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EXAMINING THE FACTORS UNDERPINNING THE DIFFERENTIAL SUCCESS OF WILD BUMBLE BEE SPECIES AND THEIR RESPONSES TO ANTHROPOGENIC CHANGE

A Thesis in

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John Cody Feuerborn

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The thesis of John Cody Feuerborn was reviewed and approved by the following:

Heather M. Hines
Associate Professor of Biology and Entomology
Thesis Co-Advisor

Christina M. Grozinger
Professor of Entomology
Thesis Co-Advisor

C. Guilherme Becker
Associate Professor of Biology

Elizabeth McGraw
Professor of Biology
Program Head

ABSTRACT

Several bumble bee species (Hymenoptera: Apidae: *Bombus* spp.) are showing significant declines in the U.S. and Europe. These declines have been associated with anthropogenic stressors such as climate change, habitat and floral resource loss, pesticide use, and pathogens and parasites. As the main pollinators in diverse natural ecosystems and efficient pollinators of many agricultural crops, bumble bee declines can negatively affect ecosystem function and agricultural production. However, not all bumble bee species are equally impacted, as some species are experiencing moderate to precipitous declines while others are stable or are even expanding their ranges. Identifying the causes of these species-specific differences in response to anthropogenic change requires understanding the diverse factors that limit these species.

This thesis examines the role of phenology, habitat, floral resource use, macronutrient preferences, and heat stress resistance on the differential success of several bumble bee species in eastern Nearctic communities. First, baseline information is obtained on phenology, habitat distributions, relative abundances, and floral visitation patterns for bumble bee species across Pennsylvania. Species vary in their preference for forest versus open habitat, phenology, and season length, with the most abundant species, *Bombus impatiens*, having the latest phenology, the longest seasonality, and broadest habitat breadth. Floral visitation patterns of bumble bees in Pennsylvania indicate that non-native legumes (white clover and crown vetch) are most visited, and there is some evidence of species specialization, such as preference for asters by *Bombus vagans* and common milkweed by *Bombus griseocollis*. Second, this thesis examines whether bumble bee species differ in their pollen macro-nutrient preferences by assessing the protein and

lipid content of pollen they collect. In field settings, species varied somewhat in protein levels collected, but P:L ratios were similar, averaging at 4.7:1. Bees collected the highest protein from the site with the highest plant diversity and highest available protein, and bees obtained higher ratios in sites in Oklahoma versus Pennsylvania due to an abundance of a protein-rich native legume, the partridge pea. Bumble bees typically collected higher protein pollen than the average of what was available from the plants flowering at the sites, while pollen lipid concentrations collected were more similar to what was available. These data suggest bumble bees selectively forage for higher protein but what they attain depends on what is available in the environment. Finally, the role of several biological factors with potential for contributing to heat stress resistance was examined in Pennsylvania bumble bees. Species that are common in warmer valleys and the lowest median latitudinal ranges were able to withstand heat stress for the longest period of time, whereas cooler-adapted boreal species were the least heat tolerant, suggesting heat tolerance is likely a key factor limiting their distribution. Many other biological factors were found to influence heat tolerance: queens were less heat tolerant than workers and males, recently eclosed adults were less heat tolerant than older bees, queens with activated ovaries were marginally less heat tolerant than queens with inactivated ovaries, larger bees were less heat tolerant in wild foragers, and higher levels of humidity decreased heat tolerance in both workers and queens.

This thesis provides detailed analysis of multiple behavioral, physiological and nutritional factors that influence how different bumble bee species respond to variation in forage quality, habitat type, and weather. The insights gained from these studies can be used to develop habitat restoration strategies to support declining bumble bee species and diverse bumble bee communities.

Table of Contents

LIST OF FIGURES

LIST OF TABLES

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PREFACE

Chapter 3 of this thesis has been published as a peer-reviewed research article in *Ecology and Evolution* (November 28, 2023) and is titled, "***Variance in heat tolerance in bumble bees correlates with species geographic range and is associated with several environmental and biological factors*****"**. This is a multi-authored work of which I am first author. My contributions to this work include carrying out the majority of thermal tolerance assays, setting up experiments, writing the original draft for the manuscript, rearing a portion of the bumble bee colonies used for assays, and collecting most wild bumble bees used for assays. Additionally, I was responsible for data curation, statistical analysis (including climate niche modeling), and the creation of tables and figures. I also trained a fellow author in queen ovary dissections and conducted some of the dissections myself. Furthermore, I assisted my PI in conceptualizing research questions and experimental design and supported the authors with manuscript review and editing.

Introduction

Bumble bees are comprised of ~270 species that are distributed throughout the Northern Hemisphere and South America where they provide vital pollination services in both natural and agricultural settings (Goulson, 2010; P. H. Williams, 1998). Bumble bees are cold adapted insects that possess facultatively endothermic thermoregulatory capabilities where they can use shivering to heat their thorax and selectively shunt their hemolymph across their body to allow them to regulate their body temperature relative to the environment. Along with their characteristically large body size and thick pile (hair), this allows these bees to be prominent pollinators of arctic, alpine, and temperate regions, although a few bumble bee species are found in warmer areas such as the tropics (Hines 2008). This also allows bumble bees to forage early in the morning at low temperatures where smaller bees would be unable to do so, regulate the temperature of their nests, incubate developing brood, and shed excess heat when thermally stressed (Goulson, 2010; Heinrich, 2004).

An estimated 87% of flowering plant species (Angiosperms) are pollinated by animals (Ollerton et al., 2011), with the main pollinators being bees (Khalifa et al., 2021). The economic value of crop pollination by bees has been notoriously difficult to assess (Danforth et al. 2019), but the most recent estimate for the U.S. indicates that wild bees, including bumble bees, contribute $3.44 billion to the U.S economy (Calderone, 2012), and wild bumble bees are one of the most important crop pollinators in the US and Europe (Goulson, 2010). Bumble bees are capable of buzz pollination (or sonication), where they contract flight muscles producing vibrations that dislodge pollen from anthers, making them more effective pollinators than honey bees for many plant species (Goulson, 2010). Solanaceous plants such as tomatoes, peppers and potatoes require sonication for pollen to be released from poricidal anthers, and managed bumble bee colonies are commonly used in the pollination of these crops (Goulson, 2010; Kwon &

Saeed, 2003). Other crops that benefit from bumble bee pollination include blueberries, cranberries, kiwifruit, field beans, cucumber, apples and pumpkin (Buchmann, 1985; Button & Elle, 2014; Goulson et al., 2008a; Khalifa et al., 2021). Bumble bees are also the most important pollinators for many wild flower species in the northern hemisphere with some plant species depending entirely on bumble bee pollination (Goulson et al., 2008a; Goulson, 2010). As social and long-season species, bumble bees can reach high abundances that have led to many plants co-evolving with these bees. Bumble bees are among the most well-connected bees in plant-pollinator interaction webs, helping to maintain plant diversity in natural ecosystems (Memmott et al., 2004), and are often the most important pollinators in forest, grassland, and montane plant communities (Neff and Simpson, 1993). These bees are typically generalists but are recognized to have preferences for certain plants, with species partitioning resources based on floral morphologies that best match their morphologies, such as tongue length (Pyke et al., 2012).

Unfortunately, many bumble bee species have declined in recent decades. Bumble bees have become increasingly vulnerable, with 26% of bumble bee species in the U.S. and 21% in Europe now listed as threatened or endangered (Cameron & Sadd, 2020), and some species experiencing widespread declines in recent decades. This has been attributed to several factors, including climate change, habitat and floral resource loss, pesticides, and increased pathogens (Goulson et al., 2015; Soroye et al., 2020; Zattara & Aizen, 2021). A recent factor found to play a large role in these declines is climate change. According to Soroye et al. (2020), climatic warming predicted declining bumble bee occupancy over time in Europe and N. America, regardless of changes in land use (but see Guzman et al., 2021). Floral resource loss seems to be main reason for declines in long-tongued bees in Europe, especially where intensive agriculture has resulted in a loss in legume rich hayfields, as legumes are favored by these bees (Goulson, 2010; Rasmont et al., 2021). In the U.S., bumble bee declines are associated with a loss in floral resources due to agricultural intensification and urban development (Glaum et al., 2017; Grixti et

al., 2009). Impacts of pesticide use on wild bumble bee populations have not been assessed for most species, but neonicotinoid use was a strong predictor in recent *B. occidentalis* declines, second to climatic warming (N. M. Williams & Hemberger, 2023). Declining species have been shown to have higher pathogen prevalence than stable species (Cameron et al., 2011) and the negative fitness effects on bumble bees indicate a role of pathogens in recent declines (Cameron & Sadd, 2020). Of course, additive and synergistic effects of these stressors likely have profound outcomes on success, but this remains to be investigated in great detail for bumble bees (Cameron & Sadd, 2020).

Not all bumble bee species, however, are equally vulnerable (Colla & Packer, 2008; P. H. Williams et al., 2009; IUCN 2024). In the eastern Nearctic faunistic zone, the most notable declines have been in three species: *B. pensylvanicus* and *B. terricola* have seen range reductions of 23% and 31%, respectively (Cameron et al., 2011; Jacobson et al., 2018) and *B. affinis* is federally listed as endangered (U.S. Fish and Wildlife Service, n.d.). The most successful species in North America, *B. impatiens,* is expanding its range, species like *B. griseocollis* and *B. bimaculatus* are more stable or increasing relative to other species, and other species are declining in parts of their historic ranges and are a listed as either stable or threatened (e.g., *B. vagans, B. terricola, B. fervidus*; Colla et al., 2012; Ghisbain et al., 2021; Jacobson et al., 2018; Martinet et al., 2021). It is argued bumble bees are more likely to decline when they have a small range (Arbetman et al., 2017), are social parasites (subgenus *Psithyrus*) that parasitize other *Bombus* species (Suhonen et al., 2015), and have a narrow diet breadth, as in long-tongued bees (Goulson, 2010). Bumble bees have also shown phylogenetic patterns in susceptibility, with the subgenera *Bombus sensu stricto*, *Thoracobombus*, *Cullumanobombus*, and *Alpinobombus* showing particularly high rates of decline while *Pyrobombus* is more robust to declines (Arbetman et al., 2017; Cameron et al., 2011).

Bumble bee success of species in the face of change most likely relates to their natural niche limitations and plasticity. Natural (before large anthropogenic effects) range size varies among species with *B. impatiens*, *B. bimaculatus*, and *B. griseocollis* having large distributions across eastern and southern North America whereas *B. sandersoni* and *B. perplexus* have smaller ranges, generally confined to more northern boreal latitudes (P. H. Williams et al., 2014). Abundance also naturally varies among species, for instance, *B. impatiens* is extremely common within its range, whereas *B. perplexus* is encountered with less frequency where it occurs (Novotny et al., 2021). Understanding the factors that limit bumble bees in their natural distribution will facilitate better understanding of the vulnerabilities they face to changing climate and anthropogenic environments.

Factors limiting bumble bee distribution

Variables influencing habitat requirements are likely a factor in limiting species distribution and range size. Many bumble bee species appear to be adapted to specific habitats, for instance, in the eastern Nearctic fauna, *B. sandersoni* are most common in forested areas, *B. griseocollis* is most common in open valleys, and *B. fraternus* is restricted to low-elevation prairies and coastal grasslands (Gratton et al., 2023). A few species are habitat generalists, for instance, *B. impatiens* occurs in low-lying valleys, forested hills, and wetlands and is one of the most common bumble bees in developed urban and agricultural sites in eastern North America (Gratton et al., 2023; P. H. Williams et al., 2014). The ability to thrive in diverse habitats may be a reason why *B. impatiens* populations have remained stable in the face of anthropogenic change while other bees that are more confined to specific habitats are likely more vulnerable to habitat loss and climate change (Ghisbain et al., 2021; Martinet et al., 2021; Novotny et al., 2021) At present it is unclear what physiological factors most explain variation in habitat needs.

One biological factor may be their nest site needs. Most bumble bees nest in cavities, often underground in abandoned burrows used by small mammals but sometimes in tree cavities, rock piles, and even manmade structures (Liczner & Colla, 2019), while some typically nest above ground in grass tussocks (Alford, 1975; Kells & Goulson, 2003). In England, the highest density of nest sites are found along fences, hedgerows, field margins and woodland edges (Kells & Goulson, 2003; Osborne et al., 2008), but modern farming practices reduce the availability of nest sites for below-ground and surface nesters as smaller fields are combined into large monocropping systems (Goulson, 2010; Kells & Goulson, 2003). Most bumble bee species show a preference for the ground position of the nest (below ground, surface, above ground), for instance, *B. impatiens* prefers to nest underground and *B. griseocollis* prefers to nest at the ground surface (Harder, 1986; Pugesek & Crone). While nesting behavior is in need of more study, most species show some flexibility in nest position (Pugesek & Crone, 2021; Liczner & Colla, 2019) and sites (i.e., agricultural, grassland, forest, urban, etc.) (Liczner & Colla, 2019).

Thermal tolerance also likely limits the distribution and success under climate change of different bumble bee species. There is growing evidence bumble bees show species-specific thermal adaptations with species and even populations within species showing different degrees of variability in heat and cold stress resistance. Research examining the impact of heat and cold tolerance on elevational differences in *B. vosnesenskii* found they are more cold limited than heat constrained as heat tolerance did not vary along these clines (Pimsler et al., 2020). Martinet et al. (2021), however, in an analysis of a subset of bumble bee species globally, found that the European bumble bees with increasing populations in the Mediterranean were the most heat stress tolerant, while species in decline and/or species inhabiting colder biotopes at higher latitudes were associated with lower heat tolerance. This suggests that heat tolerance is limiting their range and that bees with higher heat stress tolerance may be less impacted by climate change. As another line of evidence supporting the role of climate on limiting these bees, Kerr et al. (2015)

found many bumble bee species in Europe and North America are disappearing from the southern parts of their ranges in response to climatic warming but are not expanding their ranges to track warming at northern latitudes, resulting in range contraction. These climate change-induced range contractions are likely to accelerate in the future but differ among species (Sirois-Delisle & Kerr, 2018). Understanding the vulnerability of species and the role of temperature on their distributional limits requires improved understanding of the thermal limits of these bees, both by species and within species by caste, sex, and reproductive state.

Phenology may also be a factor in affecting the success and distribution of bees. Most bumble bees have an annual colony cycle where single mated queens overwinter (underground, rotted logs, etc.) from late summer/fall until the following spring, where they emerge, find a nest site and produce a colony of non-reproductive workers, who take over foraging duties and help with rearing brood. Towards the end of the colony cycle, males and new queens are produced, and newly mated queens overwinter, starting the cycle again (Alford 1975). Bumble bee species are recognized to differ in their phenology (P.H. Williams et al., 2014), but phenological comparisons across species within communities and their role in declines have not been well evaluated. Species with longer seasonality tend to have larger colonies with a high number of workers, for example tropical bumble bees achieve large perennial colonies (Sakagami, 1976), whereas short season arctic bumble bees can have just a few dozen workers (Løken, 1976). This results in evolutionary tradeoffs, as queens and males, which are the reproductive unit of the colony, are only produced late in the colony cycle and longer seasons expose these bees to more vulnerability, thus long-season bees can potentially produce more offspring only if resources are adequate to build a large colony (Persson & Smith, 2013). One consequence of having a short colony cycle is that queens may have a longer period of diapause, with some queens diapausing through the summer months. It may be, therefore, that short season, heat sensitive species are constrained to cooler climates, although no studies have confirmed this. For queens undergoing

experimental diapause regimes, warmer diapause temperatures had a negative effect on fat body content and immune function, especially in smaller queens (Vesterlund et al., 2014), so the impacts of climatic warming on diapausing queens are potentially severe. The timing of bumble bee species must also align with the timing of their food sources and may influence which habitats, given their temporal flush in flowering plants, best support a species.

A major but more complex factor that likely limits the distribution and success of bumble bee species are differences in floral host visitation preferences and nutritional intake requirements. Traditionally, bumble bees have been thought to primarily partition their resources by tongue length (Pyke, 1982), as longer tongued species most efficiently forage on longer tubed flowers and short tongued species are constrained to short corolla flowers. Declining species in Europe are noted to be long-tongued species, which forage from a narrower range of plants compared to short-tongue species and collect a high proportion of pollen from Fabaceae (Goulson, 2010; Goulson et al., 2008b; Wood et al., 2021). Bumble bee species have been recognized to occupy different parts of pollinator networks and thus vary in visited plants. For example, different bumble bee species in southern Poland were found to visit different plant families (Goulson et al., 2008b). One factor that contributes to this is floral toxins, as *B. consobrinus* is a specialist on alkaloid-rich *Aconitum* spp. (Laverty & Plowright, 1988), and *B. griseocollis* is recently found to prefer the cardenolide-laced milkweed plants (*Asclepias*) (Villalona et al., 2020). A factor recently found to likely impact floral choice is macronutrient content of pollen. Plant species differ in their pollen protein, carbohydrate and lipid concentrations (Vaudo et al., 2020 (supplementary material); Vaudo et al., 2024 (supplementary material) as well as amino acid and fatty acid and sterol profiles (Chau & Rehan, 2024). If bumble bees do actually differ in their nutritional intake targets, it is expected that they will show specific foraging patterns. Indeed, Wood et al. (2021) found a phylogenetic conservation of diet as 3 *Bombus* subgenera collected more than 50% of their pollen from Fabaceae, a plant family

with high pollen protein levels (Hanley et al., 2008). It seems important, therefore, to understand floral preferences of different bumble bee taxonomic and functional groups (e.g., tongue length) when maintaining and improving landscapes for these pollinators.

Protein-lipid ratio intake preferences of only two bumble bees have been studied to date, *B. terrestris* and *B. impatiens* (Kraus et al., 2019; Vanderplanck et al., 2014; Vaudo et al., 2017), which happen to be among the most widespread and abundant bees in their respective regions, Europe and North America (Martinet et al., 2021; P. H. Williams et al., 2014). In paired choice tests where the protein and lipids of diets were manipulated, these bumble bees preferred high protein to lipid (P:L) ratios (Vaudo et al., 2017). In field choice experiments, *Bombus impatiens* workers preferred to forage pollen from plants with the highest P:L ratio (5:1), only switching to species with lower P:L values after pollen from the preferred species had been exhausted (Vaudo et al., 2016). The degree to which other bumble bees adhere to these patterns is unknown. Evaluating these intake preferences along with the macronutrient availability of pollen resources (protein and lipid content, and protein-lipid ratios) in the landscapes these bees inhabit can provide information for optimal habitat restoration efforts. If sympatric bee species in a given landscape have similar nutritional intake targets and plant preferences, then restoration efforts should be fairly simple compared to scenarios where species have divergent nutritional preferences and/or partition floral resources. Restoration efforts will also have to account for how the nutritional availability of a landscape may change throughout the growing season, especially for bees with long seasonality. An interesting question that arises from this is whether more successful and widespread species have more variable nutritional intake. If so, this could explain why *B. impatiens* thrives in low- to high-quality sites, while other species seem to thrive only in habitats with high floral diversity (Gratton et al., 2023), another potential cause for the differential success among species.

Many of these factors likely interact in influencing the distributions and differential success of bumble bee species. For example, diapause length and phenology may be limited by thermal tolerance, nutritional stress may impact thermal stress tolerance, and species and caste may have different floral host preferences, pollen nutritional preferences, and responses to heat stress. It is the goal of this thesis is to understand which factors differ most in these bees and *how these factors relate to their distributions and success towards better predicting how bumble bee species and communities will respond in the face of anthropogenic change*. Recognizing why species show differential success will be integral to promoting healthy bumble bee communities, which will need to consider conservation approaches tailored to the needs of vulnerable species, while still supporting bees that have stable populations. It is therefore critical that these factors be studied for all bumble bees within a community, regardless of conservation status.

In Chapter 1, I seek to better describe the distribution, phenology, and floral visitation patterns of bumble bees in Pennsylvania communities as a baseline for characterizing their differences. Towards this goal I compared field-collected data on distribution, phenology, and floral preference for 9 bumble bees species observed across 32 sites distributed across Pennsylvania in 3 broad habitat ecozones (valley, edge, and forest).

In Chapter 2, I seek to understand whether or not there are differences in nutritional preferences among bumble bee species and the role habitat may play in the nutrition these bees attain. Towards this goal, I examine how pollen macronutrient preferences differ among the five most abundant bumble bee species in the Ridge and Valley region of Pennsylvania and 2 bumble bee species (1 shared with PA) in the Great Plains region of Oklahoma. I also compare pollen macronutrient preferences among queens and workers, across sites, and across the season. Finally, I examine plant visitation patterns among bumble bee species in each region by examining pollinator-plant interaction networks and whether or not bee and plant species form

distinct interaction modules within the broader network. These data will aid in recommending planting floral resources that provide optimal nutritional space for bumble bee communities.

In Chapter 3, I examine whether bumble bees of different species and with different biological traits vary in their heat tolerance, towards better understanding the importance of this trait in driving distribution and success at a community level. I use time to heat stupor (THS) assays to investigate several biological factors that contribute to heat stress tolerance in bumble bees including the role of species, caste, sex, body size, different physiological states of queens, as well as the interactive effects of heat and humidity. I also examine how each species' heat tolerance corresponds with their distribution, climatic niche, and habitat to better understand how differential responses to heat stress can impact success in the face of climate change.

Altogether, by melding information on habitat, phenology, floral visitation, thermal requirements, and nutritional needs, this thesis research provides baseline data towards better understanding the resiliency and vulnerability of different bumble bee species and will help inform strategic plans to reduce the impacts of anthropogenic change.

Chapter 1

Assessing the role of habitat distributions, phenology, and floral visitation patterns on the resilience of different bumble bee species in Pennsylvania

Introduction

There are ~270 species of bumble bees globally that typically coexist in communities of about a dozen species by partitioning resources through differences in floral preferences, habitat use, and phenology (Goulson, 2010; Pyke, 1982; Pyke et al., 2012; P.H. Williams, 1988, 1989). Anthropogenic change has been linked to declines in bumble bees, yet some species have remained stable despite these changes (Goulson et al., 2008a). Characterizing the factors that limit bumble bees is crucial in explaining differential success among species under change. To better understand these factors, I gathered baseline data on distribution, phenology, and floral resource visitation patterns for bumble bee species with varying conservation status in Pennsylvania communities.

While distributions and phenology can be gleaned from global databases such as GBIF and iNaturalist, these databases have limitations for informing local conservation. These databases are prone to human biases in the locations and species that are sampled, as many museum specimens, such as those included in GBIF, have been collected without the use of controlled sampling methods and are biased towards particular, often rare, species of interest, leaving more common species underrepresented (Johnston et al., 2020). Furthermore, global databases only report presence data, leaving nondetections to be inferred (Guzman et al, 2021).

Citizen science data, such as that included in iNaturalist, is often biased towards population centers (Kelling et al., 2019), potentially underrepresenting species that do not persist in urban environments and underrepresenting biodiversity. Compared to native bee collections-based monitoring conducted by trained volunteers, iNaturalist data reported lower native bee biodiversity over the same area and time period within Pennsylvania (Turley et al., 2024). Although global databases are useful in gleaning general species distributional patterns over time (Kerr et al., 2015; Soroye et al., 2020; P.H. Williams et al., 2014), they are less informative for understanding how species within a geographic region vary in their resource use such as through partitioning their habitat and phenology - data especially valuable for management.

Prior work sampling across the hill and valley system of central Pennsylvania (Gratton et al., 2023), found communities of bumble bees transition locally along a valley, edge (ecotone between valley and forest), to forest gradient, showing a trend of habitat sorting among these species. This research identified *B. impatiens* as a habitat generalist which may help explain why it is one of the most widespread and common bumble bees in North America, whereas less abundant species, like *B. vagans* and *B. sandersoni*, are habitat specialists, mostly found in woodland areas and thus more limited in their distributions (Gratton et al., 2023).

Phenology is known to vary in response to environmental variables, such as temperature, latitude, and elevation (Zettlemoyer & Peterson, 2021). Temperature is one of the main factors for terminating diapause in insects (Amsalem et al., 2015a), and it is known that, for some bumble bee species, queen emergence tracks spring onset (Koppel & Kerr, 2022), thus habitat type and location likely impact spring queen emergence. Furthermore, bumble bees vary in their sociality. While all social bumble bee queens generate worker brood first followed by later season reproductive brood, some only produce a small number of worker cohorts for a short phenology prior to generating males and new queens (gynes), and others generate multiple cohorts and produce larger colonies across a longer time window. Data from across North America (P.H.

Williams et al., 2014) suggests variance in phenology in the species that occur in Pennsylvania, however detailed comparisons of phenology within the same location – and thus the same seasonal conditions - are lacking.

Host plant visitation data is also useful for understanding what limits the success of these bees, as differences in visitation patterns impact how species respond to changes in land use that affect floral resources, for example, bumble bees with more specialized foraging preferences may be more impacted by floral diversity loss than more generalist bees (Goulson, 2010; Rasmont et al., 2021). Many host plant visitation studies have focused on high-quality sites (Goulson et al., 2008b; Lanterman Novotny et al., 2023) thus presenting a biased perspective on what landscapes normally have to offer these bees. Floral visitation data across a range of unmanaged habitats provide a more realistic overview of species preferences and will inform more targeted restoration efforts for specific bee-host plant communities.

Differences in floral visitation use have been recorded among species with different tongue lengths (Goulson, 2010; Inoue & Yokoyama, 2006; Pyke et al., 2012). In Pennsylvania, species vary from short to long tongue lengths with respect to other bumble bees (Harmon-Threatt & Ackerly, 2013; Michener, 2000). Therefore, some differences in visitation patterns are expected by virtue of varying tongue lengths among these bees. Generalist and specialist bumble bees may be impacted differently by reduced floral diversity (e.g., due to land use change) and host plant use may differ among bees with different phenology, which thus may be differently impacted by phenological shifts in host-plants. Species-specific floral preference data helps understand how to provide for the diversity of bees.

In this Chapter, I compile controlled field data on distributions and phenology of bumble bees across Pennsylvania from April - September, as well as floral visitation data of these bees, to better inform how these bees differently utilize resources in these communities. Altogether, these data provide information on what contributes to differential success of bumble bees in

Pennsylvania. The cross-season plant visitation data, sampled across sites of variable quality in Pennsylvania, can be used to inform what is sustaining bees in depauperate landscapes, and to guide plantings in restoration areas and pollinator gardens throughout the growing season.

