that below a certain threshold, they responded to the presence of volatiles, but they were not able to

Table II Results from the generalized linear mixed-effects models (GLMM) and linear models (GLM). Here, we include model structure with the response variable and its transformation, the fixed effects (including contrasts for categorical effects), the random effects, and the number of observations. We report the model output including the effect size, *T* value, *P* value, and marginal and conditional R^2. For GLMMs, the marginal R^2 reports the variance explained by the fixed effects, while the conditional R^2 reports the variance explained by the whole model, including random effects. Significant fixed effects are indicated in bold. (BB = foraging bumble bees, HB = foraging honey bees, Other = foraging bees that were not bumble or honey bees, VR = visitation rate, A = Asteraceae, F = Fabaceae, L = Lamiaceae)

Response	Fixed Effect	Contrast	Random effect	Observations (obs)	Family	Effect size	*T value*	*P value*	R^2m	R^2c
Effects on protein										
Protein	**Family**	**A-F**	Year	149 obs, 2 Years		84.6	5.75	**< 0.001**	0.24	0.26
		A-L				−8.22	−0.58	0.56		
	Access	**High-low**	Plot	149 obs, 20 Plots	Gaussian	27.65	1.97	**0.049**	0.03	0.11
Effects on volatiles										
Volatiles	**Family**	**A-F**				−1.37	−2.29	**0.03**	0.14	
		A-L				−0.15	−0.27	0.79		
	Access	**High-low**				−1.45	−2.97	**0.008**	0.31	
Protein vs. volatiles										
log(volatile)	Protein		Species	21 obs, 9 Species	Gaussian	−0.002	−0.64	0.78	0.008	0.78
Effects on VR										
log (all foragers VR)	**Family**	**A-F**	Plot	984 obs, 20 Plots	Gaussian	−0.36	−3.41	**< 0.001**	0.02	0.04
		A-L				0.05	0.66	0.51		
log (all foragers VR)	**Access**	**High-low**	Plot	984 obs, 20 Plots	Gaussian	−0.27	−4.13	**< 0.001**	0.02	0.04
Volatiles only										
log (other foragers VR)	log(volatile)		Plot	141 obs, 4 Plots	Gaussian	0.07	1.13	0.09	0.009	0.09
log (BB VR)	log(volatile)			72 obs	Gaussian	0.11	1.51	0.14	0.12	
	Access	**High-low**				0.83	2.6	**0.01**		
log (HB VR)	log(volatile)			34 obs	Gaussian	0.46	1.92	0.06	0.27	
	Access	High-low				0.02	0.03	0.98		
Protein only										

Table II (continued)

Response	Fixed Effect	Contrast	Random effect	Observations (obs)	Family	Effect size	*T value*	*P value*	R^2m	R^2c
log (other forager VR)	Protein		Plot	680 obs, 20 Plots	Gaussian	−0.0006	−1.3	0.19	0.06	0.07
	Access	**High-low**				−0.42	−5.74	**< 0.001**		
log (BB VR)	Protein		Plot	173 obs, 20 Plots	Gaussian	−0.0007	−0.9	0.37	0.06	0.2
	Access	**High-low**				0.39	3.15	**0.002**		
log (HB VR)	**Protein**		Plot	110 obs, 13 Plots	Gaussian	−0.001	−3.04	**0.002**	0.14	0.29
	Access	**High-low**				−1.74	−3.32	**0.0009**		
	Access: protein					0.01	2.24	**0.03**		

differentiate between individual compounds, yet as the concentrations increased, the bees were able to differentiate between compounds (Wright and Smith 2004). Although honey bees are likely deterred from visiting low-access flowers by physical restrictions (Balfour et al. 2013) (honey bees cannot buzz-pollinate, have relatively short tongues, and seem deterred by keel petals) (Giovanetti and Aronne 2013), their lack of sensitivity to lower emission levels may also explain their lower abundance on the Fabaceae. Honey bees have more olfactory receptors than solitary bees (Robertson and Wanner 2006; Karpe et al. 2017) and may be more sensitive to stronger floral signals. Much work has been done on the chemotactile sensitivity of these bees. Honey bees have been shown to be sensitive to nutritional resource quality and are able to distinguish between amino and fatty acid concentrations of pollen in behavioral assays (Ruedenauer et al. 2021). Similarly, *Bombus* can detect several amino acids via their antenna but cannot determine differences in concentrations (Ruedenauer et al. 2019), and their foraging preferences are distinct from those of honey bees (Leonhardt and Blüthgen 2012).

4.2. Effects of floral attributes on bee visitation

The relationship between bee abundance and inflorescence nutritional quality is well documented, yet it remains unclear how pollinators assess nutritional quality and make decisions to land and forage. In our study, plant species with the highest pollen protein concentrations had low volatile emission levels (and few compounds) and experienced some of the lowest overall bee visitation, abundance, and richness. In this case, it appears that these differences can be attributed to restrictive floral morphology because the accessibility of the flowers had a significant relationship with volatile emissions and pollen protein content. For example, while the Fabaceae had the highest pollen protein, many of these species have a keel petal which excluded certain pollinators. Foraging bumble bees were the primary visitors to the Fabaceae in our study,

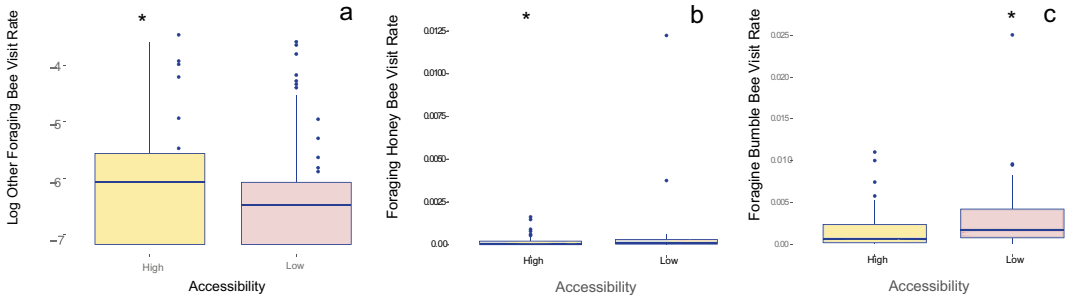

Figure 3 Box and whisker plots of visitation rates of all other foraging bees (**a**), foraging honey bees (**b**), and foraging bumble bees (**c**) to plant species grouped as either high or low accessibility. Significantly higher visitation is indicated by asterisk (*) ($P < 0.05$). The boxplots represent the median, first, and third quartiles, and the dots are outliers

likely due to this restrictive floral morphology. Notably, *S. marilandica* (Fabaceae) relies on buzz pollination, so it requires visits from bees that are capable of vibrating the anthers sufficiently (De Luca and Vallejo-Marín 2013). All of the Fabaceae species had overall low visitation rates, perhaps as a result of the combination of low emission levels and physical barriers to entry.

Foraging honey bees did not appear to be attracted to pollen protein content but preferred plants with accessible flowers and preferred the highly aromatic Lamiaceae. The preference for Lamiaceae was notable because these plants produced pollen with lower protein content, suggesting that honey bees either could not detect or were not prioritizing high-protein pollen. Instead, it is possible that Lamiaceae volatile emissions may indicate the availability or quality of nectar to visiting honey bees. However, behavioral assays would be needed to support this connection. In contrast, the visitation rate of foraging bumble bees was not significantly affected by volatile production, although other studies have shown bumble bees to be sensitive to plant volatiles (Mhlanga et al. 2021; Parachnowitsch et al. 2012). In our study, foraging bumble bee visitation rates were also not significantly correlated with pollen protein, unlike previous studies (Russo et al. 2019; Vaudo et al. 2020). The plant species in this study that had higher pollen protein were also lower-access plants (restricted physical access), but these physical barriers did not deter foraging bumble bees, which are capable of buzz-pollinating and opening keel petals to access nectar and pollen (Stout 2000). The lack of floral emissions in these plants suggests that bumble bees may rely on other cues to land and forage, such as floral morphology. In our study, foraging bumble bees had higher visitation rates on less accessible flowers, suggesting they were affected by floral morphology. However, a review by Krishna and Keasar (2018) found that while bumble bees preferred more accessible flowers with shorter corollas in laboratory studies, floral morphology had little effect on their visitation rates in the field.