Methods

Site visitation

Between April 14th and May 25th, 2022, I conducted 31 spring queen surveys at 10 sites visiting each site across three consecutive time periods (spring 1: April 14th to April 23rd, spring 2: April 24th to May 3rd, spring 3: May 5th to May 25th (Table 1a). All sites were located in Centre County, PA. Since I was interested in how distribution and phenology are influenced by habitat, sites were selected so that habitat types were roughly balanced: valley sites (n = 3), valley-forested (n = 2), edge sites (n = 2), and forested hills (n=3) (Table 1a).

Between June 1st and September 4th, 2022, with the help of Tori Strausser, an undergraduate in the Hines lab, I conducted 58 summer surveys at 30 sites over 3 time periods (Table 1b). To observe how species distributions and caste phenology change by habitat type, I chose sites that allowed us to cover as much of Pennsylvania as possible from three habitat types: valley (n = 11), edge (n = 19), and forested hills (n = 28) (Figure 1a, 1b). Sites were chosen to include: 1) a selection of sites used in previous field studies in Pennsylvania that were originally chosen for being publicly accessible, to vary in surrounding landscape conditions, and have at least some bumble bees (Gratton et al., 2023; McNeil et al., 2020), 2) a few sites of known higher quality, 3) and some additional parks and natural areas with public accessibility. Site quality was not determined *a priori* for most sites but was assessed at the time of the survey, and sites that did not have floral resources were dropped from the study. These surveys incorporated a diversity of

site types: public parks (i.e., state parks and urban parks), state game lands, gardens, and state forests (Table 1), and thus, represent typical managed and natural areas of Pennsylvania. I attempted to balance the number of sites across sample periods, however, the same sites were not sampled in each time period (Table 1) due to time restrictions. Time was most restricted during the summer 3 survey period and so only sites closer to State College, PA were sampled a third time. Since Pennsylvania is 57% forested (USDA Forest Service, 2021), sites were biased toward forested areas.

Survey protocol

Surveys were conducted by 2 people/site for 30 minutes each or 1 person/site for one hour between 9 am and 4:30 pm during weather conditions suitable for bees (low wind, temperatures between 10 and 34 °C, no rain). Bees were surveyed in an opportunistic manner, meaning we scanned all blooming plants for bees, seeking to cover the full area equally. Thus, area covered varied among sites and within single sites across time periods. If the entire survey area had been walked but time still remained, the surveyor would return to the starting point and walk the survey path again until the full hour had passed. At each site, species, sex, and caste of each bee was recorded. All bee-flower interactions were recorded with host plants identified to species when possible.

All bees were identified to species and sex/caste in the field while they were on flowers except for *B. vagans*, *B. perplexus*, and *B. sandersoni* (VSP complex), and some individuals where ID was uncertain; these individuals were brought back to the lab for identification. The timer was paused for the site sampling when bees were collected. This difference in recording may have created a slight bias in a potential repeated sampling of bees that were not collected during the survey. Species in the VSP complex are difficult to distinguish without the use of a

microscope or molecular tools (Gratton et al., 2023; Milam et al., 2020) thus requiring their collection. To avoid negative impacts on subsequent site populations, queens of these species were not sampled in the spring, thus we decided to combine the spring queen data for these bees into the VSP cryptic species complex. However, some VSP queens caught after May 30th (i.e., during the summer) were brought back to the lab for identification. 25 workers in the VSP complex were observed in the field but were not captured (e.g., observed while flying by, escaped from net, etc.) and were recorded as VSP. Collected VSP specimens were identified using DNA barcoding of the COI locus (n = 74) (procedures follow Gratton et al., 2023) or using morphological features following the methods in P.H. Williams et al. (2014) and Milam et al. (2020), with morphological concepts improved using reference specimens from DNA barcoding (n = 51). Table 2 shows the methods used to identify VSP specimens in chapter 1, chapter 2, and chapter 3.

Analysis

To reveal how each caste and species shifts across time and how this may be influenced by habitat type, species sex/caste stacked bar phenology plots were generated for each time period (spring [pooled across time periods], summer 1, summer 2, summer 3) within each habitat type. Bar plots were also made to examine queen phenology over the three spring time periods within each habitat type. For this analysis I categorized sites somewhat differently, recognizing as a distinct group sites that are in the valley but forested versus open valley sites. Since there was an imbalance of site numbers among habitat types and sites for each time period, I corrected for sampling effort with weighted counts within each habitat type-time period. Summer 1 forests had the most sites (14), so I used this as the divisor for all weighted calculations to normalize across all habitat type-time period combinations. The weighted correction matrix is presented in Table 3.

I also made phenology bar plots for each sex/caste for each species, pooling across all sites and organizing the data by day of the year. Correcting for sampling gaps, phenology curves for each sex/caste were generated from these data using the locally estimated scatterplot smoothing (loess) function in R version 4.2.1 (R Core Team, 2022) and were overlaid on top of the bar plots. A few summer VSP queens (n = 7) were identified to species but were decoupled from their IDs for the analysis and added to the spring queen VSP bar plot-line chart. Pie charts of proportional abundance were made to assess 1) overall species composition by each habitat type (valley, forest, edge) and 2) species composition by habitat type in different regions of the state.

I used host-plant interaction data to generate heat maps with the geom_tile function in R (using the ggplot2 package; Wickham, 2016) that show the proportion of plants visited by workers of each of the following species: *B. impatiens*, *B. bimaculatus*, *B. griseocollis*, *B. sandersoni*, *B. vagans*, and *B. perplexus,* which were represented by ≥ 25 observations, although the VSP complex (n = 68) was excluded since these bees were not identified to species, and host-plat interactions would not have been informative. The other three species observed (*B. ternarius, B. terricola,* and *B. fervidus*) were excluded due to low sample sizes. Floral visitation data was compiled across all sites, habitat types, and time periods. Floral visitation data was not corrected for sampling biases such as species non-detections at certain sites, habitats, or regions, nor for variance in sample sizes among species, but is intended, rather, to illustrate overall visitation patterns across the entire dataset. I also created stacked bar plots of the percentages of plant species visited by each bumble bee species where plant species are clustered by botanical family. For each bee species the proportion of the top 10 most-visited plants were differentiated and all other plants were combined into an 'other' category (except for the case of *B. perplexus,* which were only observed on 9 plants). For *B. griseocollis,* only 8 plants were differentiated as all other plants were associated with one observation each and were combined into 'other'. Finally, I created a pie chart showing the percentages of plant families visited across all bees (i.e., queens,

workers, and males of all 9 bee species across all surveys, n = 2229). Families with 10 or more observed visitations are differentiated and families with less than 10 visitations are combined into 'other'.

Results

Species abundance by habitat type:

Species showed considerable variance in relative abundance as well as variance by habitat type, showing patterns similar to that observed by Gratton et al. (2023) (Table 4, Figure 1). *B. impatiens* was by far the most abundant species across all habitat types (valley: n = 214, edge: n = 283, forest: n = 903), making up more than half of all species in all habitat types. Workers of *B. impatiens* reached highest abundances in forests, although their queens had the lowest relative abundance in forests (Figure 1, Figure 2a,c). *B. bimaculatus* was the second most abundant species in edge (n = 135) and forest (n = 299) sites, was the second most abundant species overall (n = 505) and was observed throughout the state. *B. griseocollis* was the second most abundant species in valley sites (n = 101), and the third most abundant species overall (n = 225). *B. griseocollis* was best represented in valleys (Figure 1, Figure 2a) especially in eastern parts of the state at lower elevation sites, although this species was represented in a few forested/high elevation sites. The fourth most abundant species was *B. sandersoni* (n = 68) with the majority of observations in forest sites (n = 48). *B. vagans* and *B. perplexus* observations were nearly equal (n = 30 and 27, respectively), with *B. vagan*s favoring forest sites and *B. perplexus* favoring edge sites. *B. sandersoni*, *B. vagans*, and *B. perplexus* had very low or no occurrences in valley sites and were less represented in western parts of the state (Figure 1, Figure 2a). *B. ternarius, B. terricola,* and *B. fervidus* each made up less than 15 observations with *B. ternarius*

(n=14) favoring edge sites, *B. terricola* (n=13) occurring in forest and edge sites, and *B. fervidus* (n=10) most represented in forests (Figure 1, Figure 2a), although low sample sizes for these bees may prevent drawing any definitive conclusions about habitat preferences within Pennsylvania.

Seasonality and phenology

Queens were observed with the highest frequency in spring, peaking in the third spring sampling period May 5th to May 25th, with summer-emerging queens (gynes) observed with less frequency (Figure 2a,b, Figure 3, Figure 4). Queen emergence times were fairly similar across species, although *B. impatiens* emergence declined over time relative to other species across the three sampling periods and the few *B. fervidus* and *B. terricola* were observed in the last spring sampling period (Figure. 2c). Workers peaked between mid-June and mid-August with notable differences between species (Figure 3, Figure 4). Four species have short worker seasons: *B. sandersoni* peaked on June 20th with worker production lasting 45 days, *B. bimaculatus* workers peaked on June 22nd (82 days, but 99.7% of samples observed within 46 days), *B. perplexus* workers peaked on July 4th (37 days), and *B. griseocollis* workers peaked on July 12th (41 days) (Figure 3, Figure 4). *B. impatiens,* in contrast*,* had a very long seasonality and among the latest phenology, with workers peaking on August 1st, but were still being observed in high numbers by September 4th, the last day of field observations (133+ days). Similarly, *B. vagans* workers had late phenology (peaking on August 14th) with workers still being observed in great numbers by September 4th (71+ days) (Figure 3, Figure 4). Males for *B. sandersoni, B. bimaculatus, B. perplexus*, and *B. griseocollis* all peaked in mid-July, while observations were still increasing by September 4th for *B. vagans* and *B. impatiens* (Figure 3, Figure 4).

Habitat-specific phenology:

There was evidence of delayed phenology along a habitat gradient from valley to forest. Queens came out first in valleys, which was the only location where queens were observed in Spring 1 (Figure 2c). Workers had the highest relative counts earlier in the year in valleys compared to edges and forests. This was particularly evident in *B. impatiens* and *B. bimaculatus.* In other cases, workers and males absent in late summer in valleys and edges were present in in late summer in forest sites as seen in *B. bimaculatus* and *B. vagans*, suggesting that workers and males emerge later in forested areas and that phenology shifts temporally rather than contracts across a shorter time period in these areas (Figure 2a). Finally, in the late season, males that had high counts in valleys had low counts in edges and/or forests, indicating that males are just beginning to emerge in edge/forest sites during this same time period. This was seen in *B. impatiens*, *B. griseocollis*, *B. vagans* (high male counts in edges and low counts in forests), and *B. perplexus* (Figure 2a).

Plant visitation analysis

The top five plants that workers were observed most frequently on across Pennsylvania sites were *Trifolium repens* (white clover, nonnative, Fabaceae)*, Centaurea stoebe* (spotted knapweed, nonnative, Asteraceae), *Prunella vulgaris* (common self-heal, native, Lamiaceae), *Securigera varia* (crown vetch, nonnative, Fabaceae), and *Asclepias syriaca* (common milkweed, native, Apocynaceae) (Figure 5). *B. impatiens* and *B. bimaculatus* visited the highest number of plants and showed the most similar visitation patterns (Figure 6) but were also the most abundant bee species in the study, thus biasing them to be more similar and display more diet breadth. Crown vetch made up 32% of all visitations for *B. impatiens* despite the high diversity of plants

visited by this bee. Common milkweed was almost exclusively visited by *B. griseocollis* and made up 45% of all visitations by this bee (Figure 5,6). White clover was the top plant visited by *B. sandersoni* (42%) and *B. bimaculatus* (30%). The top plant for *B. perplexus* was *Rubus phoenicolasius* (wineberry, nonnative, Rosaceae, 29%) and for *B. vagans*, the top plant was spotted knapweed (30%). Plants are arranged in order, from highest proportion of visitations across all bumble bees (top row of heat map tiles) to lowest (bottom row of heat map tiles) (Figure 5). The three plant families common to 5 of the 6 bumble bee species (workers only) were Fabaceae, Asteraceae, and Lamiaceae (*B. sandersoni* was not seen visiting Asteraceae) (Figure 6). When evaluating all bees across all surveys (workers, queens, and males of all 9 species), these three plant families made up ~76% of visitations (Fabaceae ~39%, Asteraceae ~ 22%, and Lamiaceae ~ 15%) (Figure 7). Apocynaceae (almost exclusively common milkweed) and Plantaginaceae made up made up ~5% of plant visits each, Ericaceae ~4%, Rosaceae, Caprifoliaceae and Apiaceae ~2% each, Brassicaceae ~1%, Polygonaceae ~0.5%, and 'other' ~3%. (Figure 7).

Discussion

Throughout 2022, I evaluated 41 sites across Pennsylvania and three different habitat types, totaling 89 surveys across the season for a representative sample of Pennsylvania bumble bee communities, their phenology, habitat distribution, and host-plant interactions. Overall, I found that there are differences among species in their habitat distributions consistent with prior data from central Pennsylvania (Gratton et al. 2023). This study also confirms that *B. impatiens*, *B. bimaculatus*, and *B. griseocollis* are the most successful species in terms of abundance and are all well represented in valleys where the six other species observed were relatively rare. It is notable that *B. vagans, B. sandersoni,* and *B. perplexus* were rare or absent from surveys in

eastern parts of the state even in habitats where these species are normally found (i.e., edges and forests). These are boreal species that may reach their habitat limits within Pennsylvania and thus are more susceptible to land use and climate changes. One species, *B. terricola*, has suffered recent declines across its range (Grixti et al., 2009; Jacobson et al., 2018) and the rarity of this species in my surveys is consistent with these reported declines. *B. ternarius* was also rare, but likely for a different reason, as the southern limits of this species' distribution occurs within Pennsylvania (P.H. Williams, et al., 2014) and thus this is more of an outlier species in the state. Also of note, are the species that across 2,360 total observed bumble bees, were never observed, yet have been found historically in the state. This includes namesake species *B. pensylvanicus*, *B. auricomus*, *B. affinis*, and all *Psithyrus* species (n=4); all of these species are reported as being in decline. These data confirm that these bees are now very rare in Pennsylvania. In general, edge sites showed the greatest diversity of bumble bee species, followed by forests sites and then valley sites.

When comparing workers and males among species, the most abundant species, *B. impatiens,* had the longest seasonality. Most other species had a shorter season, producing reproductives mostly by July and finishing by August. However, *B. vagans* exhibited a later phenology, distinguishing it from its comimics, a pattern not previously recognized. While *B. perplexus* and *B. sandersoni* complete worker and reproductive production in July, *B. vagans* stretches its phenology into the fall. These phenology differences will confound measures of abundance of these different species: sampling in early season will indicate that *B. perplexus* and *B. sandersoni* are more abundant, while sampling in the late season will indicate that *B. vagans* is most abundant. *B. vagans* has historically been thought to be the most abundant of the three, however, Gratton et al. (2023) showed that *B. sandersoni* is the most abundant species in central PA, which was confirmed by this broader survey of Pennsylvania. Moreover, *B. vagans* has

recently declined in certain parts of its historic range (Colla & Packer, 2008), thus these patterns should be compared more to historic data.

There was a general pattern among species of shifting phenology across different habitats. Spring queens were observed earliest in open valleys likely because these areas reach warmer temperatures earlier compared to edge and forest sites. For workers and males, there was a pattern of advancing phenology from valleys to edges to forests, indicating that the phenology of the entire colony cycle is dependent on abiotic factors like temperature, although other factors like humidity and precipitation are in need of investigation. Dates of peak queen and worker observations varied among species. However, peak male observations were tightly clustered (within 5 days) for all species except *B. impatiens* and *B. vagans*, which were still increasing by the end of the study. Although not observed in great numbers, the production of the new female reproductives (gynes or future queens) of these species likely peaked in late summer as well, especially since males are often produced before gynes (Alford, 1975; Goulson, 2010). Droughts in late summer, phenology shifts of flowering to earlier in the season, or early freezes in fall, could therefore affect the foraging ability and reproductive success of *B. impatiens* and *B. vagans*. Furthermore, floral resource diversity tends to decrease in late summer/early fall (Goulson, 2010; Yan et al., 2023), so landscapes that are already depauperate in resources will be less likely to support bees as resources begin to dwindle later in the growing season (Dolezal, et al., 2019).

To support more species, improved floral diversity and drought-tolerant floral resources in mid to late summer should be part of management schemes for these bees. Queens should benefit from establishing spring forage such as ephemerals and trees such as willows (*Salix spp.*) as these provide pollen and nectar resources and increase queen survival (Alford, 1975). Theoretically, sympatric species that differ in their phenology partition resources, as these species should have different sets of host-plants available to them for which they do not have to compete.

Therefore, bumble bee communities should be more diverse if provided diverse floral resources throughout the year.

Across all bees, the three most visited plant families were Fabaceae (legumes), Asteraceae (asters), and Lamiaceae (mints) which supports previous research that these families are highly visited by bumble bees (Goulson et al., 2005; Rosenberger & Conforti, 2020; Wood et al., 2021). The most-visited plants by workers were not plants one would recommend for pollinator gardens or pollinator habitat restoration, as most are non-native weeds (> 60% of all visitations). Across the six most common bumble bee species, two nonnative legumes and one nonnative aster were in the top 5 most visited plants. Plants in the botanical family Fabaceae made up more than 40% of plants visited by workers of 3 bumble bee species, however, only a few of these legumes were native. Many nonnative legumes and some nonnative asters (particularly plumeless thistle) have high pollen protein content and/or high protein-lipid ratios which are sought out by bumble bees (Goulson, 2010; Russo et al., 2019; Vaudo et al., 2020).

The fact that visitation on nonnatives was so high (3 of the 5 top-visited plants) is likely because these plants dominate in landscapes and thus, we should recognize that these are key support plants fulfilling the needs of bumble bees. In particular, white clover was present in most managed landscapes (i.e., state parks), filling in for the native plants lost for pollinators. While white clover may be disliked by many in their lawns, their proportional value for pollinator support compared to small high diversity gardens, is high. However, some highly visited plants such as crown vetch and spotted knapweed are invasive, and so are not recommended if they can easily be replaced by native bumble bee foraging plants (e.g., partridge pea (*Chamaecrista fasciculata*), native mints like bee balm (*Monarda fistulosa*) and mountain mints (*Pycnanthemum*)). The data show that bumble bees do utilize plants differently, although the lack of many plants to choose from made assessing visitation biases aside from a few exceptions (*Asclepias* in *B. griseocollis*, cf. Villalona et al., 2020), difficult. Future work should investigate if

improving host-plant diversity or including more native plants supports more diverse bumble bee communities.

Figures

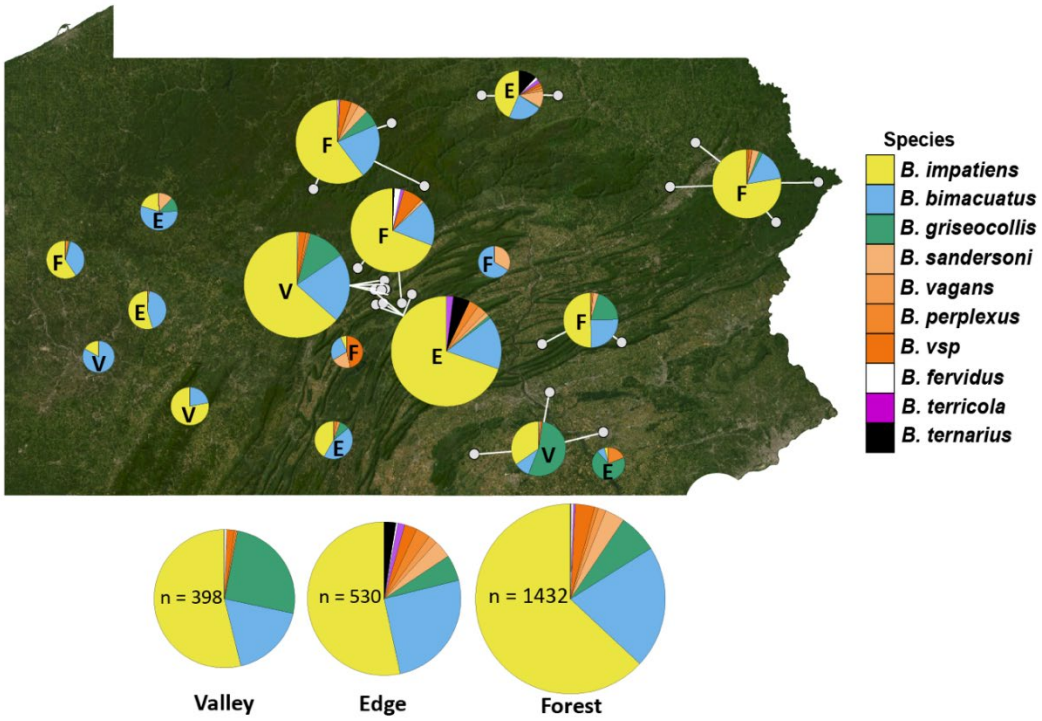

Figure 1. Distribution by habitat type for each bumble bee species. Pie charts inside of the map represent proportional abundance of bumble bee species identified across surveys within each region-habitat type. Relative pie chart size indicates how many surveys are represented for each pie chart [1-16 surveys]. Light grey dots indicate individual sites with white lines connected to pie charts that represent those sites and when not present pie charts represent only one site. Note that there is an imbalance surveys among sites (Table 1a, 1b). Letter insets represent habitat types: **V** = valley, **E** = edge, **F** = forest. VSP = *B. vagans*, *B. perplexus*, and *B. sandersoni* mimicry complex. Pie charts below the map represent species proportional abundances totaled for

each habitat type and are sized based on the number of bees observed (total sample sizes are inset).

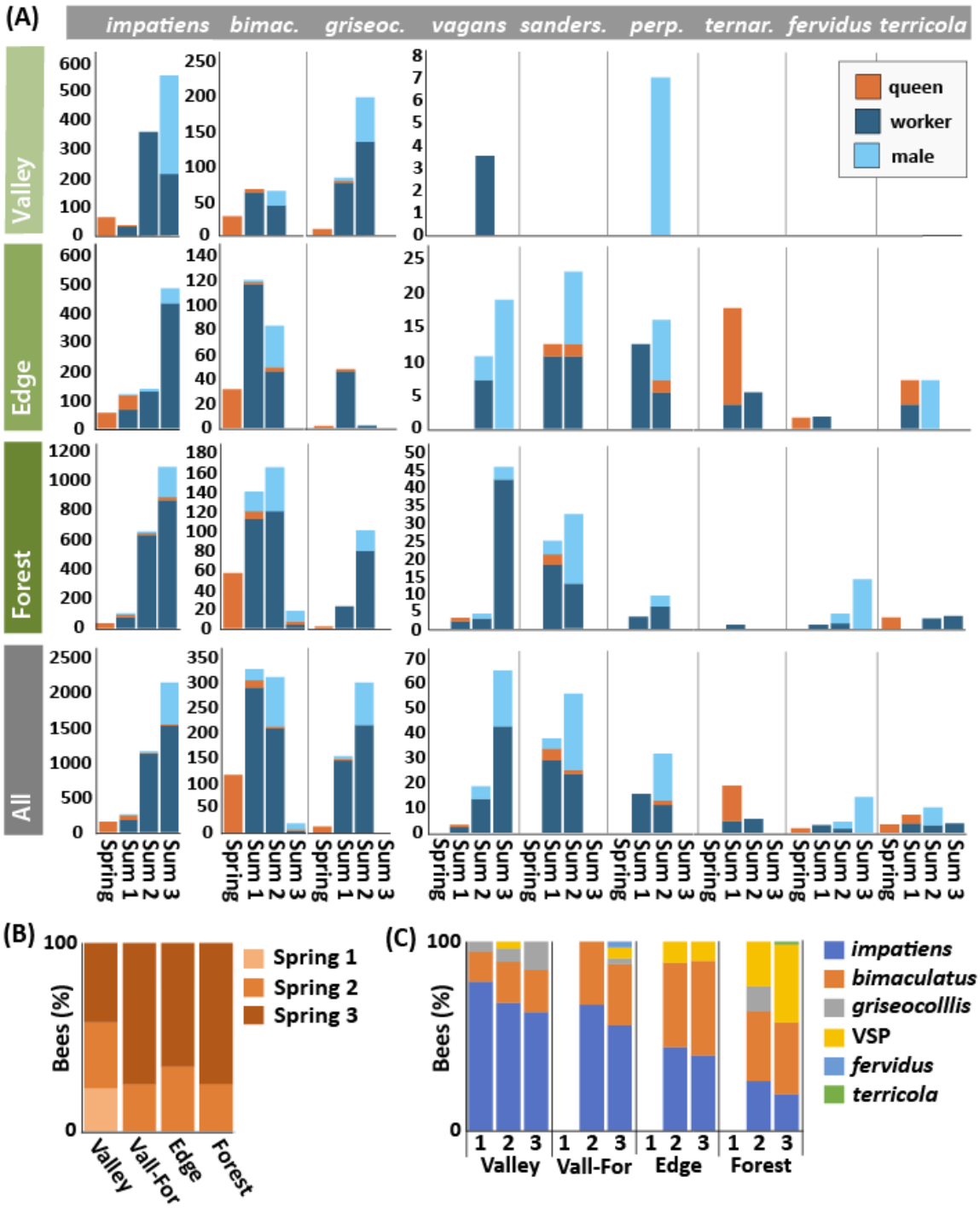

Figure 2. Variation in phenology among habitat types and throughout time. **(A)** Counts of queens, workers, and males of each species in valley, edge, and forest sites, and all habitat types combined (All) for each time period in 2022. Spring: April 14th - May 25th, summer 1 (Sum 1):

June 1st – June 19th, summer 2 (Sum 2): July 7th – July 19th, and summer 3 (Sum 3): August 8th – September 4th. bimac = *B. bimaculatus*, griseoc. = *B. griseocollis*, sanders. = *B. sandersoni,* perp. = *B. perplexus, ternar. = B. ternarius.* Imbalance of sampling effort among habitat type across time periods was corrected for using weighted counts within each habitat type-time period (Table 3). **(B)** Proportion of queens (all species combined) in each time period for each habitat type: valley, valley-forested (Val-For), edge, and forest sites. Spring 1: April 14th - April 23rd, spring 2: April 24th - May 3rd, spring 3: May 5th - May 25th. **(C)** Proportion of queens of each species among spring time periods (x-axis) within each habitat type. Note that all spring time periods are combined into one spring time period (April 14th - May 25th) in part A. Queens of *B. vagans*, *B. sandersoni*, *B. perplexus* are difficult to differentiate in the field and were not collected, thus were only identified to the VSP complex. Vall-For = forested pockets in valleys.

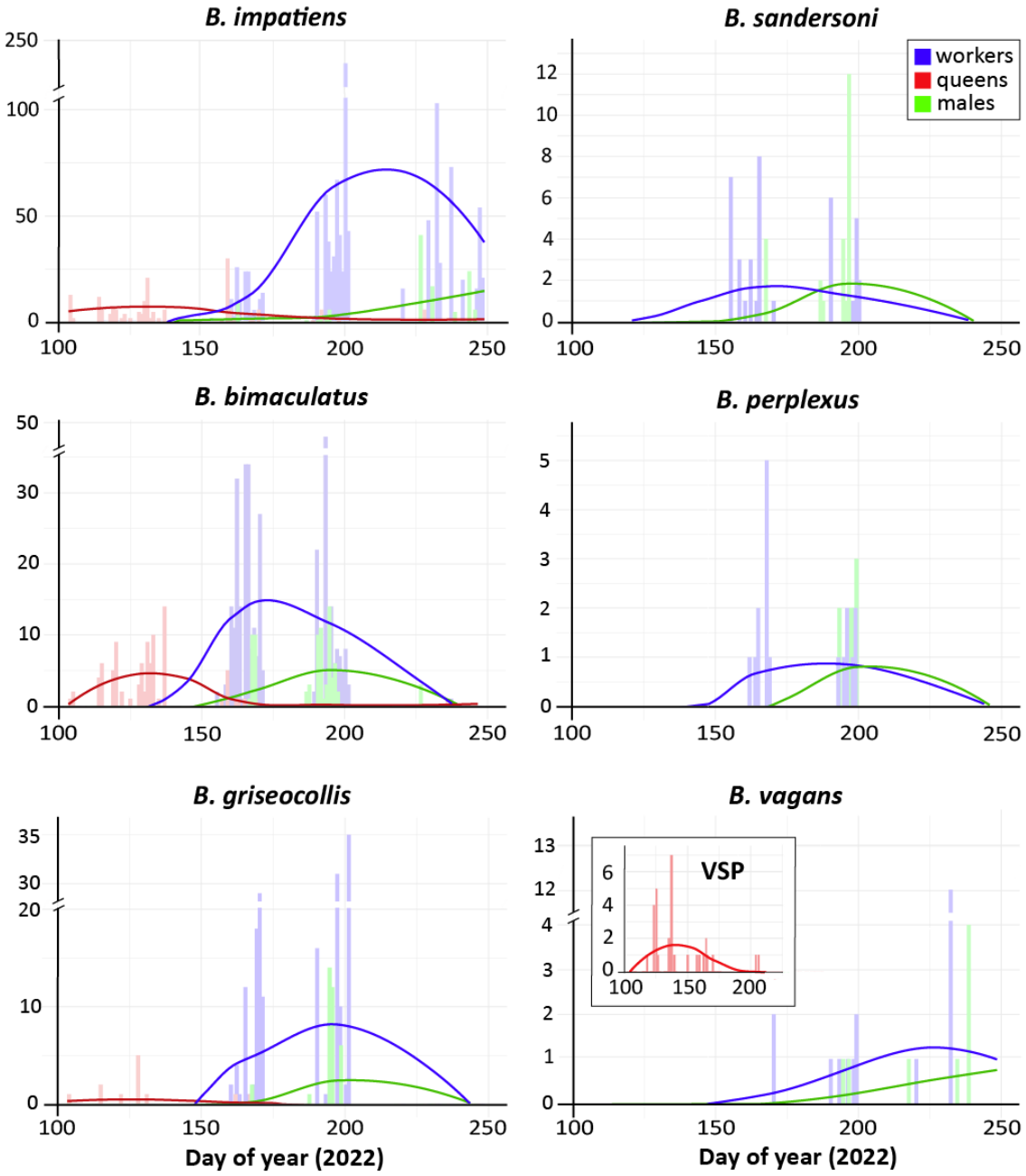

Figure 3. Phenology of queens (red lines), workers (blue lines), and males (green lines) of the 6 most-abundant species across all sites. *B. sandersoni*, *B. perplexus*, and *B. vagans* spring queens were not identified to species and were grouped into the VSP complex and given their own graph (inset inside of *B. vagans* graph). Bars show total raw counts/day for each species, and, as a

result, gaps between survey dates are shown. Day of year is shown on x-axes. Smoothed lines (loess curves) were generated based on raw counts and overlaid on the bar graphs so the first and last observation dates align with the corresponding day of year. This allowed for data interpolation for non-detections and for days on which no surveys were conducted.

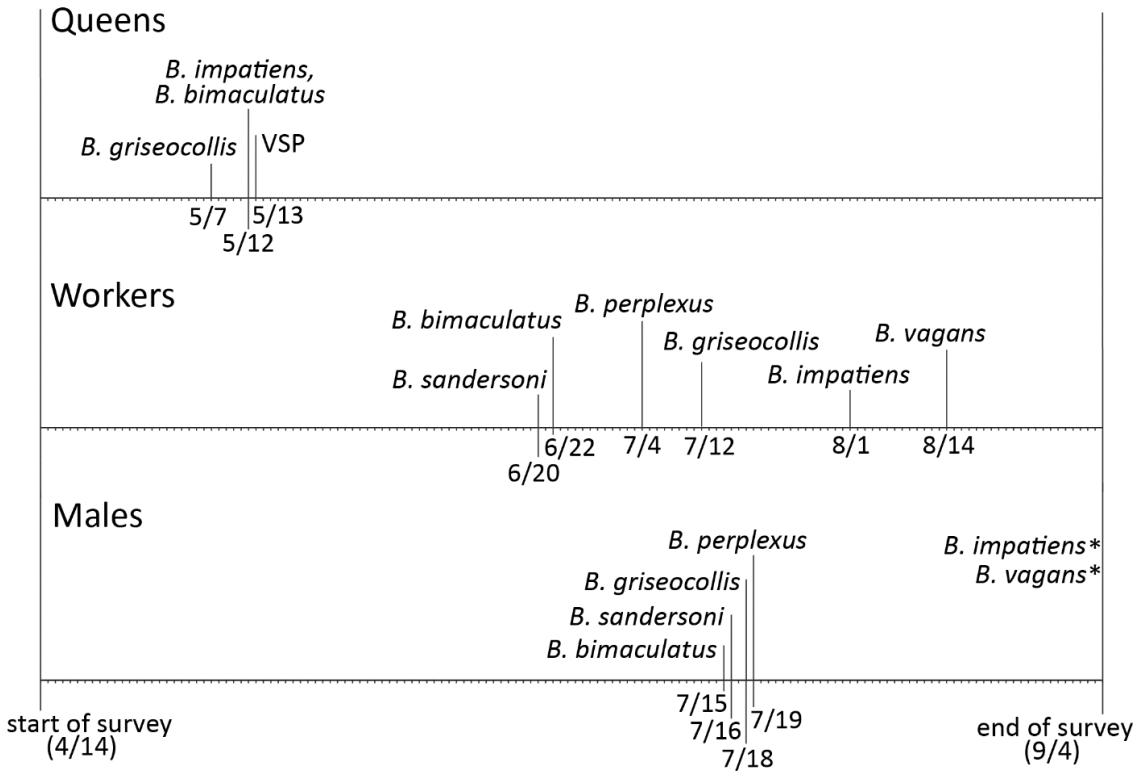

Figure 4. Peak occurrence dates for spring queens, workers, and males, based on predicted values from the loess models used in Figure 2. Only summer queens for *B. perplexus* were observed and thus peak occurrence date was not included. *Males of *B. impatiens* and *B. vagans* were still increasing by the date of the last survey, September 4th, so peak occurrence dates could not be determined.

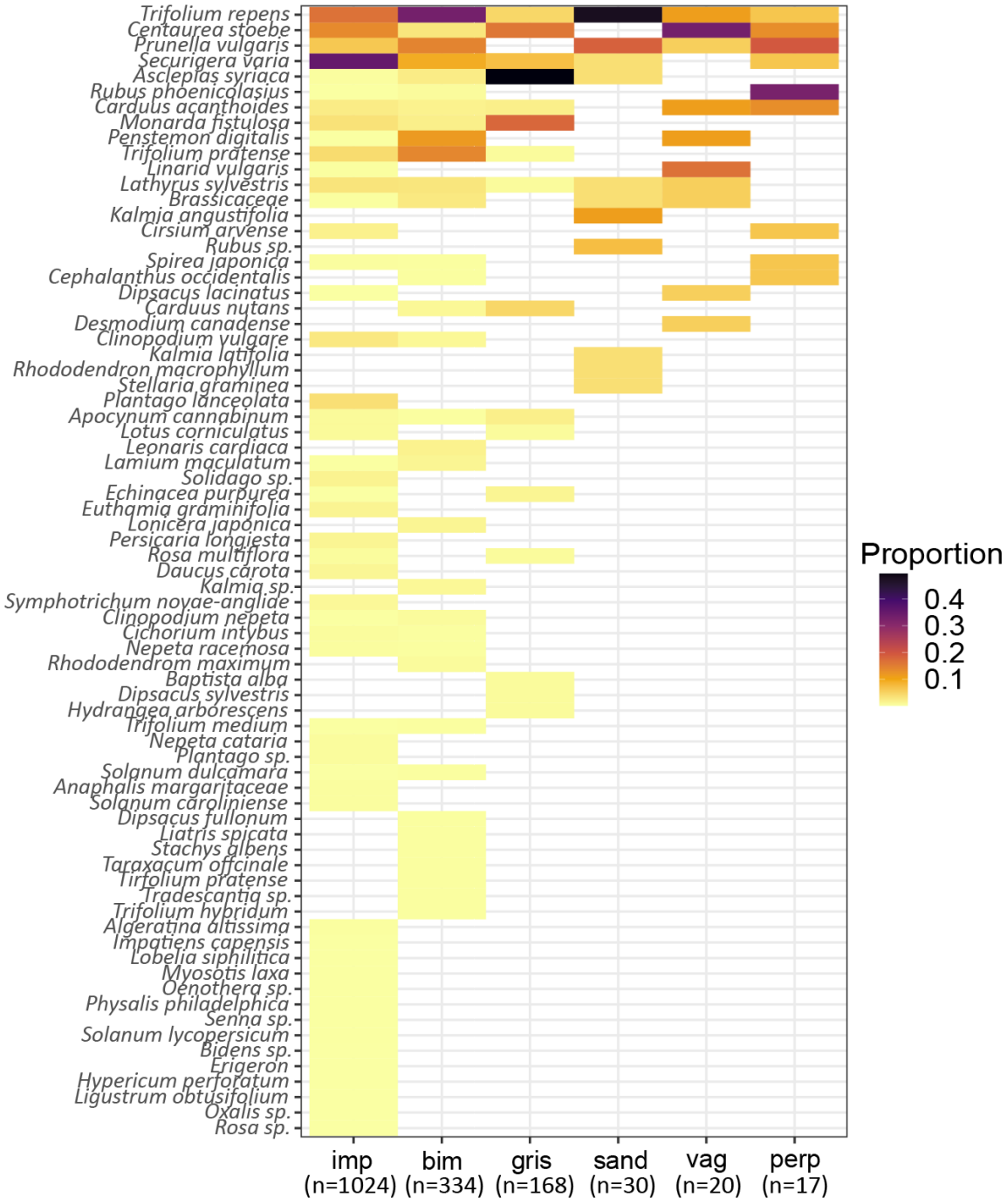

Figure 5. Heat map of worker host-plant visitations among the 6 most common bumble bee species. Data is aggregated from across all observations and sites. Colors show the proportion of plant visitations for each bumble bee species. Plants are arranged in order from top to bottom by the total proportion of visits aggregated across all bee species. Samples sizes below species labels

indicate the total number of plants visited. Plants were identified to the lowest taxonomic order possible and in a few instances, were only able to be identified to genus (n=9) or family (n=1). imp: *B. impatiens*, bim: *B. bimaculatus*, gris: *B. griseocollis*, sand: *B. sandersoni*, vag: *B. vagans*, perp: *B. perplexus*.

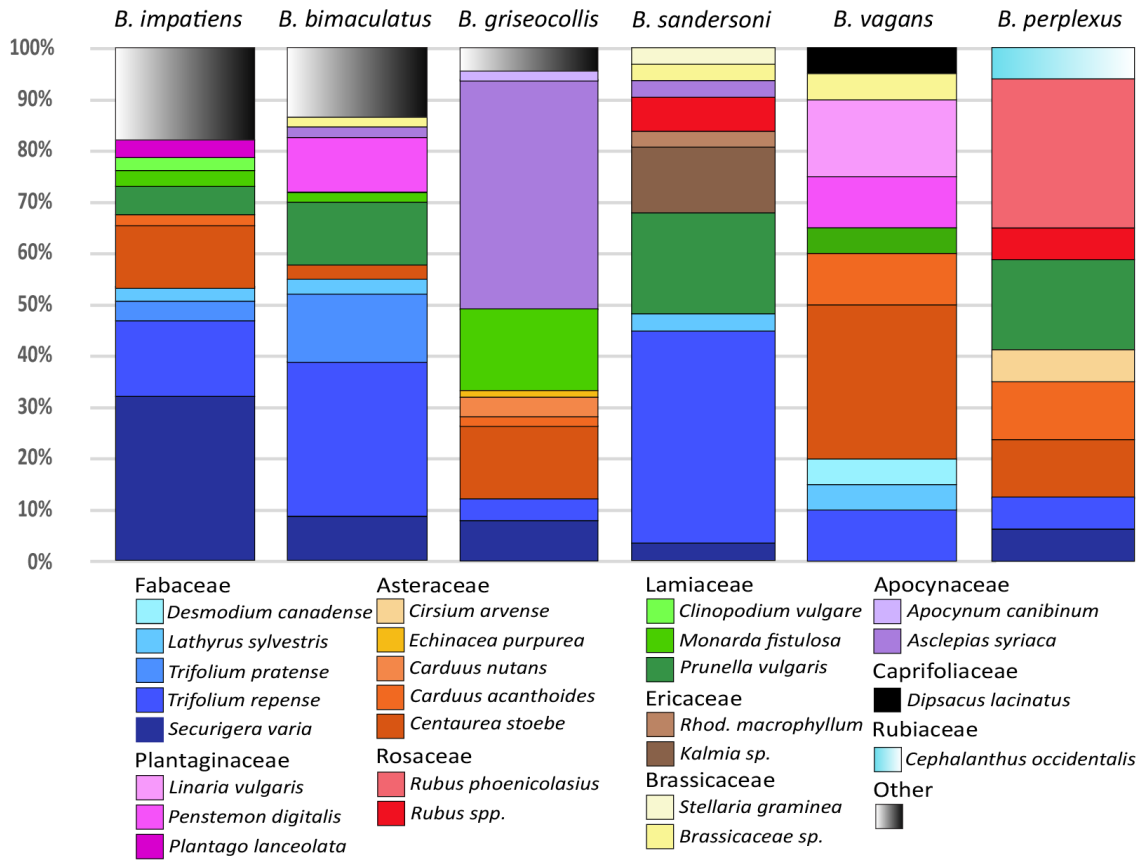

Figure 6. Plant visitation data among the 6 most abundant bumble bee species. Stacked bars show the percentage of plants visited by each species. Plant species are color coded by family. *Rhod.* = *Rhododendron*.

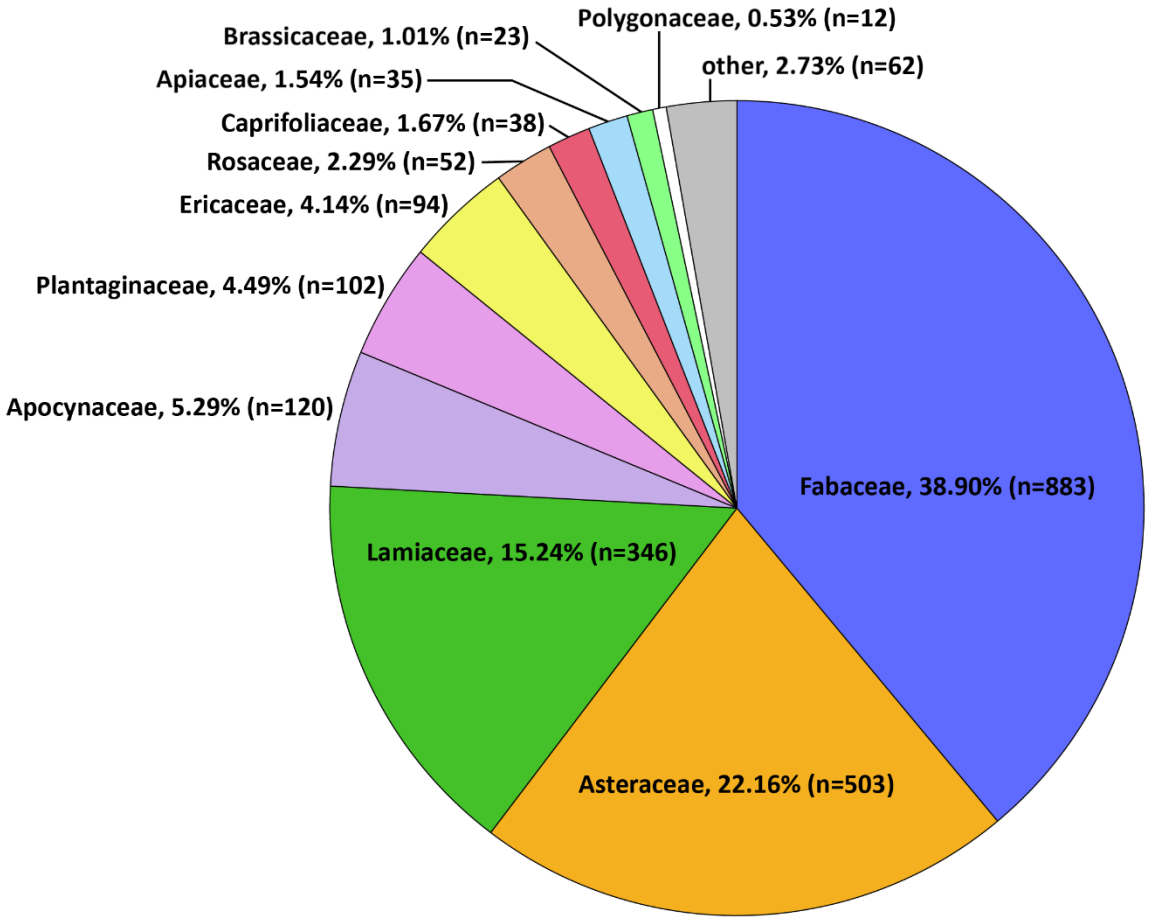

Figure 7. Percentages of visitations to each plant family aggregated across all bees from all surveys: queens, workers, and males from all 9 species. 2229 bees out of 2360 (94.4%) were recorded visiting a flower at the time of observation. Plant families with 10 or more visits are labeled, and families with fewer than 10 visits are combined into 'other'.

Tables

Table 1. Sites in the study, how many times each was surveyed, site habitat type, and site coordinates. All sites are in Pennsylvania and were surveyed during 2022. **(A)** Spring queen surveys (April 14th – May 25th). **(B)** Summer surveys (June 1st – Sept 4th).

Site	# Surveys	Habitat	lat long
(A) Spring			
Bernel Road Park	4	valley	40.85 -77.88
Penn State UP	3	valley	40.80 -77.86
Tudek Park	3	valley	40.80 -77.80
Circleville Park	3	valley-forested	40.80 -77.99
Fairbrook Park	3	valley-forested	40.72 -77.93
Colyer Lake	3	edge	40.78 -77.68
Scotia Pond	3	edge	40.80 -77.94
Bear Meadows (Rothrock SF)	3	forest	40.73 -77.75
Black Moshannon SP	3	forest	40.92 -78.06
Hyner View SP	3	forest	41.34 -77.60
(B) Summer			
Site	**# surveys**	**Habitat**	**lat long**
Bernel Road Park	1	valley	40.85 -77.88
Frick Park	1	valley	40.43 -79.91
Hummel Nature Trail	1	valley	40.27 -76.73
Ligoner Cemetery	2	valley	40.24 -79.24
Noel Dorwart Park	2	valley	40.06 -76.35
PSU Arboretum	2	valley	40.81 -77.87
PSU Fruit Farm	2	valley	39.94 -77.25
Abandoned PA Turnpike	2	edge	40.00 -78.23
Clarion County Park	2	edge	41.20 -79.44
Colyer Lake	4	edge	41.20 -79.44
Crooked Creek SP	2	edge	40.71 -79.51
Enola Grade Trail	1	edge	39.91 -76.34
Hills Creek SP	2	edge	41.81 -77.20
Mt Pisgah SP	2	edge	41.81 -76.67
Pine Grove Mills	3	edge	40.73 -77.88
Scotia Pond	1	edge	40.80 -77.94
Appalachian Trail (Weiser SF)	3	forest	40.53 -76.22
Bear Meadows	3	forest	40.73 -77.75

Benezette Elk Center	3	forest	41.32 -78.37
Black Moshannon SP	1	forest	40.92 -78.06
Camp William Penn Lk.	2	forest	41.15 -75.15
Cherry Springs SP	2	forest	41.67 -77.83
Frances Slocum SP	2	forest	41.34 -75.89
Hyner View SP	3	forest	41.34 -77.60
Lackawanna SP	2	forest	41.56 -75.71
Lily Pond	1	forest	41.36 -74.86
Moraine SP	2	forest	40.97 -80.10
Raymond B. Winter SP	1	forest	40.99 -77.15
State Game Lands 118	1	forest	40.49 -78.14
Weiser SF	2	forest	40.52 -76.78

Table 2. Methodology used to determine B. vagans, B. sandersoni, and B. perplexus (VSP mimicry complex) workers, queens, and males to species in each chapter. Barcoding: specimens were identified using the COI locus (Gratton et al., 2023). Morphology: specimens identified using morphological features – check length, pile color patterns in females, genitalia (i.e., gonostylus, penis valve, volsella) in males (P.H. Williams et al. 2014; Milam et al., 2020). Wing metrics: machine learning identification on scanned wings (Spiesman et al., 2024), run by Brian Spiesman (Kansas State Department of Entomology).

Chapter 1	**Species**	**Sex/caste**	**Barcoding**	**Morphology**
	B. vagans	worker	5	16
	B. vagans	queen	1	NA
	B. vagans	male	1	7
	B. sandersoni	worker	31	8
	B. sandersoni	queen	5	NA
	B. sandersoni	male	17	7
	B. perplexus	worker	11	6
	B. perplexus	queen	NA	1
	B. perplexus	male	3	6
Chapter 2	**Species**	**Sex/caste**	**Morphology**	**Wing metrics**
	B. vagans	worker	52	10
	B. sandersoni	worker	58	NA
Chapter 3	**Species**	**Sex/caste**	**Morphology**	
	B. vagans	worker	8	
	B. vagans	male	5	
	B. sandersoni	worker	8	
	B. sandersoni	queen	3	
	B. sandersoni	male	4	
	B. perplexus	worker	10	
	B. perplexus	queen	3	
	B. perplexus	male	4	

Table 3. Sampling effort by habitat type correction matrix used to produce the stacked bar phenology plots in figure 1a. The matrix is composed of the number of surveys for all habitat type-time periods (e.g., summer 1-valley = 6 surveys). Summer 1-Forest had the most surveys (14) and was therefore used as the dividend in the weighted correction equation. The number of surveys of the habitat type-time period being corrected for was used as the divisor. The quotient was then multiplied by the number of bees in the habitat type-time period being corrected for. As an example, the number of B. impatiens workers in summer 1-valley was 101. Therefore, the weighted correction is 14/6*101 bees = 235.67 bees.

	Valley	Edge	Forest
Spring	13	9	9
Summer 1	6	8	14
Summer 2	4	8	10
Summer 3	1	3	4

Table 4. Raw counts and raw percentages for each Bombus species by habitat type (Valley, Forest, Edge) and across habitats (2360 bees). VSP: B. vagans, B. sandersoni, B. perplexus mimicry complex.

Habitat	Species	Count	Relative abundance (%)
Valley	*B. impatiens*	214	53.77
	B. bimaculatus	71	17.84
	B. griseocollis	101	25.38
	B. sandersoni	0	0.00
	B. vagans	1	0.25
	B. perplexus	2	0.50
	VSP	7	1.76
	B. terricola	0	0.00
	B. fervidus	2	0.50
	B. ternarius	0	0.00
Edge	*B. impatiens*	283	53.40
	B. bimaculatus	135	25.47
	B. griseocollis	29	5.47
	B. sandersoni	20	3.77
	B. vagans	10	1.89
	B. perplexus	16	3.02
	VSP	14	2.64
	B. terricola	8	1.51
	B. fervidus	2	0.38
	B. ternarius	13	2.45
Forest	*B. impatiens*	903	63.06
	B. bimaculatus	299	20.88
	B. griseocollis	95	6.63
	B. sandersoni	48	3.35
	B. vagans	19	1.33
	B. perplexus	9	0.63
	VSP	47	3.28
	B. terricola	5	0.35
	B. fervidus	6	0.42
	B. ternarius	1	0.07
Total	*B. impatiens*	1400	59.32
	B. bimaculatus	505	21.40
	B. griseocollis	225	9.53

	B. sandersoni	68	2.88
	B. vagans	30	1.27
	B. perplexus	27	1.14
	VSP	68	2.88
	B. terricola	13	0.55
	B. fervidus	10	0.42
	B. ternarius	14	0.59

Chapter 2

Field-collected pollen macronutrient content is similar across bumble bee species but is site dependent

Introduction

Bumble bees are important pollinators in terrestrial ecosystems, with wild and managed populations contributing significantly to crop pollination in many parts of the world (reviewed in Goulson, 2010). However, approximately half of the studied bumble bee species in the U.S. and Europe have been showing declines in their abundance or distributions (Cameron & Sadd 2020; Colla 2012; Soroye, et al. 2020). While many bumble bee species have stable populations with some even expanding their range (e.g., *B. impatiens* and *B. terrestris*) (Colla & Packer, 2008; Herbertsson et al., 2021; Martinet et al., 2015b), others are experiencing declines over large parts of their historic ranges (e.g., *B. pensylvanicus, B. occidentalis)* (Cameron et al., 2011) or are declining in certain parts of its historic ranges (e.g., *B. vagans*) (Grixti et al., 2009). Bumble bees depend on nectar and pollen obtained from flowering plants for their food (Carnell et al., 2020) but expansion of industrialized agriculture has led to a reduction in the availability of flowering plants in agricultural landscapes (Goulson et al., 2008a; Rasmont et al., 2021) and changes in weather and climate further limit these floral resources (Quinlan et al., 2022, 2023a). Bumble bee species differ in the breadth of flowering plant species they visit (Wood et al., 2019). Differences in foraging needs may explain the success and distributions of these bees if habitats and ecozones differ in the nutritional resources they provide. Furthermore, bumble bee species that are more

specialized on certain plant taxa may be more likely to be nutritionally limited and thus more sensitive to changing land use patterns. Our understanding of how nutritional needs of different bumble bee species may impact their survival in different environments, however, is limited.