The present study offers insight into the factors that influence bumble bee and honey bee visitation. However, the sensitivity of foragers of other bee species to floral volatile emissions remains unclear. Their overall visitation rate was lower on flowers with low accessibility but was not affected by floral volatiles or pollen protein in this study. It should be noted that volatiles can also act in an antagonistic capacity as deterrents, responding to florivores or nectar robbers (Galen et al. 2011; Sasidharan et al. 2023). For example, linalool can repel ants, and isoeugenol and benzyl benzoate can repel florivires (Eilers et al. 2021; Junker and Blüthgen 2008). To protect against florivores, plants can change their emission rates and even scent composition (Kessler et al. 2019). Bees are sensitive not only to the presence of volatiles but also their relative concentrations. In *Polemonium viscosum*, *Bombus* visitors reduce their visitation in

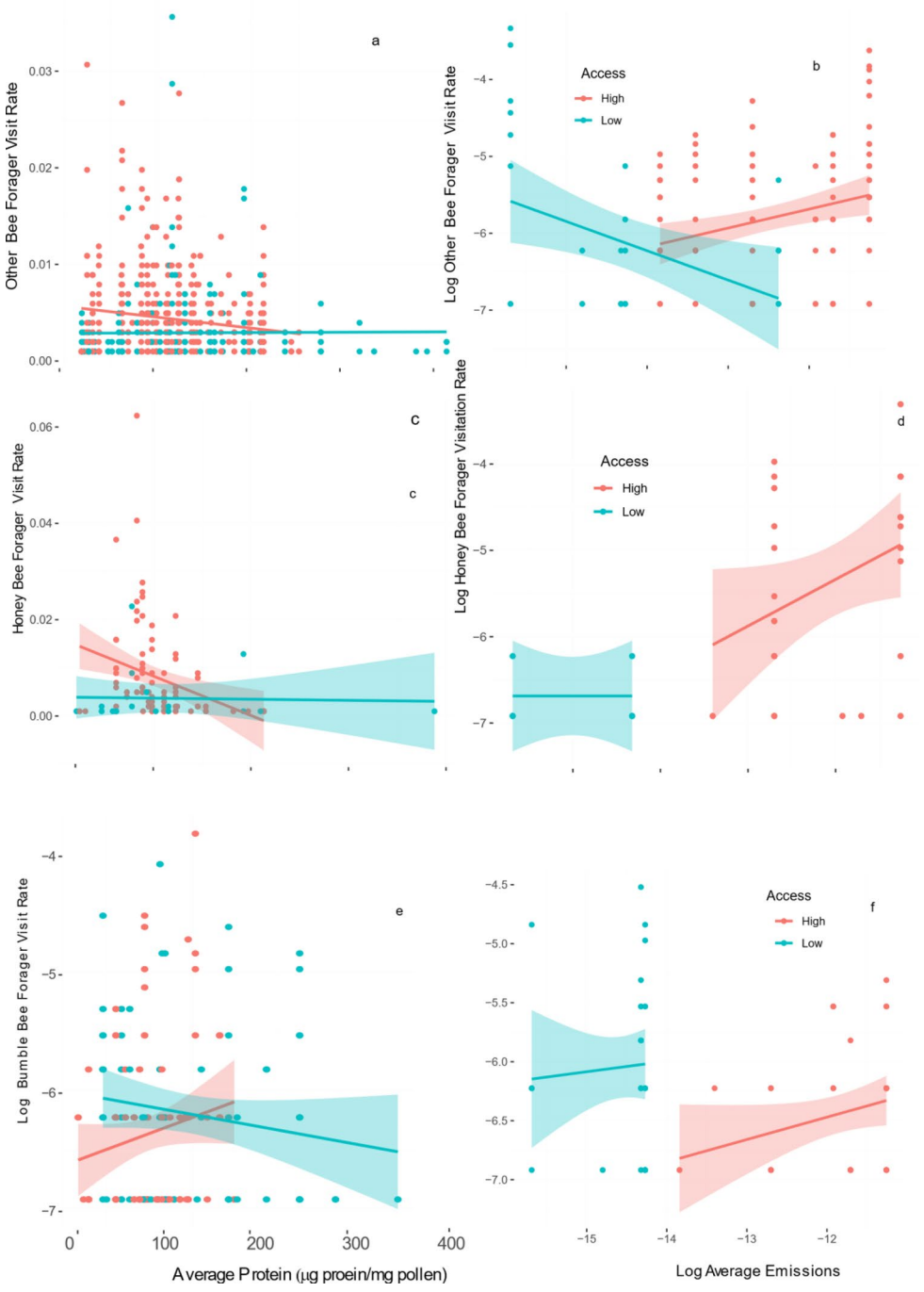

Figure 4 Visitation rates of all other foraging bees (**a**, **b**), foraging honey bees (**c**, **d**), and foraging bumble bees (**e**, **f**) compared to pollen protein content (**a**, **c**, **e**) and quantitated floral volatiles (**b**, **d**, **f**), expressed as average emissions. The accessibility of the flowers is indicated by color (red = high, blue = low)

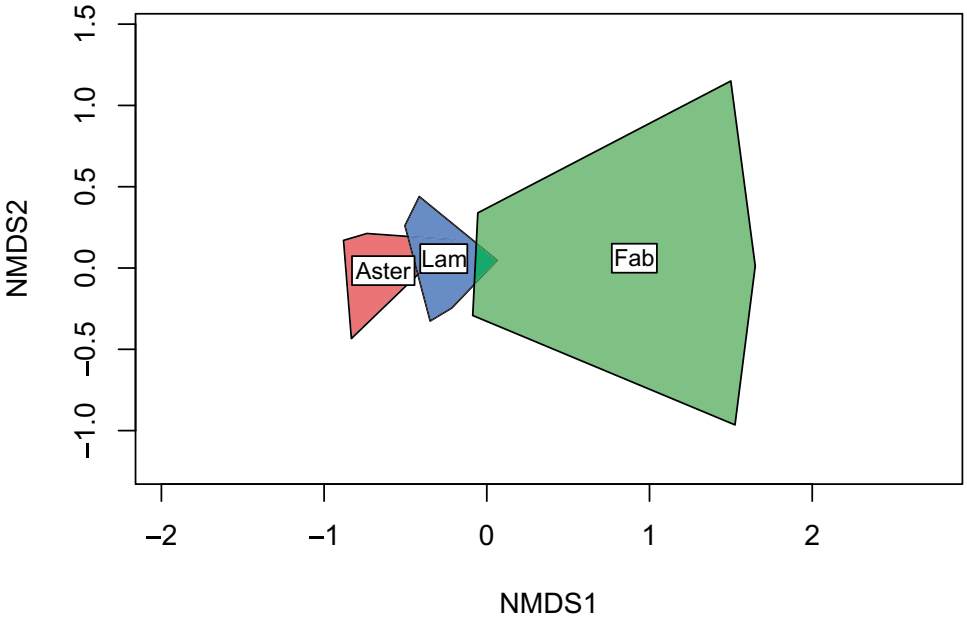

Figure 5 NMDS of bee species composition visiting species from the three plant families: Asteraceae (Aster, red), Fabaceae (Fab, green), and Lamiaceae (Lam, blue)

response to higher concentrations of 2-phenylethanol released to deter ant larcenists (Galen et al. 2011). It has been argued that the reason for floral scent complexity is to present compounds that act as attractants to some visitors or deterrents to others (Kessler et al. 2019; Euler and Baldwin 1996).