Bumble bees obtain their dietary lipids and proteins from pollen (Carnell et al., 2020; Vanderplanck et al., 2014), which serves as the primary food source for developing brood. However, concentrations of these pollen macronutrients differ among plant families (Vaudo et al., 2024, Roulston et al., 2000). For instance, pollen from plant species in the botanical families Fabaceae and Boraginaceae have high protein content relative to pollen from plant species in the families Asteraceae and Rosaceae (Hanley et al., 2008, Vaudo et al., 2024). In contrast, plants in Asteraceae tend to have higher pollen lipid concentrations than plant species in Fabaceae (Vaudo et al., 2020, 2024). Therefore, reductions in the number of floral species available to bumble bees has the potential to limit the nutritional diversity in the landscape, resulting in bees either foraging for less pollen if bees will only collect pollen with certain macronutrients (Vaudo et al., 2018) or force bee to collect pollen that is not nutritionally appropriate (Vaudo et al., 2016). Obtaining these nutritional optima does not require bees visiting plants only with preferred nutrient ratios, as bumble bees and other bees are recognized to complement nutrient intake by foraging from different floral resources that average around preferred nutritional means (Vaudo et al., 2024). Pollen mixing not only serves to balance nutritional macronutrient needs, but also to dilute or balance other compounds from these plants, some of which can be toxic (Rivest & Forrest, 2020).

Proteins and lipids are essential for bee health, larval development, and colony growth in social bees (Kämper et al., 2016; G. M. Quinlan & Grozinger, 2023; Tasei & Aupinel, 2008; Vanderplanck et al., 2014). Queenright *B. terrestris* colonies had higher colony growth rates, worker mass, pupae number, pupae wet mass, and lower worker mortality when fed pollen diets with higher protein compared to lower protein pollen diets (Watrobska et al., 2021), and *B. terrestris* micro-colonies fed pollen diets with higher protein content had increased larval weight

and decreased worker mortality (Tasei & Aupinel, 2008). Kraus et al. (2019) found that when given artificial diets, *B. terrestris* workers will regulate protein intake when brood is present but regulate lipid intake when brood is absent (suggesting workers make different choices when feeding on pollen for themselves versus collecting pollen for brood rearing in the colony), and mini colonies fed high-protein diets had the highest adult emergence rates. Studies of other bee species have also found important effects on development, health, and fitness from variation in proteins and lipids provided in pollen. Larvae of the sweat bee *Lasioglossum zephyrum* provisioned with the most protein-rich diets had the largest body sizes as adults (Roulston & Cane 2002), omega 6:3 fatty acid ratios of 1 or less improved associative learning in honey bees (Arien et al., 2018), and omega 3 polyunsaturated fatty acid deficiency decreased olfactory and tactile associative learning (Arien et al., 2015). In addition to protein and lipids, pollen sterols have been shown to be important for larval development in *B. terrestris* (Vanderplanck et al., 2014) and hormone synthesis in bees (Roulston & Cane, 2000; Vanderplanck et al., 2014) and insects in general (Patton, 1963).

Data from two of the most studied – and most widely distributed - bumble bee species, *Bombus impatiens* and *B. terrestris*, suggest they prefer to collect and consume pollen diets with a higher protein:lipid (P:L) ratio than other bee species that have been evaluated thus far, and that *B. impatiens* will selectively forage on these plants in diverse plant communities (Vaudo et al., 2016, 2018, 2020). Plants species have been found to produce pollen with a P:L range from 0.13 – 28.94 ratios, averaging at 4.64 in ecological plant communities that have been studied, whereas bumble bees seek out average ratios between 4:1 and 12.5 in the field (Vaudo et al., 2018, 2020, 2024). Forage preference tests of six plant species revealed that *B. impatiens* workers preferred to collect pollen from the flowering plant species with the highest P:L ratio among the tested plants (*Senna hebecarpa,* 4.6:1) in semi-field studies (Vaudo et al., 2016). In caged studies, where individual workers consumed the pollen themselves rather than bringing it back to the colony,

paired choice tests with nutritionally-manipulated honey bee pollen showed that *B. impatiens* workers preferred P:L ratios of 5:1 and 10:1 over 1.6:1 and 25:1 (Vaudo et al., 2016). Similarly, in paired choice tests with liquid diets (sucrose solutions with casein as a protein source and soy lecithin as a lipid source) spanning a wide range of P:L ratios, *B. terrestris* and *B. impatiens* caged workers regulated their P:L ratio intake to 14:1 and 12:1, respectively (Vaudo et al., 2017). A study providing *B. terrestris* micro-colonies with a choice of two artificial solid diets (high protein or high lipid) found that workers preferentially collected and stored diets with a somewhat lower ~3.1:1 P:L ratio regardless if brood was present or not (Kraus et al., 2019). Furthermore, although bumble bees may have preferences for proportionally high protein levels, this does not mean they can always attain these in natural environments, and thus the ratios they collect in the environment may be different. However, workers from *B. impatiens* colonies placed in the field collected pollen with an average of 4:1 P:L ratio, regardless of habitat (forest, edge, meadow) (Vaudo et al., 2018). Interestingly, this study also found that the bumble bees collected high protein content pollen earlier in the day, and then moved to lower protein pollen as the day progressed. Together, these results suggest bumble bees are prioritizing high P:L pollen when it is available but will collect pollen with lower P:L ratios as the floral community is depleted or changes over the course of a season. Notably, the nutritional content of bumble bee collected pollen tends to be higher than ratios collected by other bee species, such as *Osmia cornifrons* (~3:1 ratio) (Crone et al., 2023), *Andrena spp.* (3.5:1)*, Megachile sensu stricto spp.* (3.6:1)*, Perdita spp.* and *Agapostemon spp.* (1.2:1) (Vaudo et al., 2024).

The need for higher protein in these bees likely limits floral resource use and thus it may be expected that there has been historical selection for each species to balance their nutritional needs with the diversity of resources available to them. While the nutritional preferences of bumble bee species other than *B. impatiens* and *B. terrestris* have not been explicitly examined, previous studies have demonstrated that different bumble bee species preferentially forage on

different types of flowers, and thus they either preferentially or inadvertently may be obtaining different pollen nutritional content. For example, long-tongued bumble bee species preferentially forage on flowers with deep corollas compared to shorter-tongued bees (Inouye, 1978, 1980; P.H. Williams et al., 2014)**.** As flowers with deeper corolla tubes are predicted to produce more protein in pollen to enable the pollen tube to reach the ovules for fertilization (Roulston et al., 2000), longer-tongued bees may thus be predicted to forage on higher protein pollen. Exemplifying this, long-tongued bumble bee species disproportionately collect a higher percentage of pollen from the botanical family Fabaceae, plants which often have deep corollas (Wood et al., 2021), and Fabaceae has higher protein content (Hanley et al., 2008) and higher protein-lipid ratios compared to other plant families (Vaudo et al., 2020, 2024). Previous studies found that long-tongued species also collect pollen from fewer plant species relative to short and medium-tongued species (Wood et al., 2019), which may limit their attainable ratios. While this suggests long-tongued species may collect pollen with higher P:L ratios in the field, bumble bees may visit different plants for nectar and pollen (Brian, 1957), thus the degree to which innate differences drive preferences for pollen based on protein and lipids cannot be easily inferred from tongue length or general visitation data.

The flowering plant community can vary dramatically across habitat types, geographic regions, and seasons (Aggemyr et al., 2018; Goulson, 2010; Yan et al., 2023). Given different developmental needs across the season and shifts in floral communities, the preferred and/or attainable nutrient ratios may shift seasonally for bumble bees. Most bumble bee species are active across several weeks or months during the growing season, with foundress queens emerging early in the spring and collecting pollen for themselves and their first clutch of worker brood, and workers collecting pollen to rear developing queens and males in late summer or early fall (Goulson, 2010). A previous study examining *B. impatiens* worker-collected pollen over a 9 week period in the summer months found that the ratios did not vary with habitat (forest, edge,

meadow) but there was weekly fluctuation in nutritional content (ranging from 2.5:1 to 7:1): since these colonies were all in the social phase, the variation seemed to be primarily due to seasonal variation in surrounding floral community (Vaudo et al., 2018). Kriesell et al. (2017) found that different bumble bee species collected pollen from different flowering plants, yet all collected similar amino acid profiles, suggesting that different species may have similar nutritional requirements and can meet these using different plant species. Interestingly, the protein content of honey bee collected pollen does seem to change seasonally, with higher protein concentration in the summer and lower concentrations in the fall (Quinlan et al., 2021), which may be a reflection of changes in the overall plant community. More research is needed to understand whether there are seasonal shifts in obtained ratios in bumble bees and the factors that may drive them.

In this study I determine whether bumble bee species within an ecological community vary in their nutritional needs and whether location and season impact their attainable pollen nutritional ratios. I examined the pollen foraging preferences (plant species) and pollen nutritional preferences (protein concentrations, lipid concentrations, and protein-lipid (P:L) ratios) by evaluating pollen collected from the legs of field-caught wild foragers of five bumble bee species in Pennsylvania (*B. impatiens*, *B. bimaculatus*, *B. griseocollis, B. vagans*, and *B. sandersoni*) and two bumble bee species in Oklahoma, *B. pensylvanicus* and *B. impatiens*. These bees belong to several *Bombus* subgenera, have different tongue lengths, prefer different habitats, and vary in conservation status (Cameron and Sadd, 2019; Colla et al., 2012; Jacobson et al., 2018; P.H Williams et al., 2014) (Table 5). I examined these preferences from spring queens through to late summer workers and across multiple locations to determine if these preferences varied with background plant communities. These studies provide insights into whether landscapes similarly support the nutritional needs of diverse bumble bee species, providing information valuable for designing floral resources plantings to sustain these communities.

Methods

Bumble Bee Sampling

In Pennsylvania, pollen balls were collected in the field from the corbicula of 692 wild bumble bees representing 5 species - *B. impatiens*, *B. bimaculatus*, *B. griseocollis, B. vagans*, and *B. sandersoni* - from 5 sites. I collected pollen from 48 queens from two sites (Hyner View State Park, Colyer Lake) from May 6th and May 16th and from 552 workers from 4 sites (Colyer Lake, Rothrock State Forest, the Russell E. Larson Agricultural Research Center (Rock Springs), the Arboretum at Penn State) from June 10, 2023 to July 22nd, 2022. For queens, pollen was only collected from *B. impatiens*, *B. bimaculatus*, and *B. griseocollis* as *B. vagans* and *B. sandersoni* are difficult to distinguish in the field and I did not want to collect those queens for identification as this would prevent these foundresses from establishing colonies. Worker pollen collections were divided into four sampling periods: mid-June (sampling period A), late-June (sampling period B), early-July (sampling period C) and mid-July (sampling period D). Each site was sampled once per sampling period, except for the Arboretum at Penn State which was only sampled during the last two sampling periods. In Oklahoma, pollen was collected from 92 wild *B. impatiens* and *B. pensylvanicus* workers at 4 sites (Canadian River, Sutton Urban Wilderness, Songbird Park, Will Rogers Gardens) from a single time period from July 20th to July 26th.

The goal was to sample pollen from 12 bees per species during each survey, however, some species had low abundances at sites (i.e., *B. vagans* and *B. sandersoni*) or have relatively short colony cycles (i.e., *B. bimaculatus* and *B. griseocollis*), and therefore catching 12 workers per species per survey was not always possible. Detailed information on sampling period dates, locations, numbers of bees that pollen was collected from per species/caste and number of pooled pollen samples are provided in Table 6.

Pollen Collection in the Field

Bees were captured with insect nets while visiting host flowers, put into clean plastic vials (with holes drilled to allow air flow), and kept in a cooler containing ice packs at ~6˚C for 10-20 minutes to render the bee immobile without harming them. The host flower each bee was caught from was identified to species with the "SEEK by iNaturalist" app (*Seek by iNaturalist · iNaturalist*, n.d.) when ID was unknown and recorded for bee-host plant visitation analysis. Only 2.5% of bees were captured while not visiting flowers (e.g., netted while flying, crawling on the ground, etc.).

While bees were immobilized, pollen was gently scraped from both of their corbiculae with forceps and placed in a 1.7 ml sterile centrifuge tube. Forceps were cleaned with ethanol and a Kimwipe™ between bees. All workers were identified to species and released after pollen collection except for *B. vagans* and *B. sandersoni* workers. These bees were brought back to the lab for identification under a microscope using the malar space distinctions outlined by Milam et al. and reinforced by a reference collection of barcoded bees from Gratton et al. (2023). However, a subset of these bees (11) could not be clearly identified using malar space features, and thus identification was performed using scanned wings and the machine learning identification technique of Spiesman et al. (2024), a technique shown to be highly accurate for these species.

Vials containing pollen were kept on ice and transported to the lab where they were stored at -20˚C until further processing. Pollen loads were combined from two bees of the same species at each site per sampling period. Therefore, pollen collected from 12 bees for a particular species at a particular site and sampling period resulted in 6 pollen samples after combining. Pooling was performed to ensure there was enough pollen for protein-lipid (P:L) assays and metabarcoding from the same sample. Pollen loads of equal size (or of similar size if there was no exact match) were combined so that each pollen load contributed an equal proportion of

macronutrients and DNA for P:L and metabarcoding analysis, respectively. Combined pollen loads (hereafter "pollen sample") were homogenized in a 1.5 mL centrifuge tube with a plastic centrifuge pestle for 30 seconds.

Prior to P:L analysis, ~2 mg (wet weight) subsample per pooled sample was then collected for DNA metabarcoding (hereafter "metabarcoding sample"). Oklahoma samples were lyophilized first and then a ~2 mg dry weight sample was taken per pooled sample for DNA metabarcoding. Metabarcoding data will be performed separately and thus not reported further. The remaining pollen sample, used for P:L ratio analysis, was lyophilized for 12 hours and stored at -20˚C. I used the Bradford protein assay procedure to quantify protein concentration and the sulfo-phospho-vanillin assay (SPVA) method to quantify lipid concentrations following the same protocol described by Vaudo et al. (2020) with the following modifications: 1) For protein analysis, I used between 0.5 and 0.75 mg (instead of 1 mg) of pollen to ensure samples were in the linear range for quantification as early assays revealed higher levels reached a point of saturation in the standard curve. 2) I only ran one biological replicate per pollen sample per assay type instead of three (as in Vaudo, et al. 2020) since many of the pollen samples were 4 milligrams or less and thus, after removing material for metabarcoding, two or more biological replicates were often not possible. For each pollen sample, I calculated the protein concentration (ug protein/mg pollen), lipid concentration (ug lipid/mg pollen), and P:L ratio by dividing protein concentration by lipid concentration. In total, I conducted 344 protein and 344 lipid analyses.

Plant Surveys

I performed plant surveys at each site to analyze the pollen ratios collected by the bees compared to known ratios of species present at the site. For this all blooms in the survey site were counted and identified to species when possible, using the 'SEEK by iNaturalist' app when

necessary to confirm species. Inflorescences made of a head (capitulum) (many Asteraceae), head-like clusters (*Trifolium spp.*, *Pycanthemum spp., Plantago spp.,* etc.), and cyanthiums (e.g., *Zinnia, Croton*) were considered one floral unit as tiny individual florets on these plants were nearly impossible to count or estimate. Individual flowers on all other inflorescence types (raceme, corymb, spike, umbel, etc.) were considered single floral units. Table 7 lists each plant species recorded during the surveys and what is considered one floral unit for each species.

Statistical analysis

Pollen macronutrients

Comparisons among species, site, and sampling period in PA workers

All statistical analyses were carried out in R version 4.2.1 (R Core Team, 2022). For the worker bumble bees collected in Pennsylvania, I conducted multiple analyses of variance (ANOVAs) to compare the effects of species, site, and sampling period on protein concentration (Pc), lipid concentration (Lc), and P:L ratio followed by a Tukey's HSD test for multiple comparisons. Shapiro-Wilks tests revealed that none of the residuals were normally distributed. However, variances (standard deviation) for each species were approximately equal as confirmed by a Levene's test using the 'car' package (Fox & Weisberg, 2019), so ANOVA using non-transformed data was considered the best option as opposed to a nonparametric test. One additive and three interactive models were compared for each nutrition metric, and model selection was performed using Akaike's Information Corrected Criterion adjusted for small sample size (AICc) using the AICcmodavg package (Mazerolle, 2017) (Table 8). Models were chosen with the lowest Delta AICc score. Although this study included repeated measures (i.e., surveys) at each

site, sampling period was included as a fixed effect and not as a random effect since it was expected that different foraging bees were being sampled at each sampling period and that there was high plant community turnover between sampling periods at each site, and I hypothesized that this turnover might influence pollen macronutrients over time. Finally, a second version of each analysis was conducted after removing samples from the Arboretum at Penn State. This adjustment was made because *B. vagans* and *B. sandersoni* were not observed at this site. The aim of this second analysis was to compare pollen from sites where all 5 bumble bee species were represented.

Comparisons among PA queens and between PA queens and workers

For queens, the effects of species and site on Pc, Lc, and P:L ratio were tested using ANOVAs for each. Protein and lipid data for queens had equal variance (Levene's test) and normal residuals (Shapiro-Wilks test), while P:L ratio residuals were not normal but species had equal variance. I also tested for the effect of species and caste on pollen nutrition by comparing spring queens and workers combined across time periods for *B. impatiens*, *B. bimaculatus* and *B. griseocollis*. This analysis was only performed for the Colyer Lake site since this was the only site where pollen samples were obtained for both castes for all three species. AICc was used for model selection between an additive and interactive model (Table 8).

Comparisons between species and sites in Oklahoma

For worker bumble bees collected in Oklahoma, I tested for the effects of species and site on P:L, Pc, and Lc with ANOVA followed by Tukey's HSD. AICc was used for model selection between an additive and interactive model (Table 8).

Comparisons between Oklahoma and Pennsylvania workers of B. impatiens

To understand if there was a regional effect on pollen nutrition, I used t-tests to look for an effect of origin (state) on pollen collected by *B. impatiens* workers from Oklahoma and Pennsylvania. Due to collinearity between bee origin and location (sites within a state) for each nutritional metric, as identified using the 'vif' function from the 'car' package (Fox & Weisberg, 2019), I only looked at the effect of origin on mean Pc, Lc, and P:L ratio. Separate t-tests for were conducted for Pc, Lc and P:L ratio.

Evaluation of nutritional flexibility across B. impatiens

I also investigated how *B. impatiens* varies in nutritional preference across location and time. For this analysis, I used an ANOVA, followed by a Tukey HSD test, to test for the additive effects of site and sampling period on Pc, Lc, and P:L ratio of collected pollen. Additive models were chosen as no interactive models produced significant interactions between site and sampling period.

Evaluation of realized versus available nutrition for B. impatiens workers

To assess if bumble bees selectively forage for protein and lipids, at each site, I compared P:L ratios, protein concentrations (Pc) and lipid concentrations (Lc) in plants visited by *B. impatiens* to overall pollen P:L/Pc/Lc available in the community. When calculating collected macronutrients, I used the same protocol as in Vaudo et al. (2020), however, to avoid saturation of protein with Bradford reagent, I used less pollen for protein assays than what was recommended in the protocol (0.5 – 0.75 mg compared to 1 mg), and thus, I likely achieved

higher protein values for the same plant species/genera than what Vaudo had calculated. Therefore, to obtain values for collected macronutrients, I calculated Pc, Lc, and P:L ratio from plants that bees were observed visiting using pollen metrics from Vaudo et al. (2020, 2024) instead of my own collected pollen macronutrient data. Although relying solely on visitation data may not fully represent the diversity of pollen species in each sample, it offers a consistent approach by using a single pollen metric dataset. The only exception where I did not use Vaudo's data to calculate pollen macronutrient content was for the plant *Chamaecrista fasciculata* (partridge pea, Fabaceae) on which visitation rate was 100% and 92% by *B. impatiens* at two Oklahoma sites. All pollen loads collected from bees visiting this plant species were identical in color and texture, and therefore, were assumed to all be *C. fasciculata* pollen (most other pollens collected from a single plant species were not consistent and/or uniform in color and texture, indicating that pollen was from various plant species). Thus, I used my protein concentration values from this pollen to calculate *C. fasciculata* protein contribution to the landscape and collected protein by bees visiting this plant. This was done since the Pc value for *C. fasciculata* from Vaudo et al. (2020) was much lower than bee-collected *C. fasciculata* pollen in my study (173.55 vs 474 ug/mg, respectively). Since visitation to this plant significantly influenced regional differences in protein and P:L ratio collection (see results below), it was important to ensure that its contribution to available nutrients was consistent with my own pollen assays.

I chose to only use *B. impatiens* for this analysis as this was the only species that was equally represented across all sites/sampling periods (i.e., 12 bees per survey) for Pennsylvania data and all sites for Oklahoma data (each Oklahoma site was only sampled one time). The proportion of blooms for each plant species of interest relative to the total amount of blooms at each survey site-date was multiplied by its corresponding P:L ratio, Pc, and Lc (P:L/Pc/Lc) and the products were summed to give the available P:L/Pc/Lc. As an example, to calculate total

available P:L, Total P:L $= \sum_{i=1}^{n} \left(\frac{\text{Bloom count}_i}{\text{Total bloom count}} \times \text{P: L}_i \right)$. This allowed me to compare the total P:L/Pc/Lc available at each site to the mean P:L/Pc/Lc of the visited plants. For available P:L/Pc/Lc, I used data for the lowest taxonomic order possible. Most plants in my plant surveys were identified to species, but often a particular species was not available from the reference databases, in which case I used the P:L/Pc/Lc from the corresponding genus. If there were more than one species listed in a genus, I used the mean P:L/Pc/Lc of all species in that genus. If a genus was not in the reference database, I used the mean P:L/Pc/Lc for the family. If the family was not in the reference data base, I used mean P:L/Pc/Lc for the closest related family in the reference data base. If there was no intra-order family P:L/Pc/Lc available, the plant was removed. No emissions were made for visited plants as all had matches in the reference database. Vaudo et al. (2024) found a strong phylogenetic correlation in P:L ratios, Pc, and Lc which justifies this use of this approach. This approach exhibits bias in that not all flower units produce the same quantity of pollen and visitation is impacted by other factors besides pollen ratios, however, this serves as a best approximation of what is available in the floral community to assess whether bumble bees show any preference when collecting pollen from the surrounding floral community or simply collect pollen based on availability. A paired Wilcoxon rank-sum test was used to compare total available P:L/Pc/Lc at each site/sampling period with mean collected P:L/Pc/Lc for each site/sampling period. Since the calculated available P:L/Pc/Lc at each site/sampling period were the summed products of the relative plant proportions and calculated P:L/Pc/Lc for the plants, no standard deviation could be calculated for total P:L/Pc/Lc availability and so a non-parametric analysis was considered appropriate. A paired version of this test was used since the samples were related (i.e., non-independent) as the P:L/Pc/Lc of bee-collected pollen was likely influenced by the availability of P:L/Pc/Lc in the landscape (e.g., bees may collect higher P:L ratios when in landscapes with higher available P:L ratios).

Visitation Analysis

To assess whether bumble bee species differ in their utilization of floral species at a given site, heat maps were generated showing the proportion of plants visited by each bee species at each site (sampling period was pooled for this analysis). Separate heat maps were generated for Pennsylvania bees (including queens) and Oklahoma bees.

As another metric for whether species utilized these communities differently, the 'bipartite' package in R was used to generate plant-pollinator interaction networks (Dormann et al., 2023). I examined visitation of individual PA workers for this analysis. The Arboretum site was excluded as *B. vagans* and *B. sandersoni* were not present at this site as I wanted to look at networks based on sites where all species were present. The modularity of the network was computed using the 'computeModules' function (Dormann et al., 2023), and a likelihood score was generated based on how well the module represented the modular organization of the network. To determine statistical significance, the likelihood score of the realized module was compared against a probability distribution of null model likelihood scores (n = 500). For this, the bipartite function nullmodel was used followed the method vaznull (Dormann et al., 2023).

Results

Protein analysis

PA workers:

Mean pollen protein concentration (Pc) for PA workers was 304.60 ± 55.66 [sd], with a minimum of 107.01 and a maximum of 500.66. *B. vagans* had the highest Pc (324.2 ± 60.8),

followed by *B. griseocollis* (314.50 ± 57.24), *B. bimaculatus* (312.08 ± 50.89), *B. impatiens* (295.51 ± 55.88), and *B sandersoni* (285.85 ± 58.03).

An ANOVA analysis using all the samples showed significant differences in Pc by species ($F_{4,252} = 3.39$, $p = 0.01$), location ($F_{3,252} = 6.08$, $p < 0.001$), and the interaction of species and location ($F_{10,252} = 2.49$, $p = 0.008$) (Table 9a). Pairwise comparisons among species (Tukey's HSD Test) revealed that *B. vagans* and *B. sandersoni* were significantly different ($p = 0.044$), and *B. impatiens* and *B. vagans* were nearly different ($p = 0.078$). Pairwise comparisons among locations revealed that pollen collected from the Arboretum had significantly higher protein content than Colyer Lake ($p = 0.027$), Rock Springs ($p < 0.001$), and Rothrock ($p = 0.020$) and 5 pairwise comparisons were significant for species-site interactions (Figure 8a and 8b, Table 10a). Sampling period did not have an effect on Pc ($F_{3,251} = 0.72$, $p = 0.538$).

When I removed the Arboretum so that only four sites that included all species and all sampling periods were part of the analysis, there was a statistically significant effect of species on Pc ($F_{4,233} = 4.91$, $p < 0.001$) (Table 9b). Pairwise comparisons showed *B. vagans* had higher pollen Pc than *B. impatiens* ($p = 0.005$) and *B. sandersoni* ($p = 0.033$), and *B. bimaculatus* > *B. impatiens* ($p = 0.019$). There was no effect of sampling period ($F_{3,233} = 0.41$, $p = 0.749$) and a trend towards an effect of site ($F_{4,233} = 2.44$, $p = 0.090$) (Figure 8c, Table 10b). One *B. sandersoni* pollen sample had unusually low protein (107.01 ug/mg) but removing this observation and rerunning the analysis had no effect on results.

PA queens:

Mean Pc (ug/mg) for PA queens was 287.42 ± 40.00 [sd], with a minimum of 184.67 and a maximum of 355.07. *B. griseocollis* had the highest Pc (309.24 ± 38.02), followed by *B. impatiens* (287.37 ± 39.25), and *B. bimaculatus* (276.58 ± 42.53).

An ANOVA analysis showed that the interaction of species and location had a significant effect on mean Pc ($F_{1,19} = 8.92$, $p = 0.008$), however, no pairwise comparisons were significant (Tukey HSD). No effect of species (*B. impatiens*, *B. bimaculatus*, and *B. griseocollis*) ($F_{2,19} = 1.17$, $p = 0.331$) or site ($F_{1,19} = 0.03$, $p = 0.856$) was detected, although *B. griseocollis* queens were not found at one of the two sites (Hyner View State Park) (Table 9c).

When comparing queens and workers at Colyer Lake, the interaction between species and caste had a significant effect on mean Pc (*B. impatiens*, *B. bimaculatus*, and *B. griseocollis*) ($F_{2,65} = 5.71$, $p = 0.005$), and there was a trend towards significance for species ($F_{2,65} = 2.74$, $p = 0.072$), but no effect of caste (Table 9d). Pairwise comparisons revealed that *B. bimaculatus* workers had a higher mean Pc (337.28 ± 44.26) than *B. bimaculatus* queens (237.49 ± 49.51) ($p = 0.009$) and *B. impatiens workers* (290 ± 48.88) ($p = 0.018$) (Figure 9, Table 10c).

OK workers:

Mean pollen Pc (ug/mg) for OK workers was 417.8 ± 92.23 [sd], with a minimum of 205.1 and a maximum of 634.7. Pollen collected by *B. impatiens* and *B. pensylvanicus* was not different in Pc ($F_{1,42} = 1.35$, $p = 0.252$). There was a significant effect of site ($F_{3,42} = 8.31$, $p < 0.001$) (Table 9e), with the two sites where bees primarily visited the legume *Chamaecrista fasciculata* (Canadian River and Songbird Park) having higher Pc than where bees primarily visited plants in the family Solanaceae (Sutton Park) and plants in the family Lamiaceae (Will Rogers Botanical Garden) (Figure 10, Table 10d).

OK and PA B. impatiens workers:

B. impatiens workers from OK had a significantly higher mean Pc (430.87 ± 108.28) than PA workers (295.51 ± 55.88) (t (25.29) = 5.79, CI = 87.23 -183.50, p < 0.001) (Figure 11a).

Lipid analysis

PA workers:

Mean pollen Lc (ug/mg) for PA workers was 73.20 ± 23.56 [sd], with a minimum of 16.93 and a maximum of 182.68. *B. vagans* had the highest Lc (75.90 ± 21.61), followed by *B. griseocollis* (75.34 ± 18.39), *B. bimaculatus* (74.46 ± 23.61), *B. sandersoni* (72.30 ± 27.28), and *B. impatiens* (69.94 ± 26.21).

ANOVA analysis revealed that sampling period had a significant effect on Lc ($F_{3,262}$ = 4.42, p = 0.005) (Table 9a), with period C (80.43 ± 22.38), having a higher Lc than period A, (66.09 ± 20.03) (p = 0.004), with period B (72.33 ± 26.90) and D (71.18 ± 22.02) not being significantly different from A or C (Table 10e). Species ($F_{4,252}$ = 0.71, p = 0.587) and site ($F_{3,252}$ = 0.50, p = 0.682) did not have a significant effect (Figure 12a, Table 9a).

When the Arboretum site was removed, sampling period remained significant ($F_{3,233}$ = 4.16, p = 0.007) (Table 9b), with period C (80.63 ± 22.27) having a higher Lc than period A, (66.09 ± 20.03) with no significant effect of species ($F_{4,233}$ = 0.72, p = 0.582) and site ($F_{2,233}$ = 0.356, p = 0.701) (Figure 12b, Table 10f).

PA queens:

Mean Lc (ug/mg) for PA queens was 71.75 ± 21.44 [sd], with a minimum of 26.53 and a maximum of 117.44. *B. griseocollis* had the highest Lc (80.39 ± 21.23), followed by *B. impatiens* (71.74 ± 26.95), and *B. bimaculatus* (67.43 ± 10.54). An ANOVA.

When analyzing mean Lc in pollen collected by these queens, I found no difference among species ($F_{2,20} = 0.53$, $p = 0.599$) but a trend towards a significant effect of site ($F_{1,20} = 3.78$, $p = 0.066$) (Table 9c). Colyer Lake had pollen with higher Lc (80.22 ug/mg ± 22.89) than the forested mountain site Hyner View State Park (61.73 ug/mg ± 14.98), but these sites were not significantly different (Tukey test: $p = 0.094$). I found no difference in mean Lc when comparing workers and queens for three species at Colyer Lake (*B. impatiens*, *B. bimaculatus*, and *B. griseocollis*) ($F_{5,65} = 0.72$, $p = 0.609$) (Table 9d).

OK workers:

Mean pollen Lc (ug/mg) for OK workers was 84.07 ± 34.53 [sd], with a minimum of 33.09 and a maximum of 179.31. Neither species ($F_{1,42} = 0.44$, $p = 0.512$) nor site ($F_{3,42} = 1.93$, $p = 0.140$) had a significant effect on Lc (Table 9e).

OK and PA B. impatiens workers:

OK *B. impatiens* did not have a significantly higher mean Lc (80.74 ± 33.30) than PA *B. impatiens* (69.94 ± 26.21) ($t(29.87) = 1.44$, CI = -4.54 - 26.14, $p = 0.161$) (Figure 11b).

P:L analysis

PA workers:

Mean pollen P:L for PA workers was 4.66 ± 1.88 [sd], with a minimum of 1.08 and a maximum of 12.98 across samples, and generally exhibited high variance across samples. There was a narrow range of overall means across species from 4.5:1- 4.8:1 P:L. *B. impatiens* had the highest P:L ratio (4.78 ± 1.96), followed by *B. bimaculatus* (4.71 ± 2.04), *B. sandersoni* (4.63 ± 2.13), *B. vagans* (4.63 ± 1.66), and *B griseocollis* (4.47 ± 1.59).

P:L ratio was significantly influenced by sampling period ($F_{3,252} = 3.77$, $p = 0.011$) and the interaction of species and site ($F_{10,252} = 2.28$, $p = 0.014$) but not by species alone ($F_{4,252} = 0.27$, $p = 0.90$) or site ($F_{3,252} = 0.15$, $p = 0.93$) (Figure 13a Table 9a). Means by sampling period also showed a fairly narrow range of 4.14 – 4.99 P:L. The earliest sampling period (A: 4.99 ± 1.91), had a significantly higher mean P:L than the third sampling period (C: 4.14 ± 1.99) ($p = 0.024$), and there was a near significant difference in mean P:L between sampling period B (4.88 ± 2.11) and C ($p = 0.05$) (Figure 13a, Table 10g). Mean P:L for sampling period D was 4.80 ± 1.99.

When the Arboretum site was removed (Figure 13b) the results were the same: P:L ratio was significantly influenced by sampling period ($F_{3,225} = 3.22$, $p = 0.024$) and the interaction of species and site ($F_{8,225} = 2.31$, $p = 0.021$) but not by species alone ($F_{4,225} = 0.37$, $p = 0.83$) or site ($F_{2,225} = 0.16$, $p = 0.85$) (Table 9b). The earliest sampling period (A: 4.99 ± 1.91), had a significantly higher mean P:L than the third sampling period (C: 4.08 ± 1.51) ($p = 0.029$), and there was a near significant difference in mean P:L between sampling period B (4.88 ± 2.11) and C ($p = 0.056$) (Figure 13b, Table 10h)**.**

PA queens:

Mean pollen P:L for PA queens was 4.38 ± 1.61 [sd], with a minimum of 2.63 and a maximum of 9.40. *B. impatiens* had the highest P:L (4.59 ± 2.04), followed by *B. bimaculatus* (4.21 ± 1.03), and *B. griseocollis* (4.11 ± 1.37).

When analyzing mean P:L in pollen collected by these queens, I found no difference among species ($F_{2,20} = 0.52$, $p = 0.820$) or between sites ($F_{1,20} = 2.35$, $p = 0.141$) (Table 9c). I found no significant difference in mean P:L when comparing workers and queens for three species at Colyer Lake (*B. impatiens*, *B. bimaculatus*, and *B. griseocollis*) ($F_{5,65} = 3.93$, $p = 0.217$) (Table 9d). The average P:L for all queens was 3.93 ± 1.52 and for workers was 4.76 ± 1.68.

OK workers:

Mean pollen P:L for OK workers was 5.76 ± 2.50, with a minimum of 1.80 and a maximum of 13.41. There was no difference in mean pollen P:L between *B. impatiens* (5.96 ± 2.29) and *B. pensylvanicus* (5.57 ± 2.72) ($F_{1,42} = 0.30$, $p = 0.59$), but a trend for an effect of site: Will Rogers Gardens (4.26 ± 2.01), Sutton Urban Wilderness (6.64 ± 3.14), Canadian River (5.91 ± 1.72), and Songbird Park (6.31 ± 2.58) ($F_{3,42} = 2.26$, $p = 0.096$) (Table 9e).

OK and PA B. impatiens workers:

OK *B. impatiens* had a significantly higher mean P:L (5.96 ± 2.29) than PA *B. impatiens* (4.78 ± 1.96) ($t(31.42) = 2.26$, CI = 5.96 - 4.78, $p = 0.031$) (Figure 11c).

Evaluation of *B. impatiens* worker nutritional flexibility across sites and time

B. impatiens Pc varied significantly by site (ANOVA: $F_{3,77} = 6.91$, $p < 0.001$) (Table 11a). Pairwise comparisons revealed that pollen collected from the Arboretum (355.24 ± 77.74 [sd]) had higher Pc content than pollen collected at Colyer Lake (290.18 ± 44.88), Rock Springs (288.49 ug/mg ± 46.02), and Rothrock (278.07 ± 40.27) with no other pairwise differences between sites (Figure 14a, Table 12a).

B. impatiens Lc varied significantly by sampling period ($F_{3,77} = 3.59$, $p < 0.017$) (Table 11b). However, pollen Lc collected from sampling period C had only nearly significantly higher Lc (80.40 ug/mg ± 26.86) than sampling period D (50.10 ug/mg ± 15.28) ($p = 0.056$) with no other pairwise differences (Figure 14b, Table 12b).

B. impatiens P:L varied significantly by site ($F_{3,77} = 4.07$, $p = 0.010$) (Table 11c). Pollen collected by *B. impatiens* from Rock Springs had significantly higher P:L (5.59 ± 2.58) than Rothrock (3.96 ± 1.44) with no other pairwise differences (Figure 14c, Table 12c). *B. impatiens* P:L also varied significantly by sampling period ($F_{3,77} = 4.06$, $p = 0.010$) (Table 11c). Pollen collected from sampling period D had significantly higher P:L (5.57 ± 2.37) than sampling period C (3.99 ± 1.60) with no other pairwise differences (Figure 14d, Table 12d).

Collected vs. available macronutrients in B. impatiens workers:

Across all surveys, median available Pc was 203.56 (IQR 75.83) and collected Pc was 225.00 (IQR 93.80) (Figure 15a). Median available Lc was 44.50 (IQR 10.65) and collected Lc was 39.46 (IQR 16.61) (Figure 15b). Median available P:L was 5.63 (IQR 2.59) and collected P:L 6.58 was (IQR 2.13) (Figure 15c). Results of the Wilcoxon rank-sum paired test indicated that collected Pc was significantly higher than available Pc ($V = 171$, $p = 0.014$), but no difference

between collected Lc and available Lc ($V = 90$, $p < 0.588$), and no difference between collected vs available P:L ($V = 150$, $p = 0.0967$. Collected vs available macronutrients for each survey (site/sampling period) is plotted in Figure 16: Pc (A), Lc (B), and P:L (C). In these figures, collected Pc, Lc, and P:L are shown as violin plots as they represent the distribution of collected Pc, Lc, and P:L values at each site, however, available Pc, Lc, and P:L are shown as points since each survey is the sum of the product of bloom proportions and calculated Pc, Lc, and P:L.

Plant visitation

In Pennsylvania, bee species foraged most frequently and consistently across sites on legume species *Trifolium repens* and *Securigera varia. B. griseocollis* was the only bee found to forage on milkweed while *B. vagans* foraged more than the other bee species on *Carduus acanthoides* (Figure 17). Plants are arranged in order, from highest proportion of visitations across all bumble bees (top row of heat map tiles) to lowest (bottom row of heat map tiles).

The bee-plant interaction network for PA workers is shown in Figure 18. Most bumble bee species were in distinct modules except for *B. bimaculatus* and *B. griseocollis* which shared a module (Figure 19), with *Securigera varia* as the most-visited plant for these two bees. *B. impatiens* also visited *S. varia* with high frequency, but instead showed modularity centered around *Plantago lanceolata*. *B. sandersoni* showed modularity centered around *Trifolium repens*, and *B. vagans* showed modularity centered around *Carduus acanthoides.* The observed module likelihood score was 0.226, implying moderate structure to the interaction network. The distribution of the null model likelihood scores ($n = 100$) had a median of 0.093 and did not overlap with the observed likelihood score, resulting in a p-value of 0 (Figure 20), indicating less structure in the null modules compared to the observed module.

The bee-plant heat map (Figure 21) shows that the top two most-visited plants by *B. impatiens* and *B. pensylvanicus* were *Chamaecrista fasciculata* (Partridge pea) and *Solanum elaeagnifolium* (Silverleaf nightshade). Both species also heavily visited mint plants (Lamiaceae) at the Will Rogers Garden site.

Discussion

In this study, I evaluated the foraging and nutritional preferences of six bumble bee species, across habitats, castes, time periods and geographic ranges. Overall, for the five co-foraging bumble bee species in PA, the average P:L ranged from 4:5-4.8:1, and this was slightly higher for the two species in OK (5.6-6:1), and thus, although some constituents were significantly different by species, no strong difference in P:L was observed. These ratios are consistent with prior ratios obtained from plant-pollen collection studies on *B. impatiens* and suggests that many bumble bee species may have similar nutritional needs. There was high variance across the samples collected from individual bees within a species, suggesting that colonies of foraging bumble bee workers may achieve their nutritional optima via averaging through mixed sampling across ratios. Interestingly, bees collected similar average ratios across sites and collection periods, despite quite different average ratios being offered by the plant communities, suggesting bumble bees forage selectively to achieve nutritional optima. Although effects were not large, there was variation in geographic region, time period, and site that suggest floral availability impacts the nutritional outcomes these bees can achieve and that what is available in a plant community may not always be what the bees most prefer. Proteins showed the most significant differences across sites and species and were highest at sites with high available protein and/or high plant diversity, while lipids only differed by time period. This suggests bumble bees may be selectively prioritizing protein content in the pollen they collect, which is

consistent with other studies that indicate that bees collect higher protein content pollen earlier in the day (Vaudo et al., 2014, 2018).

Nutritional and foraging preferences of *Bombus* workers.

There was some variation in protein content of pollen collected by different species, with *B. vagans* collecting pollen with the highest protein concentration and *B. impatiens* the lowest in Pennsylvania sites, though both *B. impatiens* and *B. pensylvanicus* collected pollen with similar nutritional content in Oklahoma. Bees foraging in the Arboretum at Penn State – a botanical garden with high plant diversity – and in two sites in Oklahoma with high abundance of a legume (partridge pea) - collected pollen with significantly higher quantities of protein than at other sites, suggesting that when given the opportunity, bumble bees will collect pollen with higher protein concentrations. Bumble bees across sites and regions tended not to collect pollen with protein concentrations below 200 ug/mg, despite most of the plants in the study sites having pollen protein concentrations below this value. This suggests that bumble bees may have a minimum protein concentration preference, providing further evidence for selective foraging in bumble bees.

Previous studies with *B. impatiens* showed that they prefer flowers with higher P:L ratios in foraging choice assays (Vaudo et al., 2016, 2017) and *B. terrestris* microcolonies provided with pollen with higher protein content have improved colony growth and health metrics (Tasei & Aupinel, 2008). Thus, these results suggest that *B. impatiens* collected higher protein when possible, given the floral plant community but was flexible to collect pollen with lower protein at poorer sites. Overall, *B, impatiens* has a broad distribution and is found in the most diverse types of habitats, including urban and degraded habitats (Gratton 2023), thus, it is possible that the ability to forage on low-quality pollen allows this species to thrive in these different

environments. The degree of lability of other species in the face of these preferences is in need of study.

For Pennsylvania bees, there was no significant differences in P:L among species and sites, no differences among queens, nor any differences between workers and queens. This suggests that these different bumble bee species have similar target P:L ratios. The mean P:L ratio for PA workers across species was 4.5-4.8:1, similar to ratios Vaudo et al. (2017, 2018, 2020) previously found for *B. impatiens*. One of the sampled species, *B. griseocollis*, belongs to a separate subgenus (*Cullumanobombus*) than the other four (*B. impatiens, B. bimaculatus, B. vagans,* and *B. sandersoni* (*Pyrobombus*, Table 5), and thus there does not appear to be phylogenetic variation in P:L ratio, but more studies should be conducted across a broader range of subgenera. Likewise, *B. impatiens* and *B. pensylvanicus* (*Thoracobombus*) at Oklahoma sites showed no difference in P:L ratio. However, Oklahoma *B. impatiens* had higher P:L than Pennsylvania *B. impatiens*, which appears to be driven by higher protein pollen collected at Oklahoma sites rather than lower lipid collection.

Depending on the composition of nutrients in the floral community, bees may be able achieve their P:L intake target by foraging pollen from a single plant species if the species happens to provide the target P:L. Alternatively, bees may be required to forage pollen from multiple plant species with various pollen and lipid concentrations, that, when combined in the right proportions, provide the target P:L (Vaudo et al., 2024). My plant visitation data suggests that bees may be achieving these targets from only a few species per site, but that they visit different species across sites to attain this. Future analyses using pollen DNA metabarcoding will give more insight into diet breadth of each bee species.

Nutritional and foraging preferences of Bombus queens and among castes.

Bumble bee colonies are initiated by a single foundress queen, and, once the first clutch of workers are produced, the workers continue foraging and brood provisioning (reviewed in Amsalem et al. (2015b)). Once the colony becomes large enough, the workers begin rearing the next generation of queens and males. In bumble bees, queens have larger bodies than workers (P.H. Williams et al., 2014). Queen-destined larvae are fed for longer to achieve this larger size (Alford, 1975) and may also be fed different glandular secretions (Franco et al., 2023). Protein levels in collected pollen did not shift by season, but there was a small increase in lipids during the third time period. This suggests species do not adjust pollen quality substantially or consistently across their colony cycle as they transition to queen production.

Workers reared by the founding queens are typically smaller than workers reared by other workers later in the colony cycle, which is likely because a single queen cannot collect as many resources as multiple workers (Alford, 1975) but could be a result of different nutritional optima. I found no differences for any nutritional metric among *B. impatiens, B. bimaculatus*, and *B. griseocollis* queens, indicating no species differences among queen nutritional intake. Moreover, the spring queens collected similar P:L ratios at the Colyer Lake site compared to workers suggesting there are no differences in nutritional foraging preferences. There were, however, differences in protein levels when comparing spring queens and workers, with *B. bimaculatus* spring queens collecting lower Pc than *B. bimaculatus* workers. This may be a result of protein availability rather than preference as spring floral resources at Colyer Lake were less diverse with lower bloom abundance compared to summer or because sample sizes for queens at Colyer Lake *B. bimaculatus* queens (pooled pollen samples) were rather small (n=3). Future studies could examine more carefully whether queens may forage differently, such as in prioritizing pollen quantity over quality to quickly achieve an initial set of workers.

Plant visitation analysis

Pennsylvania workers heavily foraged on *Securigera varia* (crown vetch) and *Trifolium repens* (white clover), both members of the family Fabaceae which has relatively high protein content (Hanley et al., 2008). However, at the Colyer Lake site, *B vagans* mainly foraged on *Carduus acanthoides* (spiny plumeless thistle), though this plant made up no more than 3% of plants visited by the other 4 bee species at this same site. *C. acanthoides* is highly attractive to many bee species and is known to have relatively high pollen protein content, even higher than many legumes (Russo et al., 2019) and may help explain why *B. vagans* collected the highest protein when removing the Arboretum from the protein analysis. Interestingly, all three of these most-visited plants are not native to North America (Bethany Bradley et al., 2024) and two are considered invasive in Pennsylvania (*Invasive Plant Fact Sheets*, n.d.). The most visited native species in the data set was *Asclepias syriaca* (Apocynaceae, common milkweed) and was exclusively visited by *B. griseocollis,* supporting previous field observations that have confirmed specialization on this plant (Villalona et al., 2020). However, bumble bees are known to only collect nectar from common milkweed since this plant does not produce loose pollen, but rather pollinia (pollen masses contained in waxy sacs) (Heinrich, 1976a). In other instances, it could not be easily determined from visitation data whether bees were collecting pollen, nectar, or both. Regardless, bumble bees generally formed distinct host-plant interaction modules, suggesting these bees have different resource needs, although resource partitioning cannot be ruled out. In the future, I plan to conduct metabarcoding on pollen samples to determine species composition within each pollen sample. This will give insight into whether bumble bees collect pollen from additional plant species that could not be observed in my field studies, such as trees or tall shrubs.

In Oklahoma, bees seemed to prefer the native legume *Chamaecrista fasciculata* (partridge pea) as it made up 52% of the plant community at the Canadian River site but made up 100% of the bee visits. At Songbird Park, it made up 10% of the plant community but 67% of bee visits. This species belongs to Fabaceae (a botanical family favored by bumble bees) and, based

on my data, has high protein content relative to most plant species (Vaudo 2020, 2024). This plant species is native to large parts of the U.S., including Oklahoma. Further studies will need to determine if bumble bees prefer native Fabaceae over nonnative Fabaceae when both are blooming simultaneously in the landscape.

Value of landscape planning for bumble bee nutrition

Overall, my results indicate that all six of the studied bee species have similar nutritional needs, and thus can be supported by similar plant communities. When a higher diversity of plants (e.g., the Arboretum) or higher quantity of high-protein pollen producing plants (e.g., Canadian River) were available, bees collected pollen with higher protein concentrations. Interestingly, while bees did select for higher protein pollen when available, bee-collected pollen macronutrients exhibited high variance, and bees of the same species often foraged on a variety of plant species within the same survey. This behavior suggests that bumble bees mix pollen types with different macronutrient values to achieve a balanced diet, a strategy also supported by other studies (Vaudo et al., 2017, 2024). Thus, to support bumble bee foraging and habitat, plant communities should be designed to include both nutritionally diverse floral resources and plants with high protein content and high P:L ratios. Moreover, several of the bee species showed preferences for specific floral species, such as *B. vagans* visiting *C. acanthoides* and *B. griseocollis* visiting *A. syriaca*, and thus these plant species should be included to support the individual needs of these species.

Given the similar nutritional needs and preferences of the 6 bumble bee species included in my study, it remains to be determined if and how nutritional ecology underlies the variation in outcomes for the different species. For example, in Oklahoma, both *B. impatiens*, a highly abundant species with an expanding range, and *B. pensylvanicus*, a species with a dramatically

declining range, collected pollen from largely the same plant species and with the same nutritional content, achieving higher ratios than bees in Pennsylvania. However, it remains to be tested if certain bumble bee species avoid foraging from low-quality sites (e.g., very low overall available pollen protein concentrations, low plant diversity, etc.), thereby limiting where they can exist. Lab and field experiments that test pollen choice on bumble species other than *B. impatiens* (see Vaudo et al., 2016, 2018, 2020) and the effect of pollen quality on colony health outcomes will give insight into optimal macronutrient ratios for these bees and the plants that provide them.

Conclusion

This study is the first to examine pollen macronutrient preferences across multiple wild bumble bee species, including one declining species, compare queen versus worker foraging differences, and is the first to investigate nutritional differences in a single species inhabiting distinct and distant regions. This work has shown that species have similar nutritional preferences but that attained nutrients can shift across seasonal and regional plant communities likely depending on plant availability. There was some variation across species in terms of protein content, with the most common/expanding species (*B. impatiens*) collecting pollen with higher protein content at sites where the plant community was more diverse and/or had plants with high protein content pollen. The most preferred plants for all the studied species were in the Fabaceae family, a family with high pollen protein content. Adding more legumes into landscapes should therefore help support bumble bee communities. Interestingly, most of the preferred plants were non-natives, and further comparisons should be conducted between native and non-native plant species to determine how to best structure a plant community to support diverse bumble bee and other insect species. Given the modularity in the pollinator-plant interaction networks, and the

fact that specific bumble bee species showed preferences for certain plant species, it is important to cultivate diverse host-plant communities that meet the pollen nutritional needs of bees, allow bees to balance plant pollen toxicity, and provide adequate nectar resources. Finding springtime plants with high protein pollen might be especially helpful for queens who must provision the first 2-3 batches of workers by themselves. Moreover, ensuring that high protein plants are available late in the late season will benefit gynes preparing to diapause as high protein lipid ratio pollen diets have been shown increase survival in post-diapause queens (Treanore, et al., 2023), and these plants should also benefit bumble bees with longer colony cycles and, although the availability of high pollen protein later in the year needs further assessment across landscapes. This work provides baseline data on protein, lipid, and P:L ratio preferences in bumble bees and will benefit efforts to create and maintain pollinator-friendly plant communities centered around pollen nutrient availability.