Moreover, we have not resolved the question of how bees in general detect plants with high-protein pollen. Instead, volatile cues, including relative emissions or compound composition, may indicate other nutritional qualities, such as nectar carbohydrates or pollen fatty acid levels. Thus, our findings do not suggest that floral volatiles act as an "honest signal" for pollen quality. However, the pattern we observed of low accessibility, low volatile emission, and higher pollen quality suggests plants that restrict access to their high-quality rewards may be investing less in floral volatile signals. It is possible therefore that the absence of a floral signal may indicate quality to insect visitors. The opposite was also true: plants with accessible flowers had high volatile emissions with comparatively lower pollen quality, suggesting an investment in signaling as a means to entice visitors. Studies have shown that systems with diverse pollinators may also have more complex floral diversity as compared to those with lower bee diversity and abundance (Krishna and Keasar 2018). This may suggest that plants restrict pollinator access with implications for complex co-evolutionary relationships (Krishna and Keasar 2018; Rusch et al. 2016).

The role of floral accessibility in mediating these cues suggests it may be important to include other plant families in which nutritional quality and accessibility vary; these could also be explored to establish broader trends in volatile nutritional signaling. It is possible that foragers cued in on attractants over long distances and that the effects of specific volatiles were masked once pollinators arrived at the field site. To elucidate the role of specific compounds, future studies could focus on the individual components of floral scent on pollinator choice and behavior while foraging. Finally, it is possible that this picture may not be complete; foraging pollinators

have highly sensitive olfactory systems and may detect compounds emitted at concentrations too low for our instrumentation to detect.

5. CONCLUSIONS

Bees rely on flowering plants as the sole source of their nutrition, obtaining proteins and lipids from the pollen and carbohydrates from the nectar. Though bee fitness is tightly tied to the protein content of the pollen they collect and consume, their ability to detect which inflorescences are producing high-quality pollen is still unknown. In our study, we showed no relationship between floral volatile production and pollen protein. Instead, volatiles seemed to be unrelated to pollen protein levels. Moreover, the accessibility of the floral morphology related significantly with both the floral volatiles and pollen protein. For example, bumble bees preferentially visited low-access flowers, which produced high-protein pollen. Our study has broad implications for the mutualism between plants and their pollinating insects, illustrating that plants with high-protein pollen likely target specific pollinators by physically restricting access to floral resources.

SUPPLEMENTARY INFORMATION

The online version contains supplementary material available at https://doi.org/10.1007/s13592-023-01045-5.

ACKNOWLEDGEMENTS

We would like to thank the University of Tennessee (UT), the UT Institute of Agriculture, the UT Gardens, and the UT Ag Research and Education Centers, including the Plateau AgResearch Center, the Organic Crops Unit, and the Forest Research and Education Center, for allowing us to do research on their lands, especially K. Hoyt, B. Simpson, W. Hitch, H. Jones, J. Newburn, and W. Lively. We would like to thank J. Martin for their help with the greenhouse plants. We would also like to thank D. Matheson and S. Collins for field assistance and N. Oldham for assisting with hoverfly identification. Thanks also go to S. Droege for lending his expertise in bee identification. We are also grateful to Bayer, for the Feed-A-Bee grant that provided funding for the plants in our Feed-A-Bee research plots. Thank you to the Department of Ecology & Evolutionary Biology at the University of Tennessee for the support of this project.

AUTHOR CONTRIBUTION

AFM, LR, and FC conceived the ideas and designed the methodology; AFM, KM, XC, and AK collected the data; AFM and LR analyzed the data; AFM and LR led the writing of the manuscript. All authors contributed to drafts and gave approval.

FUNDING

Funding was provided by Bayer, Feed-A-Bee grant to LR, which provided funding for the plants in our research plots.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CODE AVAILABILITY

The codes used to analyze these results are available from the corresponding author upon reasonable request.

DECLARATIONS

Ethics approval Not applicable.

Consent to participate The study did not include human subjects, so consent to participate was not applicable.

Consent for publication All authors consent to publication.

Conflicts of interest The authors declare no competing interests.

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