Figures

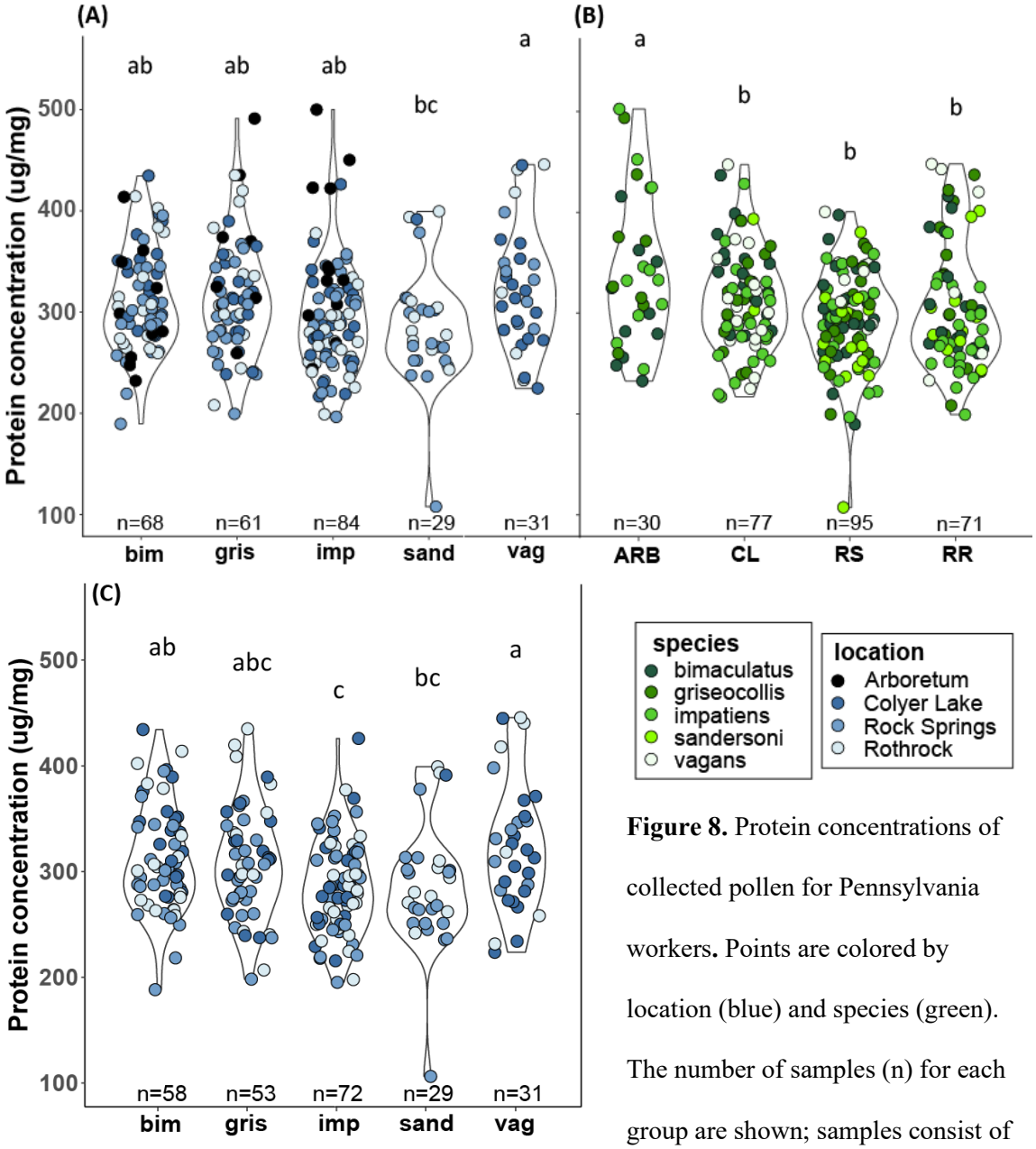

Figure 8. Protein concentrations of collected pollen for Pennsylvania workers**.** Points are colored by location (blue) and species (green). The number of samples (n) for each group are shown; samples consist of pollen balls from two individuals. Species differences-Arboretum included **(A)** site differences-Arboretum included **(B)**, and species differences-Arboretum excluded **(C)**. Significance letters

represent pairwise differences among species or sites (Tukey HSD). Two species, *B. vagans* and *B. sandersoni*, were not observed at the Arboretum. When the Arboretum was included, *B. vagans* and *B. sandersoni* were different and the Arboretum had higher protein than all other sites, which were not different from each other. When the Arboretum was excluded, there were no differences between sites, but there were differences between *B. vagans* and *B. sandersoni*, *B. vagans* and *B. impatiens*, and *B. bimaculatus* and *B. impatiens*, thus, there were more species differences when including sites in which all species were observed. See Results section for details about statistical analyses of site and species differences.

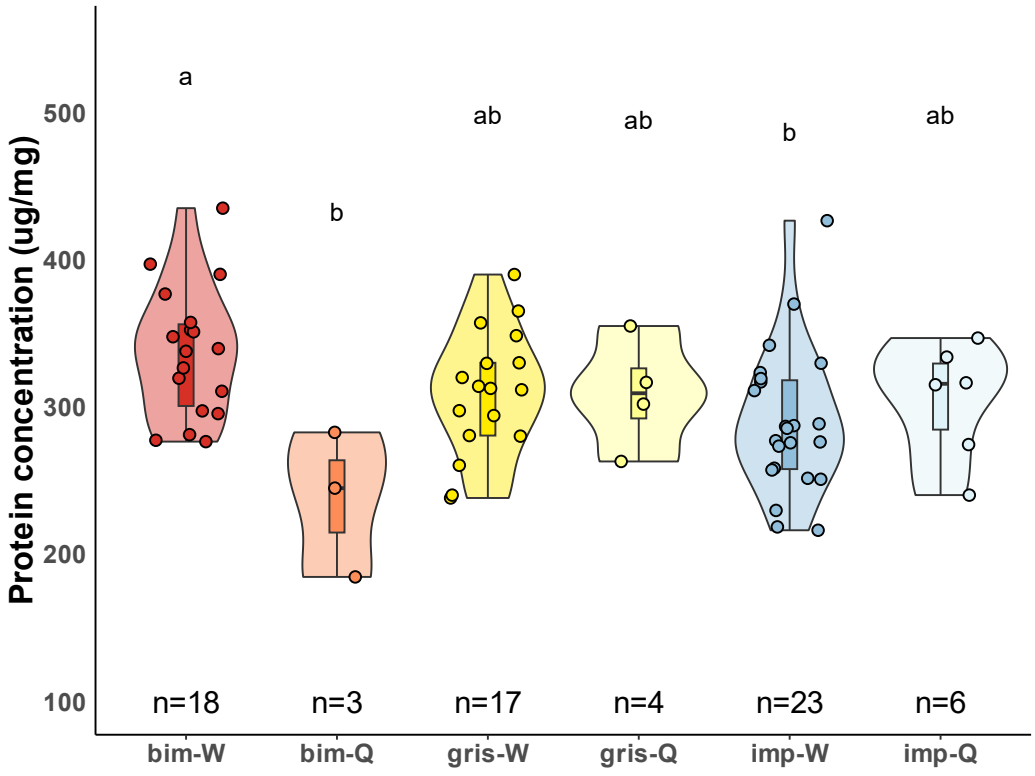

Figure 9. Protein concentrations of pollen collected by spring queens and summer workers. Queens were collected in spring (May 8 – May 11) and workers collected in the summer (June 11 – July 16). Bees were collected at a single site in Pennsylvania, Colyer Lake. Abbreviations are as follows: bim = *B. bimaculatus*, gris = *B. griseocollis*, imp = *B. impatiens*, W = worker, Q = queen. Significance letters represent pairwise differences among groups (Tukey HSD).

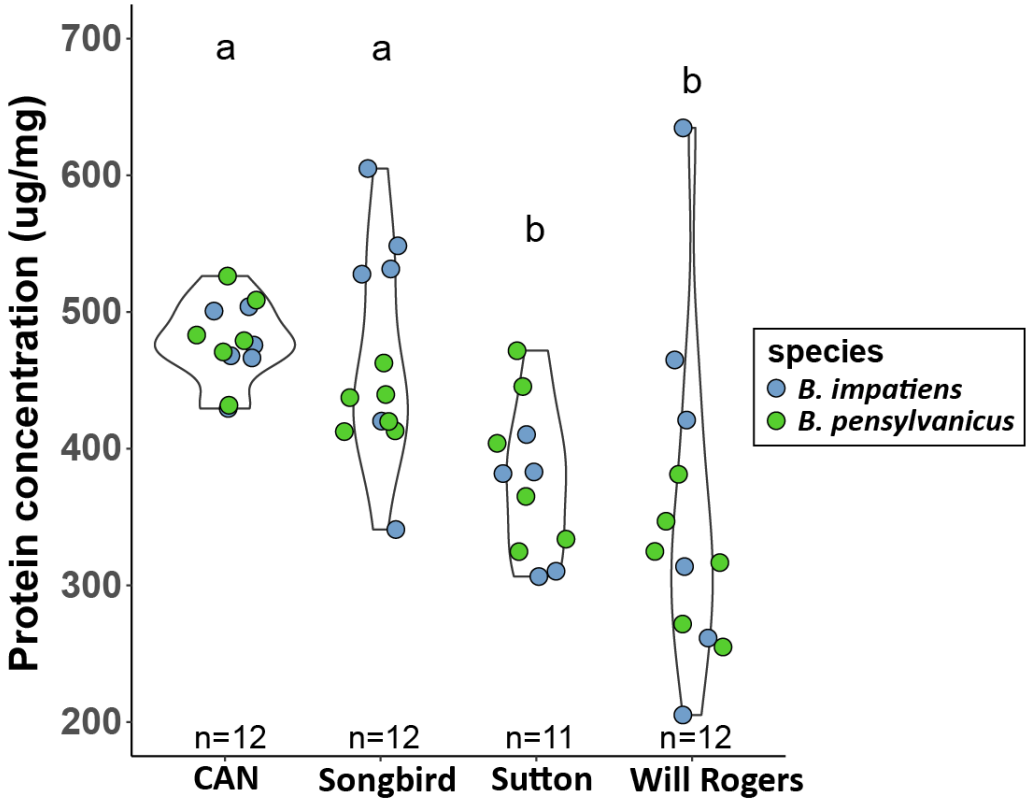

Figure 10. Protein concentrations of collected pollen for Oklahoma workers. Points are colored by species at the four sites. The number of samples (n) for each group are shown; samples consist of pollen balls from two individuals. Significance letters represent pairwise differences among groups (Tukey HSD). CAN = Canadian River, Songbird = Songbird Park, Sutton = Sutton Urban Wilderness, Will Rogers = Will Rogers Gardens.

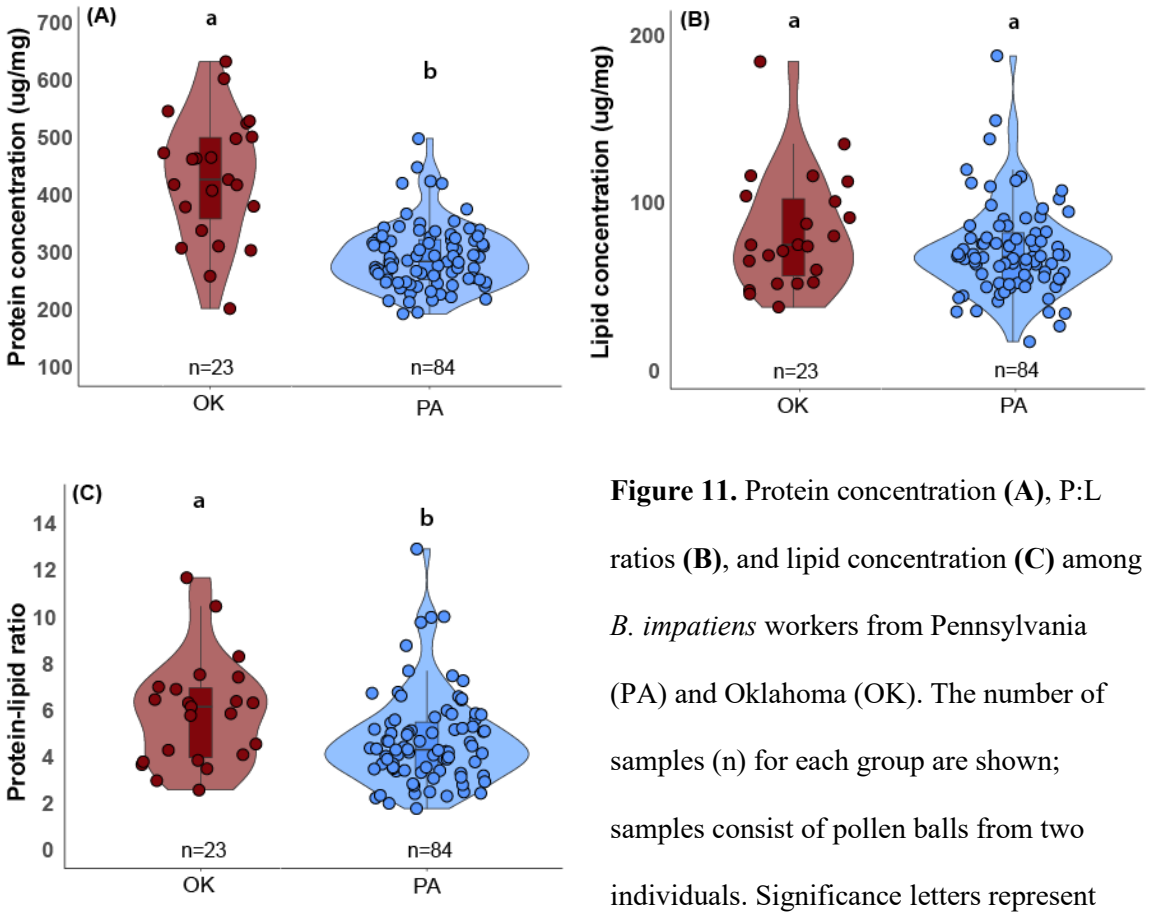

Figure 11. Protein concentration **(A)**, P:L ratios **(B)**, and lipid concentration **(C)** among *B. impatiens* workers from Pennsylvania (PA) and Oklahoma (OK). The number of samples (n) for each group are shown; samples consist of pollen balls from two individuals. Significance letters represent pairwise differences among groups (Tukey HSD).

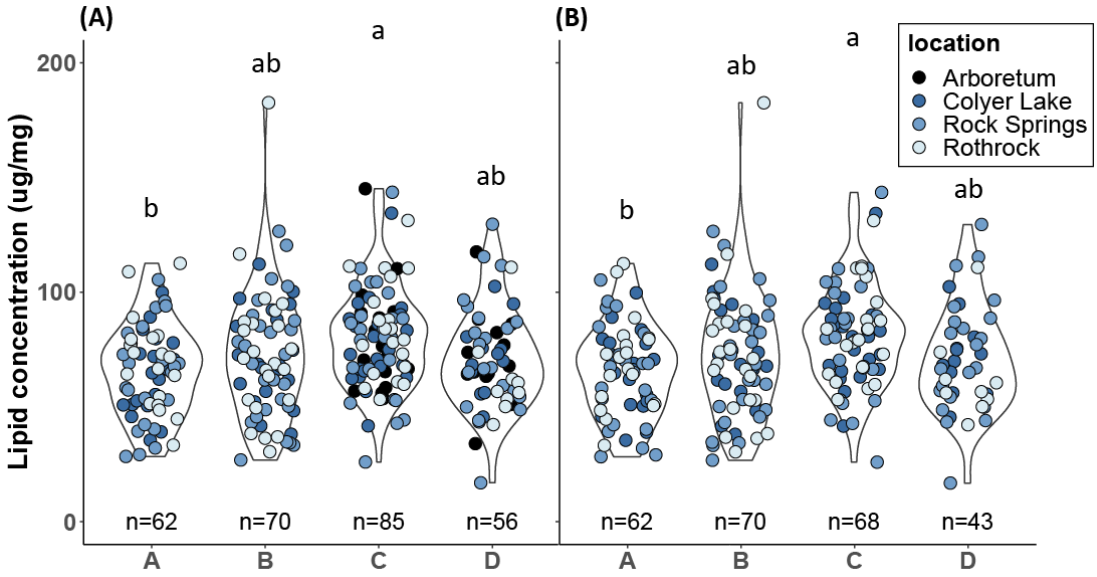

Figure 12. Lipid concentration in pollen collected by Pennsylvania workers. The data is provided for each of the four samples periods (A-D). In **Figure 12a**, all four sampling sites are included, while in **Figure 12b**, the Arboretum site is excluded, as two species, *B. vagans* and *B. sandersoni*, were not observed at the Arboretum. The number of samples (n) for each group are shown; samples consist of pollen balls from two individuals. Letters represent significant differences among sampling periods (Tukey HSD). There was no significant effect of site or species (data not shown). See Results for more details about the statistical analyses.

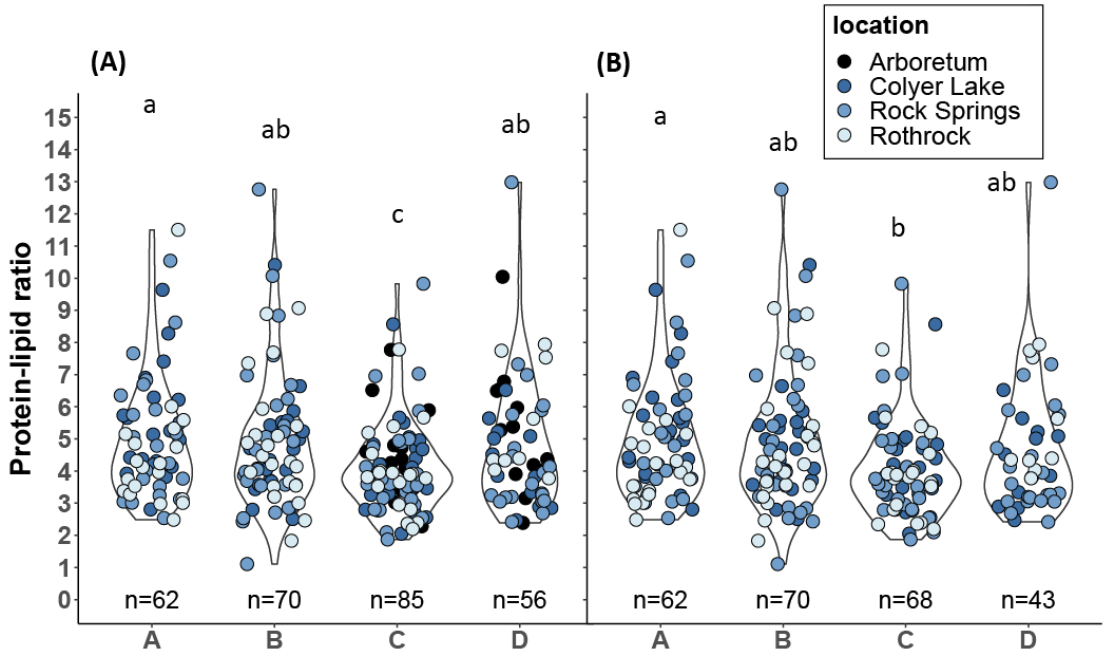

Figure 13. P:L ratios among Pennsylvania workers. The data is provided for each of the four samples periods (A-D). In **Figure 13a**, all four sampling sites are included, while in **Figure 13b**, the Arboretum site is excluded, as two species, *B. vagans* and *B. sandersoni*, were not observed at the Arboretum. The number of samples (n) for each group are shown; samples consist of pollen balls from two individuals. Letters represent significant differences among sampling periods (Tukey HSD). In both 13a and 13b, there was no significant effect of site or species alone (data not shown), but there was an interactive effect of site and species (data shown in Table 9a, 9b). See Results for more details about the statistical analyses.

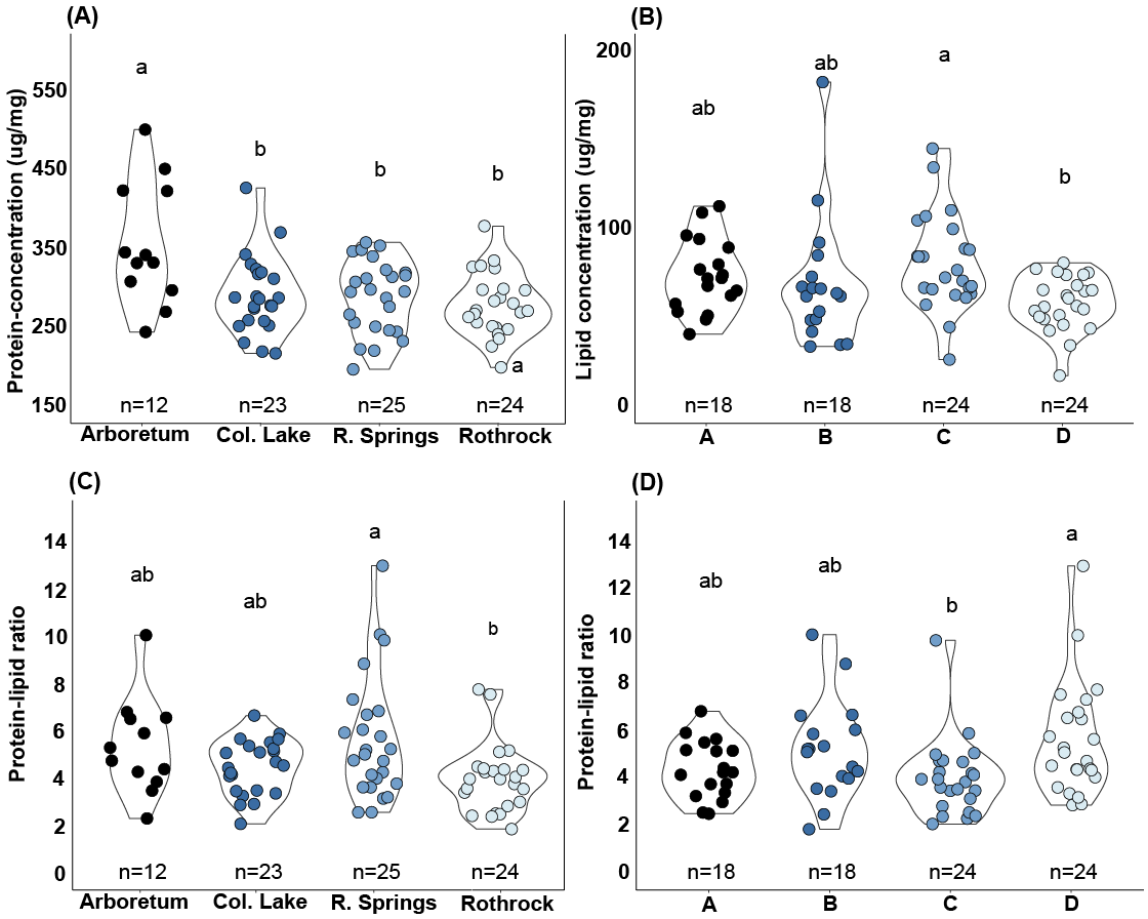

Figure 14. Evaluating nutritional flexibility in *B. impatiens*. The figure shows the results of *B. impatiens* collected pollen nutrition as protein concentration by site **(A)**, lipid concentration by sampling period **(B)**, P:L ratio by site **(C)**, and P:L ratio by sampling period **(D)**. The number of samples (n) for each group are shown; samples consist of pollen balls from two individuals. Significance letters represent pairwise differences among species (Tukey HSD).

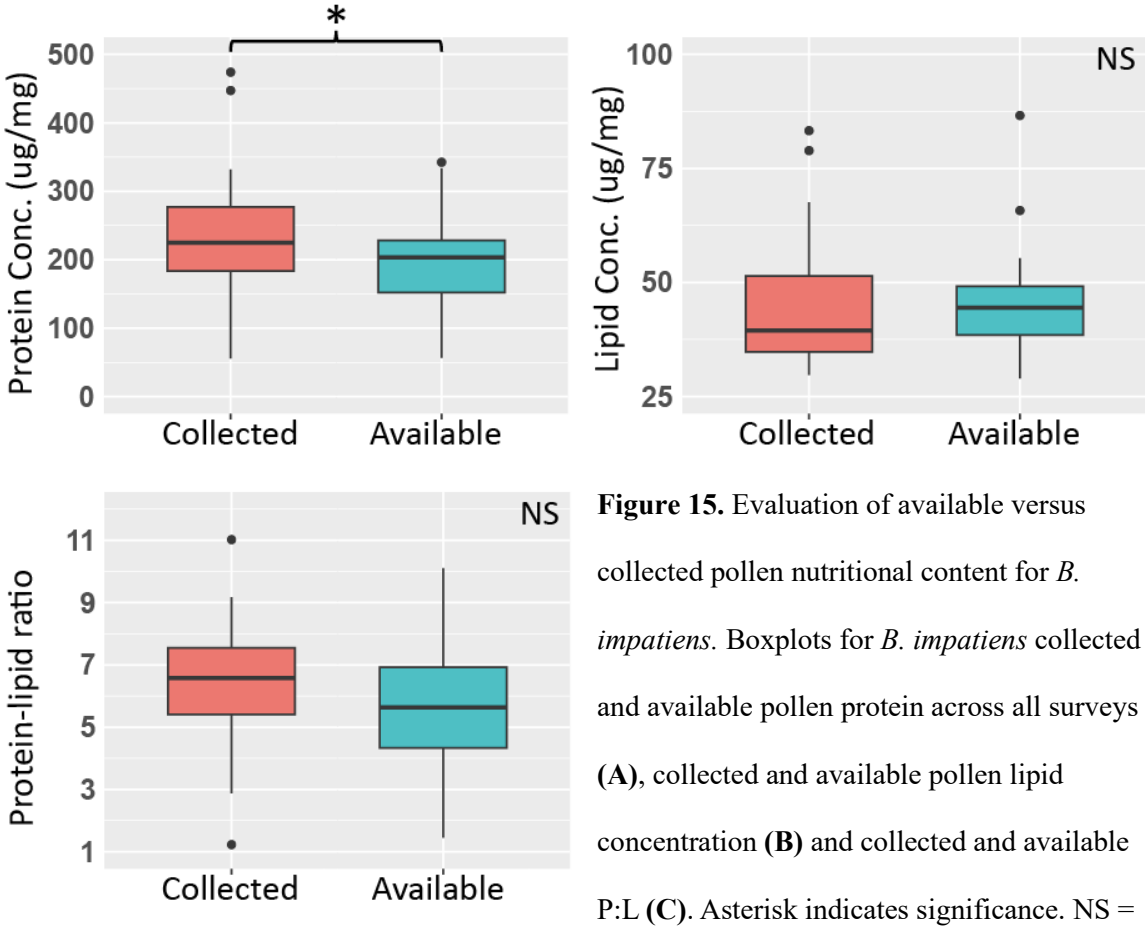

Figure 15. Evaluation of available versus collected pollen nutritional content for *B. impatiens.* Boxplots for *B. impatiens* collected and available pollen protein across all surveys **(A)**, collected and available pollen lipid concentration **(B)** and collected and available P:L **(C)**. Asterisk indicates significance. NS = not significant.

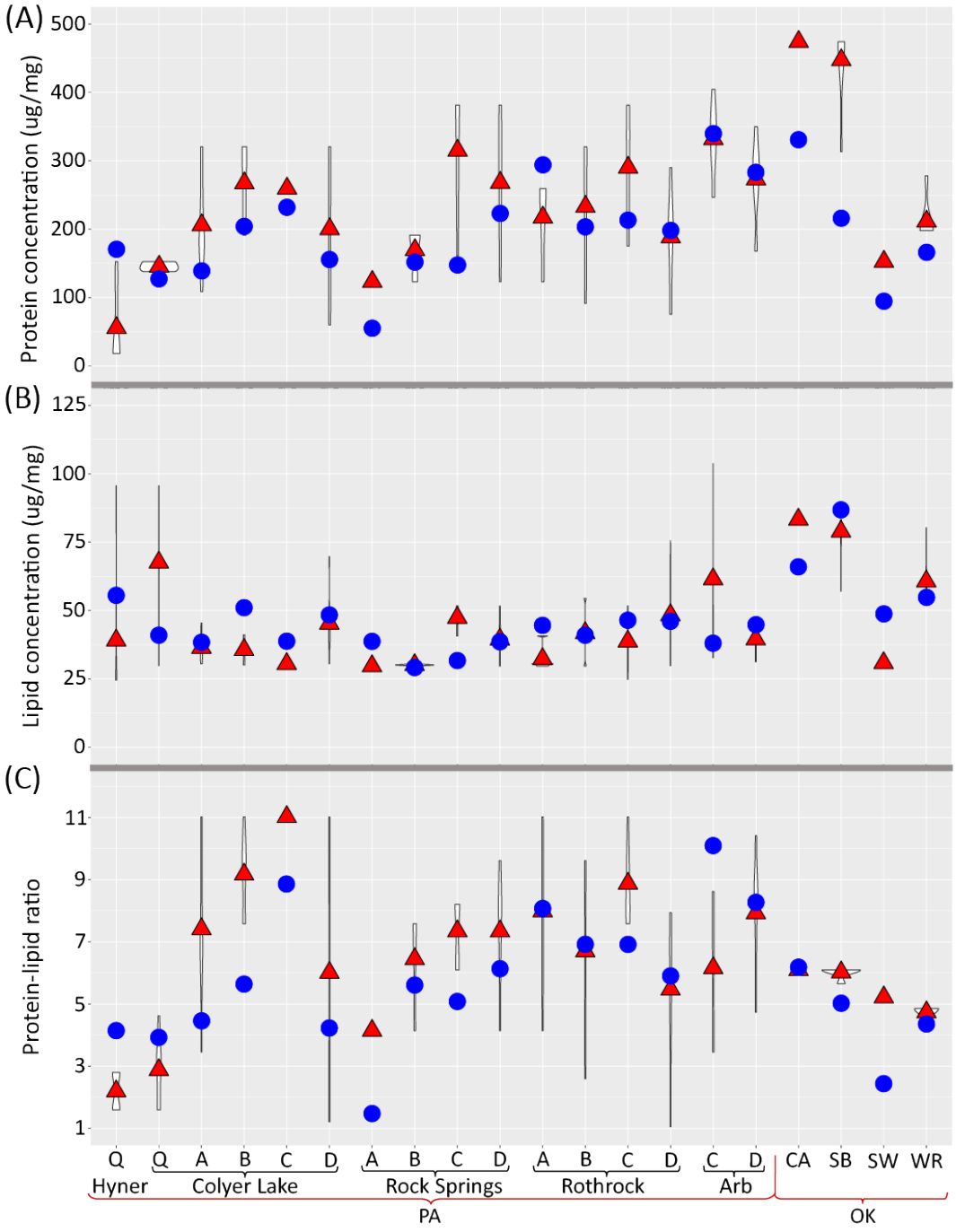

Figure 16. Available pollen macronutrients for each survey vs collected macronutrients by *B. impatiens* queens and workers. Protein concentration (Pc) **(A)**, lipid concentration (Lc) **(B)**, and P:L ratio **(C)**. The proportion of blooms for each plant species of interest relative to the total amount of blooms at each survey site-date was multiplied by its corresponding pollen Pc, Lc, and

P:L ratio (inferred from Vaudo eta al. (2020, 2024) and the products were summed to give the available macronutrients (blue dots). Violin plots show the distribution of collected Pc, Pc, and P:L ratio by B. impatiens at each site. Red triangles represent mean collected Pc, Lc, and P:L ratio. The sites are split by state, then by site, and finally by time period. Hyner = Hyner View State Park, Arb = the Arboretum at Penn State, Q = queens (spring), and A, B, C, and D refer to the summer sampling periods in Pennsylvania. For the Oklahoma sites, CA = Canadian River, SB = Songbird Park, SW = Sutton Urban Wilderness, and WR = Will Rogers Garden.

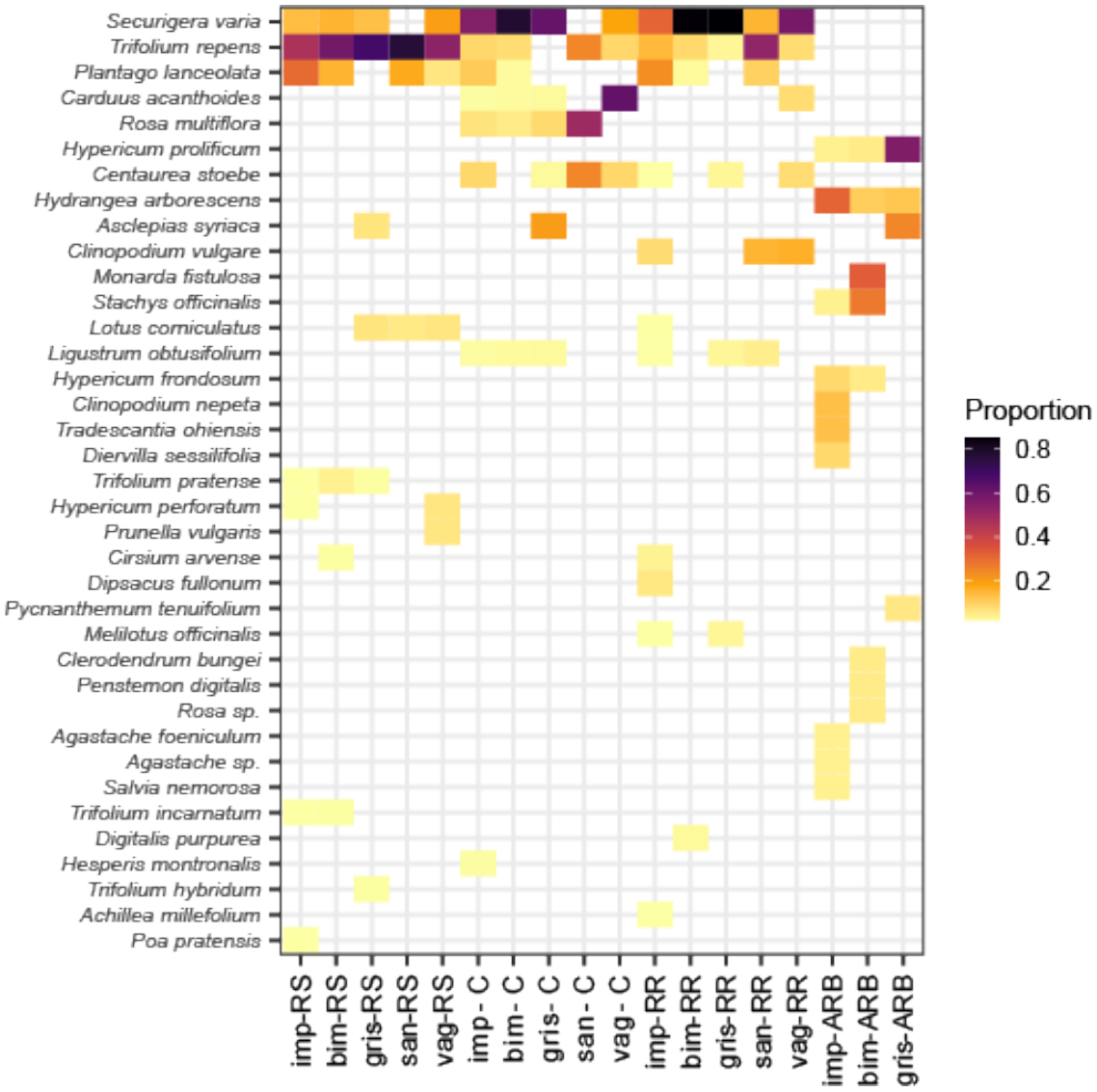

Figure 17. Heat map of plant-visitations for PA workers based on observed bee-plant interaction data. Bee-plant interaction data from all summer sites are shown. Pollen was collected from bees that were foraging. Bees were netted from host flowers and each host flower was identified in the field. Proportions are calculated for each combination of species and site. imp = *B. impatiens,* bim = *B. bimaculatus,* gris = *griseocollis,* sand = *B.* sandersoni, vag = *B.* vagans, RS = Rock Springs Research Farm, CL = Colyer Lake, RR = Rothrock State Forest, ARB = Arboretum.

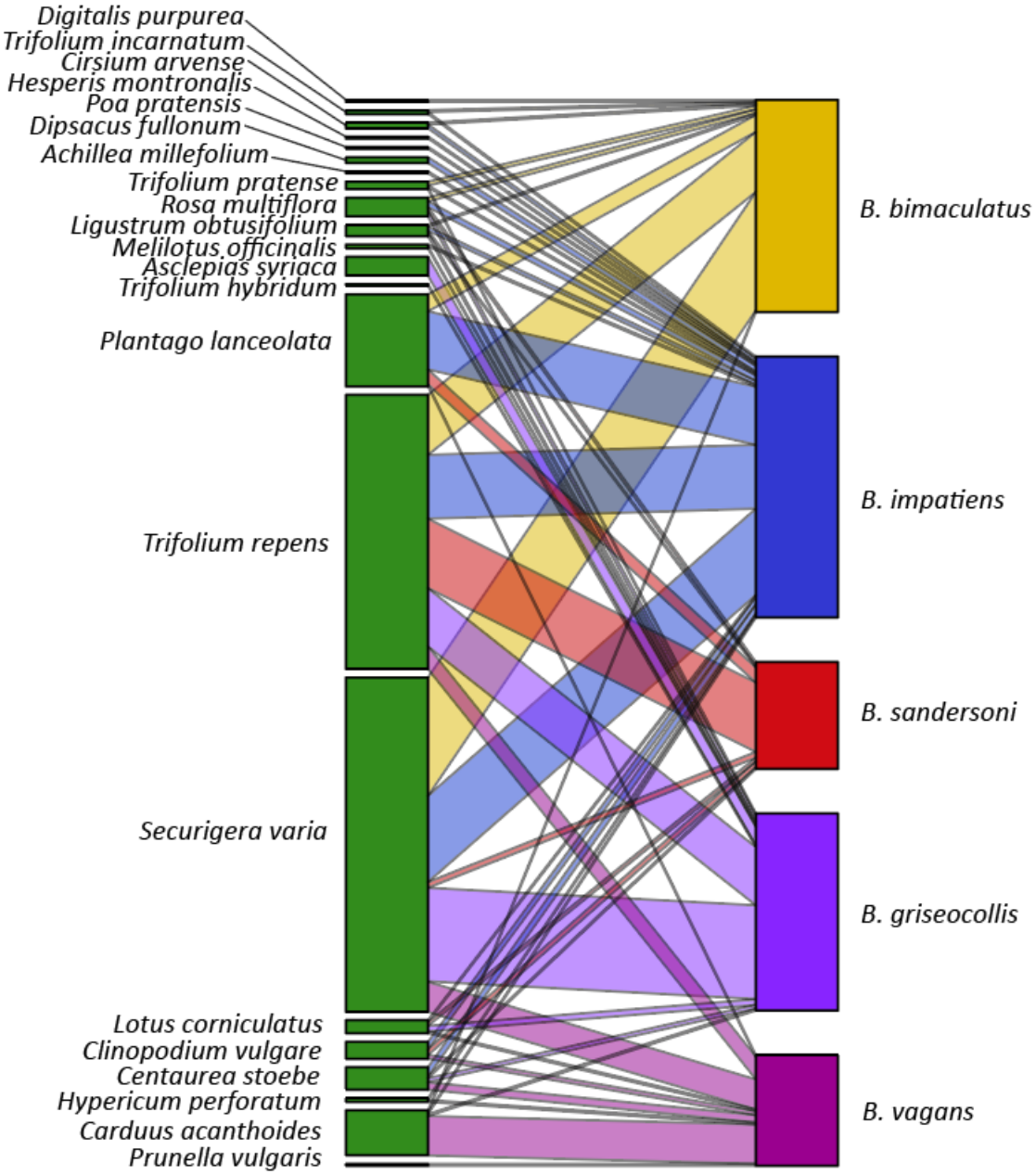

Figure 18. Plant-pollinator visitation network for Pennsylvania *Bombus* species based on observed bee-plant interaction data**.** Only workers were used in the analysis. The Arboretum site was excluded to reduce sampling bias as *B. vagans* and *B. sandersoni* were not present at this site. Including only sites where all species were present made it possible to compare visitation patterns for each bee species across sites.

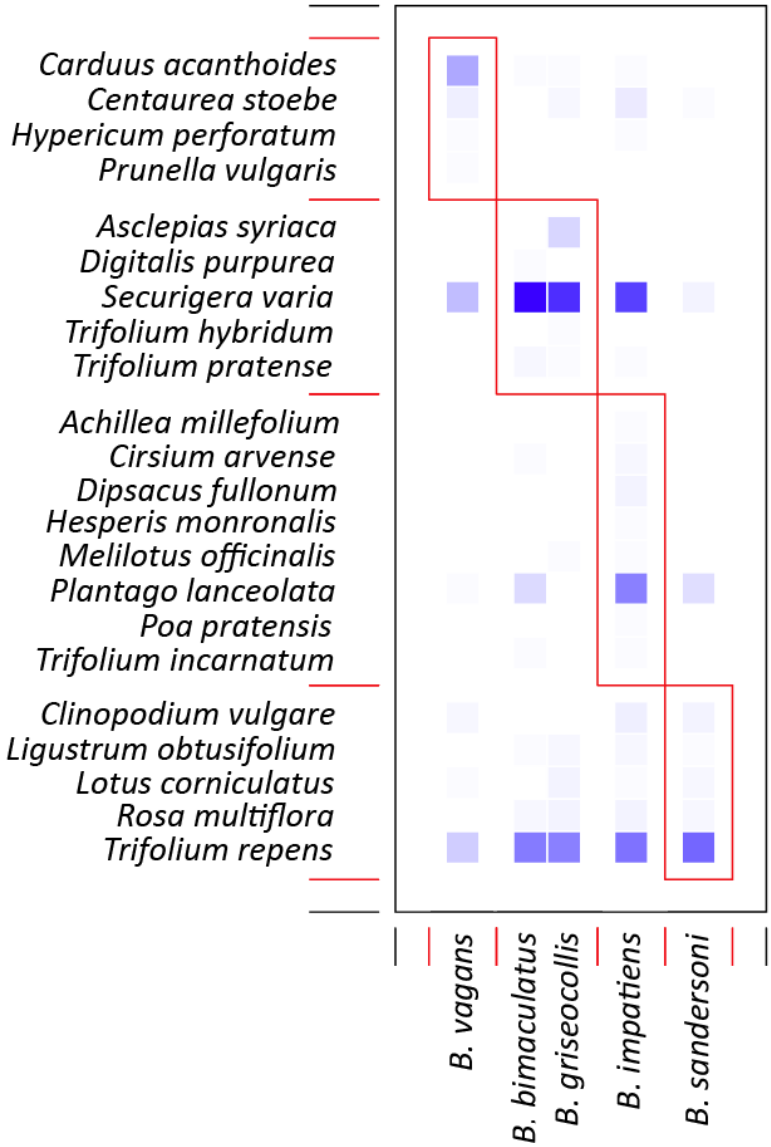

Figure 19. Visitation modules for bee-plant interactions based on the interaction network for Pennsylvania *Bombus* workers in Figure 19. Blue squares represent observed interactions between a bumble bee species and a plant species. The darkness of the square represents the frequency of visitation.

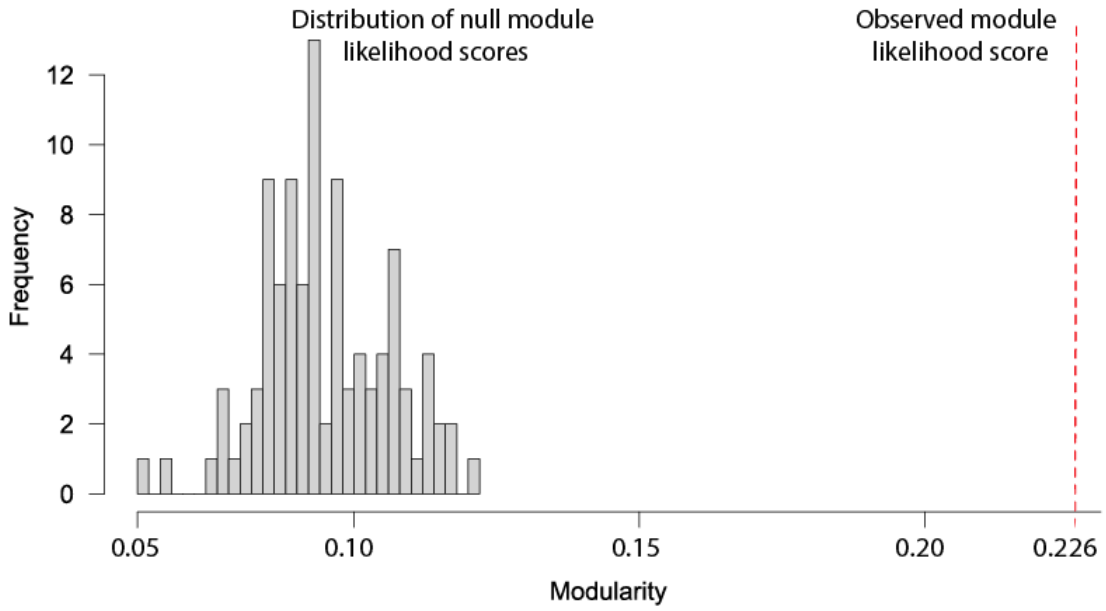

Figure 20. The likelihood of the observed module based on bee-plant interaction network for Pennsylvania workers vs the distribution of null model likelihood scores (n=500). The observed module interaction score was 0.226, and the median of the null model distribution was 0.093. The observed likelihood score did not overlap with the null model likelihood score distribution, indicating that there is more structure in the observed module than in the null model simulations.

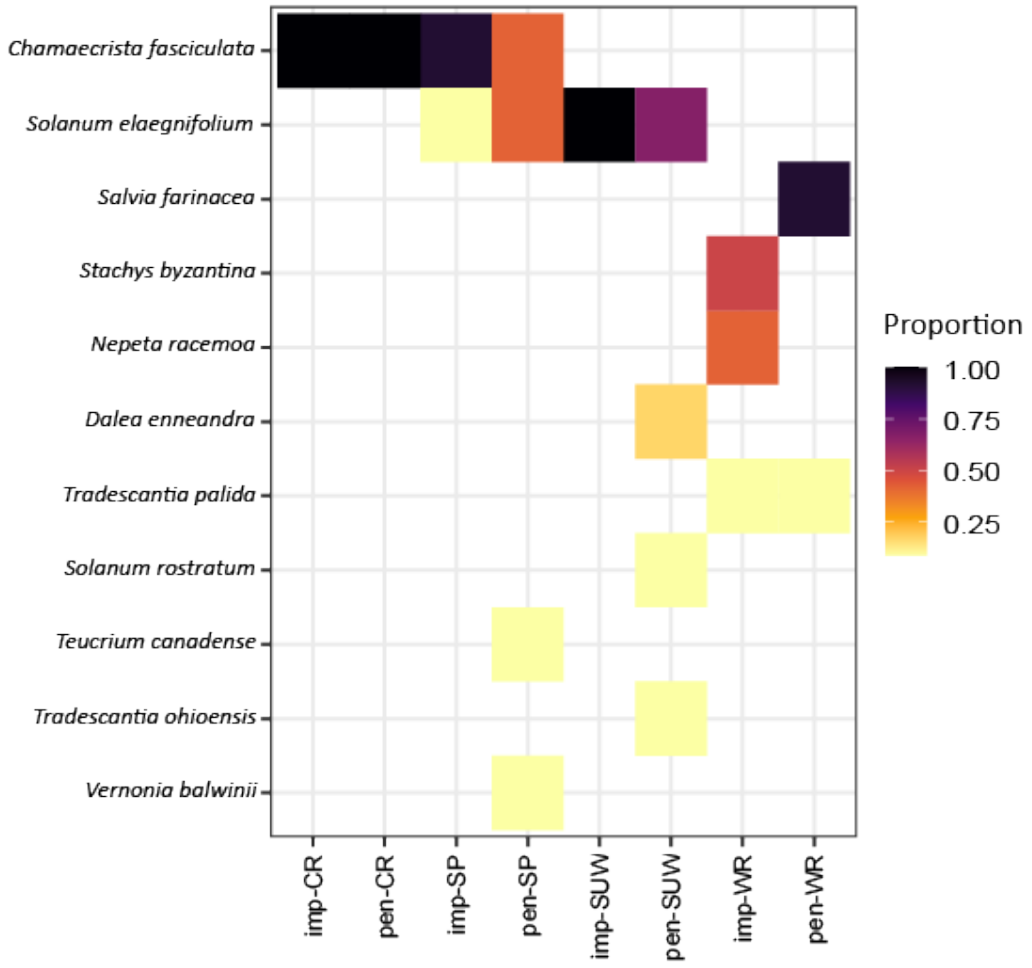

Figure 21. Heat map of plant-visitations for OK workers based on observed bee-plant interaction data from all 4 sites. Pollen was collected from bees that were foraging. Bees were netted from host flowers and each host flower was identified in the field. Proportions are calculated for each combination of species and site. imp = *B. impatiens,* pen = *B. pensylvanicus,* CR = Canadian River, SP = Songbird Park, SUW = Sutton Urban Wilderness, WR = Will Rogers Gardens.

Tables

Table 5. Summary table of Bombus species examined in the study: subgenera, tongue length, habitat, and conservation status.

species	subgenera	Tongue length	habitat	Conservation status
B. impatiens	*Pyrobombus*	med.	varied	Stable/increasing
B. bimaculatus	*Pyrobombus*	med.	edge/ecotonal	Stable/increasing
B. sandersoni	*Pyrobombus*	med.	forest	Stable/decreasing in parts of range
B. vagans	*Pyrobombus*	med-long	forest	Stable/decreasing in parts of range
B. griseocollis	*Cullumano-bombus*	short	valleys/plains/low lying areas in mountainous west	stable
B. pensylvanicus	*Thoracobombus*	long	plains	declining

Table 6. Sampling periods, dates, locations, numbers of bees that pollen was collected from per species/caste and number of pooled pollen samples. Pollen from two bees were pooled for each pollen sample so there are always half the number of pollen samples for the corresponding number of bees. PA = Pennsylvania, OK = Oklahoma.

Species	Caste	Date	State	Site	Sampling period	#Bees	#Pollen Samples
B. bimaculatus	queen	5/8-5/10/2023	PA	Colyer Lake	NA	6	3
B. bimaculatus	queen	5/6-5/16/2023	PA	Hyner View SP	NA	10	5
B. griseocollis	queen	5/8-5/10/2023	PA	Colyer Lake	NA	8	4
B. impatiens	queen	5/8-5/11/2023	PA	Colyer Lake	NA	12	6
B. impatiens	queen	5/6/2023	PA	Hyner View SP	NA	12	6
B. bimaculatus	worker	7/6/2023	PA	Arboretum	C	10	5
B. bimaculatus	worker	7/17/2023	PA	Arboretum	D	10	5
B. bimaculatus	worker	6/11/2023	PA	Colyer Lake	A	12	6
B. bimaculatus	worker	6/24/2023	PA	Colyer Lake	B	12	6
B. bimaculatus	worker	7/5/2023	PA	Colyer Lake	C	12	6
B. bimaculatus	worker	6/10/2023	PA	Rock Springs	A	12	6
B. bimaculatus	worker	6/21/2023	PA	Rock Springs	B	12	6
B. bimaculatus	worker	7/4/2023	PA	Rock Springs	C	12	6
B. bimaculatus	worker	7/15/2023	PA	Rock Springs	D	10	5
B. bimaculatus	worker	6/15/2023	PA	Rothrock	A	10	5
B. bimaculatus	worker	6/28-6/29/2023	PA	Rothrock	B	12	6
B. bimaculatus	worker	7/10/2023	PA	Rothrock	C	12	6
B. griseocollis	worker	7/6/2023	PA	Arboretum	C	12	6
B. griseocollis	worker	7/17-7/18/2023	PA	Arboretum	D	4	2
B. griseocollis	worker	6/11/2023	PA	Colyer Lake	A	12	6
B. griseocollis	worker	6/24-6/25/2023	PA	Colyer Lake	B	8	4
B. griseocollis	worker	7/5-7/6/2023	PA	Colyer Lake	C	12	6
B. griseocollis	worker	7/16/2023	PA	Colyer Lake	D	2	1
B. griseocollis	worker	6/10/2023	PA	Rock Springs	A	8	4
B. griseocollis	worker	6/21/2023	PA	Rock Springs	B	12	6
B. griseocollis	worker	7/4/2023	PA	Rock Springs	C	12	6

B. griseocollis	worker	7/15/2023	PA	Rock Springs	D	12	6
B. griseocollis	worker	6/15-6/17/2023	PA	Rothrock	A	10	5
B. griseocollis	worker	6/28-6/29/2023	PA	Rothrock	B	12	6
B. griseocollis	worker	7/10-7/11/2023	PA	Rothrock	C	6	3
B. impatiens	worker	7/6/2023	PA	Arboretum	C	12	6
B. impatiens	worker	7/17/2023	PA	Arboretum	D	12	6
B. impatiens	worker	6/11/2023	PA	Colyer Lake	A	12	6
B. impatiens	worker	6/24/2023	PA	Colyer Lake	B	12	6
B. impatiens	worker	7/5/2023	PA	Colyer Lake	C	10	5
B. impatiens	worker	7/16/2023	PA	Colyer Lake	D	12	6
B. impatiens	worker	6/10/2023	PA	Rock Springs	A	12	6
B. impatiens	worker	6/21/2023	PA	Rock Springs	B	12	6
B. impatiens	worker	7/4/2023	PA	Rock Springs	C	14	7
B. impatiens	worker	7/15/2023	PA	Rock Springs	D	12	6
B. impatiens	worker	6/15/2023	PA	Rothrock	A	10	5
B. impatiens	worker	6/28/2023	PA	Rothrock	B	12	6
B. impatiens	worker	7/10/2023	PA	Rothrock	C	14	7
B. impatiens	worker	7/22/2023	PA	Rothrock	D	12	6
B. sandersoni	worker	6/11/2023	PA	Colyer Lake	A	2	1
B. sandersoni	worker	7/16/2023	PA	Colyer Lake	D	2	1
B. sandersoni	worker	6/10/2023	PA	Rock Springs	A	10	5
B. sandersoni	worker	6/21/2023	PA	Rock Springs	B	10	5
B. sandersoni	worker	7/4/2023	PA	Rock Springs	C	10	5
B. sandersoni	worker	7/15/2023	PA	Rock Springs	D	4	2
B. sandersoni	worker	6/15-6/17/2023	PA	Rothrock	A	6	3
B. sandersoni	worker	6/28-6/29/2023	PA	Rothrock	B	8	4
B. sandersoni	worker	7/10-7/11/2023	PA	Rothrock	C	6	3
B. vagans	worker	6/11-6/13/2023	PA	Colyer Lake	A	4	2
B. vagans	worker	6/24-6/25/2023	PA	Colyer Lake	B	10	5
B. vagans	worker	7/5/2023	PA	Colyer Lake	C	12	6
B. vagans	worker	7/16-7/19/2023	PA	Colyer Lake	D	8	4

B. vagans	worker	6/10/2023	PA	Rock Springs	A	2	1
B. vagans	worker	6/21/2023	PA	Rock Springs	B	6	3
B. vagans	worker	7/4/2023	PA	Rock Springs	C	2	1
B. vagans	worker	7/15/2023	PA	Rock Springs	D	6	3
B. vagans	worker	6/28-6/29/2023	PA	Rothrock	B	2	1
B. vagans	worker	7/10-7/11/2023	PA	Rothrock	C	4	2
B. vagans	worker	7/22/2023	PA	Rothrock	D	6	3
B. impatiens	worker	7/24/2023	OK	Canadian River	NA	10	5
B. impatiens	worker	7/15/2023	OK	Songbird Park	NA	12	6
B. impatiens	worker	7/21-7/22/2023	OK	Sutton	NA	10	5
B. impatiens	worker	7/26/2023	OK	Will Rogers	NA	12	6
B. pensylvanicus	worker	7/24/2023	OK	Canadian River	NA	12	6
B. pensylvanicus	worker	7/15/2023	OK	Songbird Park	NA	12	6
B. pensylvanicus	worker	7/21-7/22/2023	OK	Sutton	NA	12	6
B. pensylvanicus	worker	7/26/2023	OK	Will Rogers	NA	12	6

Table 7. Plant species from floral surveys in Pennsylvania and Oklahoma and their corresponding countable floral units.

Pennsylvania	**Pennsylvania**	**Oklahoma**
Plant species	**Plant species**	**Plant species**
Floral unit = 1 capitulum	**Floral unit=1 flower**	**Floral unit = 1 capitulum**
Achillea millefolium	*Pastinaca sativa*	*Achillea millefolium*
Achillea sp.	*Penstemon digitalis*	*Achillea sp.*
Carduus acanthoides	*Potentilla recta*	*Aphanostephus sp.*
Centaurea stoebe	*Potentilla sp.*	*Asteraceae*
Chrysanthemum sp.	*Ranunculus sp.*	*Bradburia pilosa*
Cirsium arvense	*Rhododendron maximum*	*Coreopsis tinctoria*
Dahlia pinnata	*Rosa multiflora*	*Croptilon*
Echinacea purpurea	*Rosa sp.*	*Erigeron sp.*
Erigeron spp.	*Rubus idaeus*	*Erigeron strigosus*
Lapsana communis	*Rubus phoenicolasius*	*Gaillardia aestivalis*
Leucanthemum sp.	*Salvia gregii*	*Gaillardia pulchella*
Leucanthemum vulgare	*Salvia nemorosa*	*Helianthus annuus*
Rudbeckia fulgida	*Salvia yangi*	*Helianthus maximiliani*
Tagetes erecta	*Sambucus sp.*	*Heliopsis helianthoides*
Taraxacum officinale	*Saponaria officinalis*	*Lactuca serriola*
Taraxacum sp.	*Scutellaria incarnata*	*Liatris aspera*
Floral unit = 1 flower	*Solidago sp.*	*Ratibida columnifera*
Abelia x grandiflora	*Teucrium canadense*	*Ratibida pinnata*
Acer ginnala	*Tradescantia ohiensis*	*Rudbeckia hirta*
Agastache foeniculum	*Vaccinium angustifolium*	*Thelesperma filifolium*
Agastache sp.	*Verbascum thapsis*	*Verbesina encelioides*
Alliaria petiolata	*Verbena simplex*	*Vernonia baldwinii*
Anemonastrum canadense	*Verbena stricta*	**Floral unit = 1 cyanthium**
Barbarea sp.	*Veronica persica*	*Zinnia sp.*
Barbarea vulgaris	*Vicia sativa*	*Croton capitatus*
Berberis thunbergii	*Vicia villosa*	*Croton monanthogynus*
Betonica officinalis	**Floral unit = 1 head-like inflorescence**	**Floral unit = 1 flower**
Borago officinalis	*Dipsacus fullonum*	*Euphorbia marginata*
Chicorium intybus	*Lotus corniculatus*	*Argemone polyanthemos*
Cleome hassleriana	*Medicago lupulina*	*Buddleja sp.*
Clinopodium nepeta	*Plantago lanceolata*	*Callirhoe involucrata*
Clinopodium vulgare	*Plantago sp.*	*Castilleja indivisa*
Convolvulus arvensis	*Poa pratensis*	*Cestrum aurantiacum*
Coreopsis sp.	*Prunella vulgaris*	*Chamaecrista fasciculata*

Cornus sp.	*Pycnanthemum incanum*	*Coleus sp.*
Crateagus sp.	*Pycnanthemum tenuifolium*	*Commelina communis*
Daucus carota	*Securigera varia*	*Commelina erecta*
Dianthus armeria	*Sedum sarmentosum*	*Cornus sp.*
Diervilla sessilifolia	*Silene latifolia*	*Dalea enneandra*
Digitalis purpurea	*Trifolium aureum*	*Dalea lanata*
Elaeagnus umbellata	*Trifolium campestre*	*Dalea multiflora*
Fragaria sp.	*Trifolium dubium*	*Desmanthus illinoensis*
Gaillardia sp.	*Trifolium hybridum*	*Desmodium sessilifolium*
Galium sp.	*Trifolium incarnatum*	*Foeniculum vulgare*
Helanthius sp.	*Trifolium pratense*	*Hibiscus sp.*
Hesperis montronalis	*Trifolium repens*	*Indigofera miniata*
Hydrangea arborescens		*Lagerstroemia indica*
Hydrangea quercifolia		*Lantana sp.*
Hypericum androsaemum		*Linum sulcatum*
Hypericum perforatum		*Lythrum alatum*
Hypericum prolificum		*Melilotus albus*
Lamium purpereum		*Nepeta racemosa*
Lantana camara		*Oenothera rhombipetala*
Lathyrus sylvestris		*Pedioemlum tenuiflorum*
Ligustrum obtusifolium		*Physalis angulata*
Linaria vulgaris		*Salvia farinacea*
Lonicera morrowii		*Salvia greggii*
Malus sp.		*Salvia guaranitica*
Melilotus albus		*Salvia sp.*
Melilotus officinalis		*Solanum dimidiatum*
Melissa officinalis		*Solanum elaeagnifolium*
Monarda fistulosa		*Solanum rostratum*
Nepeta cataria		*Stachys bizantina*
Nepeta racemosa		*Teucrium canadense*
Nepeta sp.		*Tradenscantia pallida*
Oenothera lindheimeri		*Tradescantia ohiensis*
Oenothera sp.		*Verbena stricta*

Table 8. AICc table for linear models for each for pollen macronutrient comparison category: 1) species, site, and sampling period in PA workers, 2) species and site for PA queens (protein, lipid, and P:L), species and caste for PA workers and queens, 3) species and location for OK workers. For each category, the model with the lowest Delta AICc score was retained for analysis. +ARB: the analysis was conducted with pollen samples from the Arboretum at Penn State. -ARB: the analysis was conducted without pollen samples from the Arboretum at Penn State.

Model	K	AICc	Delta AICc	ModelLik	AICcWt	LL	Cum. Wt
PA worker protein + ARB							
species * location + samp. period	22	2969.31	0.00	1.00	0.81	-1460.63	0.81
species + location + samp. period	12	2972.18	2.86	0.24	0.19	-1473.49	1.00
species * samp. period + location	24	2985.43	16.11	0.00	0.00	-1466.29	1.00
species * location * samp. period	60	3022.97	53.66	2.23E-12	1.80E-12	-1434.22	1.00
PA worker protein - ARB							
species + location + samp. period	11	2592.91	0.00	1.00	0.78	-1284.88	0.78
species * location + samp. period	19	2596.05	3.14	0.21	0.16	-1277.31	0.95
species * samp. period + location	23	2598.33	5.42	0.07	0.05	-1273.63	1.00
species * location * samp. period	54	2632.83	39.92	2.14E-09	1.68E-09	-1246.53	1.00
PA worker lipid + ARB							
species + location + samp. period	12	2506.42	0.00	1.00	0.94	-1240.61	0.94
species * samp. period + location	24	2512.29	5.88	0.05	0.05	-1229.73	0.99
species * location + samp. period	22	2516.19	9.77	0.01	0.01	-1234.07	1.00
species * location * samp. period	60	2565.48	59.06	1.49E-13	1.41E-13	-1205.48	1.00
PA worker lipid - ARB							
species + location + samp. period	11	2235.27	0.00	1.00	0.94	-1106.06	0.94
species * location + samp. period	19	2241.07	5.81	0.05	0.05	-1099.83	0.99

species * samp. period + location	23	2245.13	9.86	0.01	0.01	-1097.04	1.00
species * location * samp. period	54	2288.57	53.31	2.66E-12	2.51E-12	-1074.49	1.00
PA worker P:L + ARB							
species * location + samp. period	22	1130.82	0.00	1.00	0.60	-541.39	0.60
species + location + samp. period	12	1131.67	0.85	0.66	0.39	-553.23	0.99
species * samp. period + location	24	1139.97	9.15	0.01	0.01	-543.57	1.00
species * location * samp. period	60	1190.92	60.10	8.90E-14	5.35E-14	-518.20	1.00
PA worker P:L - ARB							
species * location + samp. period	19	1013.93	0.00	1.00	0.61	-486.26	0.61
species + location + samp. period	11	1014.86	0.93	0.63	0.39	-495.86	1.00
species * samp. period + location	23	1030.04	16.11	0.00	0.00	-489.50	1.00
species * location * samp. period	54	1072.04	58.11	2.41E-13	1.48E-13	-466.22	1.00
PA queen protein							
species * location	6	249.89	0.00	1.00	0.94	-116.47	0.94
species + location	5	255.52	5.63	0.06	0.06	-121.09	1.00
PA queen lipid							
species + location	5	222.36	0.00	1.00	0.79	-104.51	0.79
species * location	6	225.03	2.67	0.26	0.21	-104.04	1.00
PA queen P:L							
species + location	5	100.18	0.00	1.00	0.85	-43.43	0.85
species * location	6	103.59	3.41	0.18	0.15	-43.33	1.00
PA worker vs queen protein							
species * cate	7.00	751.62	0.00	1.00	0.96	-367.92	0.96
species + caste	5.00	758.25	6.63	0.04	0.04	-373.66	1.00
PA worker vs queen lipid							
species + caste	5.00	635.14	0.00	1.00	0.88	-312.11	0.88
species * cate	7.00	639.19	4.05	0.13	0.12	-311.71	1.00
PA worker vs queen P:L ratio							
species + caste	5.00	279.25	0.00	1.00	0.77	-134.17	0.77
species * cate	7.00	281.65	2.39	0.30	0.23	-132.94	1.00
OK worker protein							
species + location	6.00	548.93	0.00	1.00	0.89	-267.41	0.89
species * location	9.00	553.05	4.12	0.13	0.11	-265.09	1.00
OK worker lipid							
species + location	6.00	472.93	0.00	1.00	0.92	-229.41	0.92
species * location	9.00	477.74	4.81	0.09	0.08	-227.44	1.00
OK worker P:L ratio							
species + location	6.00	225.34	0.00	1.00	0.77	-105.62	0.77
species * location	9.00	227.78	2.44	0.29	0.23	-102.46	1.00

Table 9. ANOVA summary table for pollen macronutrient comparisons. Pennsylvania workers with Arboretum included (A), PA workers with Arboretum excluded (B), PA queens (C), PA workers vs queens (D), and Oklahoma workers (E). Boldfaced p-values indicate statistical significance ($\alpha = 0.05$).

Variable	Factor	DF	F_value	Pr
(A) PA workers + Arboretum				
Protein	Species	4	3.39	**0.010**
	Site	3	6.08	**<0.001**
	Sampling period	3	0.42	0.740
	Species*site	10	2.49	**0.007**
	Residuals	252	NA	NA
Lipid	Species	4	0.71	0.587
	Site	3	0.50	0.682
	Sampling period	3	4.42	**0.005**
	Residuals	262	NA	NA
Protein-lipid ratio	Species	4	0.26	0.905
	Site	3	0.14	0.934
	Sampling period	3	3.59	**0.014**
	Residuals	262	NA	NA
(B) PA workers - Arboretum				
Protein	Species	4	4.91	**0.001**
	Site	3	0.41	0.749
	Sampling period	2	2.44	0.090
	Residuals	233	NA	NA
Lipid	Species	4		0.582
	Site	2		0.701
	Sampling period	3		**0.007**
	Residuals	233	NA	NA
Protein-lipid ratio	Species	4	0.37	0.831
	Site	2	0.16	0.853
	Sampling period	3	3.22	**0.024**
	Species*site	8	2.31	**0.021**
	Residuals	225	NA	NA
(C) PA queens				

Protein	Species	2	1.17	0.331
	Site	1	0.03	0.856
	Species*site	1	8.92	**0.008**
	Residuals	19	NA	NA
Lipid	Species	2	0.53	0.599
	Site	1	3.78	0.066
	Residuals	20	NA	NA
Protein-lipid ratio	Species	2	0.20	0.820
	Site	1	2.35	0.141
	Residuals	20	NA	NA
(D) PA queens vs workers				
Protein	Species	2	2.74	0.072
	Caste	1	1.70	0.197
	Species*Caste	2	5.71	**0.005**
	Residuals	65	NA	NA
Lipid	Species	2	0.31	0.735
	Caste	1	2.31	0.134
	Residuals	67	NA	NA
Protein-lipid ratio	Species	2	1.30	0.281
	Caste	1	2.37	0.129
	Residuals	67	NA	NA
(E) OK workers				
Protein	Species	1	1.35	0.252
	Site	3	8.31	**<0.001**
	Residuals	42	NA	NA
Lipid	Species	1	0.44	0.512
	Site	3	1.93	0.140
	Residuals	42	NA	NA
Protein-lipid ratio	Species	1	0.30	0.590
	Site	3	2.26	0.096
	Residuals	42	NA	NA

Table 10. Tukey HSD summary table for pollen macronutrient comparisons. PA workers, protein, Arboretum pollen samples included **(A)**, PA workers, protein, Arboretum pollen samples excluded **(B)**, PA workers vs queens, protein **(C)**, OK workers, protein **(D)**, PA workers, lipid, Arboretum included **(E)**, PA workers, lipid, Arboretum excluded **(F)**, PA workers, P:L, Arboretum included **(G)**, PA workers, P:L, Arboretum excluded **(H)**. Only Tukey tables based on ANOVA significance and at least one significant pairwise difference are shown. Boldfaced p-values indicate statistical significance ($\alpha = 0.05$). imp = B. impatiens, bim = B. bimaculatus, gris = griseocollis, sand = B. sandersoni, vag = B. vagans, pen = B. pensylvanicus, RS = Rock Springs Research Farm, CL = Colyer Lake, RR = Rothrock State Forest, ARB = The Arboretum at Penn State, CR = Canadian River, SP = Songbird Park, SUW = Sutton Urban Wilderness, WR = Will Rogers Garden

(A) Pa workers, Protein (Arboretum included)					
Comparison	**Protein diff. [95% CI]**	**p adj**	**Comparison**	**Protein diff. [95% CI]**	**p adj**
PA workers			**Species*location**		
vag-sand	38.36 [0.69, 76.02]	**0.044**	imp:RR-bim:RS	-18.12 [-73.58, 37.34]	1
vag-imp	28.69 [-1.94, 59.33]	0.078	vag:RS-gris:ARB	-29.75 [-124.78, 65.28]	1
sand-gris	-28.66 [-61.54, 4.22]	0.12	vag:RR-gris:RR	28.51 [-64.23, 121.25]	1
sand-bim	-26.23 [-58.56, 6.10]	0.172	sand:RR-sand:CL	-44.05 [-191.27, 103.17]	1
imp-gris	-18.99 [-43.52, 5.53]	0.211			
imp-bim	-16.56 [-40.35, 7.22]	0.313	sand:CL-bim:ARB	43.95 [-103.27, 191.17]	1
vag-bim	12.13 [-19.46, 43.72]	0.829	sand:RR-vag:RS	-26.86 [-117.01, 63.30]	1
sand-imp	-9.66 [-41.06, 21.73]	0.916	vag:RS-bim:ARB	26.76 [-63.40, 116.91]	1
vag-gris	9.70 [-22.46, 41.85]	0.922	imp:RR-gris:RS	-16.20 [-72.30, 39.90]	1
gris-bim	2.43 [-23.28, 28.14]	0.999	bim:CL-gris:ARB	-23.07 [-103.83, 57.69]	1
Location			vag:RS-imp:ARB	-24.59 [-111.34, 62.16]	1
RS-ARB	-46.12 [-74.86, -17.38]	**< 0.001**	bim:RR-gris:RS	17.25 [-44.12, 78.63]	1
RR-ARB	-32.21 [-62.09, -2.33]	**0.029**	gris:RS-vag:CL	-16.57 [-77.94, 44.81]	1
CL-ARB	-31.28 [-60.82, -1.75]	**0.033**	sand:RR-gris:RR	-21.09 [-99.79, 57.60]	1

RS-CL	-14.83 [-35.88, 6.21]	0.265	gris:RR-bim:ARB	20.99 [-57.70, 99.69]	1
RR-RS	13.91 [-7.62, 35.43]	0.341	gris:RS-gris:CL	-16.11 [-77.49, 45.26]	1
RR-CL	-0.93 [-23.51, 21.65]	1	sand:CL-gris:CL	37.15 [-104.94, 179.23]	1
Sampling period			vag:CL-sand:CL	-36.69 [-178.77, 105.39]	1
B-A	9.04 [-14.89, 32.97]	0.763	bim:RR-sand:CL	-36.01 [-178.09, 106.08]	1
C-B	-7.89 [-30.04, 14.26]	0.793	bim:CL-imp:ARB	-17.91 [-88.74, 52.92]	1
D-A	4.86 [-20.44, 30.15]	0.96	bim:RR-bim:RS	15.34 [-45.45, 76.13]	1
D-B	-4.19 [-28.79, 20.42]	0.971	vag:RS-gris:CL	19.95 [-61.53, 101.44]	1
D-C	3.71 [-19.91, 27.32]	0.977	bim:RS-vag:CL	-14.65 [-75.44, 46.14]	1
C-A	1.15 [-21.77, 24.07]	0.999	vag:RS-vag:CL	19.50 [-61.99, 100.99]	1
Species*location			bim:RS-gris:CL	-14.20 [-74.99, 46.59]	1
sand:RS-imp:ARB	-86.98 [-158.64, -15.32]	**0.003**	bim:RR-vag:RS	-18.81 [-100.30, 62.67]	1
imp:RR-imp:ARB	-76.87 [-144.06, -9.67]	**0.008**	vag:RR-vag:RS	22.75 [-79.90, 125.39]	1
sand:RS-gris:ARB	-92.14 [-173.63, -10.65]	**0.01**	imp:RS-bim:ARB	-14.94 [-86.05, 56.18]	1
sand:RS-bim:CL	-69.07 [-133.35, -4.79]	**0.021**	sand:RR-imp:RS	14.84 [-56.28, 85.95]	1
imp:RR-gris:ARB	-82.02 [-159.62, -4.43]	**0.026**	imp:RR-imp:CL	-11.54 [-67.00, 43.92]	1
imp:RR-bim:CL	-58.96 [-118.22, 0.31]	0.053 •	gris:RR-gris:CL	14.19 [-54.41, 82.78]	1
imp:RS-imp:ARB	-66.29 [-133.04, 0.46]	0.054 •	gris:RR-vag:CL	13.74 [-54.86, 82.33]	1
imp:CL-imp:ARB	-65.33 [-133.01, 2.36]	0.073 •	imp:RR-imp:RS	-10.58 [-64.89, 43.74]	1
vag:RR-sand:RS	85.13 [-5.12, 175.39]	0.092	imp:CL-bim:ARB	-13.98 [-85.97, 58.02]	1
imp:RS-gris:ARB	-71.45 [-148.65, 5.76]	0.11	sand:RR-imp:CL	13.88 [-58.12, 85.87]	1
imp:CL-gris:ARB	-70.49 [-148.50, 7.53]	0.135	gris:RR-bim:RR	13.05 [-55.55, 81.64]	1
gris:RS-imp:ARB	-60.66 [-128.87, 7.55]	0.154	gris:RR-bim:CL	-12.45 [-80.18, 55.28]	1
bim:RS-imp:ARB	-58.75 [-126.43, 8.94]	0.186	vag:RR-bim:CL	16.06 [-73.53, 105.66]	1
vag:RR-imp:RR	75.02 [-11.73, 161.77]	0.192	vag:RR-vag:CL	42.25 [-48.00, 132.50]	0.981
gris:RS-gris:ARB	-65.82 [-144.29, 12.65]	0.238	gris:RR-gris:RS	30.30 [-34.68, 95.28]	0.982
gris:RR-sand:RS	56.62 [-11.97, 125.22]	0.264	sand:RS-bim:RS	-28.24 [-89.03, 32.55]	0.982
imp:RS-bim:CL	-48.38 [-107.13, 10.37]	0.268	vag:RR-bim:RR	41.56 [-48.69, 131.81]	0.984

bim:RS-gris:ARB	-63.90 [-141.92, 14.11]	0.277	vag:RS-gris:RS	36.07 [-42.40, 114.54]	0.984
imp:CL-bim:CL	-47.42 [-107.23, 12.39]	0.336	sand:RR-bim:CL	-33.54 [-108.50, 41.42]	0.988
vag:RS-sand:RS	62.39 [-19.10, 143.88]	0.402	bim:CL-bim:ARB	33.44 [-41.52, 108.41]	0.989
vag:RR-imp:RS	64.44 [-21.96, 150.84]	0.454	gris:RR-bim:RS	28.39 [-36.04, 92.82]	0.99
vag:RR-imp:CL	63.48 [-23.65, 150.61]	0.501	vag:RS-bim:RS	34.15 [-43.86, 112.17]	0.991
imp:RR-gris:RR	-46.51 [-110.43, 17.41]	0.503	sand:RS-gris:RS	-26.32 [-87.70, 35.06]	0.993
gris:RS-bim:CL	-42.75 [-103.16, 17.65]	0.558	gris:RR-gris:ARB	-35.52 [-119.75, 48.72]	0.994
bim:RS-bim:CL	-40.84 [-100.65, 18.98]	0.627	imp:RS-sand:CL	-58.89 [-198.55, 80.78]	0.994
imp:RR-vag:RS	-52.27 [-129.87, 25.32]	0.651	gris:CL-bim:CL	-26.64 [-90.92, 37.64]	0.995
vag:RR-gris:RS	58.81 [-28.72, 146.35]	0.656	sand:CL-imp:CL	57.93 [-82.19, 198.04]	0.995
bim:RR-sand:RS	43.57 [-21.62, 108.77]	0.665	vag:CL-bim:CL	-26.19 [-90.47, 38.09]	0.996
sand:RS-vag:CL	-42.89 [-108.08, 22.31]	0.693	gris:RR-imp:ARB	-30.36 [-105.13, 44.41]	0.996
vag:RR-bim:RS	56.90 [-30.23, 144.03]	0.705	bim:RR-bim:CL	-25.50 [-89.78, 38.78]	0.997
sand:RS-gris:CL	-42.43 [-107.63, 22.76]	0.711	bim:RR-imp:RS	22.88 [-36.87, 82.63]	0.998
sand:RR-imp:ARB	-51.45 [-132.83, 29.93]	0.757	gris:RS-sand:CL	-53.26 [-193.63, 87.11]	0.998
imp:ARB-bim:ARB	51.35 [-30.03, 132.73]	0.759	imp:RS-vag:CL	-22.19 [-81.94, 37.56]	0.999
sand:RR-gris:ARB	-56.61 [-146.77, 33.54]	0.767	bim:RS-sand:CL	-51.34 [-191.46, 88.77]	0.999
gris:ARB-bim:ARB	56.51 [-33.64, 146.67]	0.769	imp:RR-sand:RS	10.12 [-50.14, 70.37]	1
gris:CL-imp:ARB	-44.55 [-116.21, 27.11]	0.781	gris:RR-sand:CL	-22.96 [-166.63, 120.72]	1
vag:CL-imp:ARB	-44.10 [-115.76, 27.57]	0.795	imp:RS-bim:RS	-7.54 [-62.46, 47.37]	1
gris:CL-gris:ARB	-49.71 [-131.19, 31.78]	0.807	gris:RS-bim:ARB	-9.31 [-81.80, 63.18]	1
bim:RR-imp:ARB	-43.41 [-115.07, 28.25]	0.816	sand:RR-gris:RS	9.21 [-63.28, 81.70]	1
vag:CL-gris:ARB	-49.25 [-130.74, 32.24]	0.818	bim:RS-imp:CL	6.58 [-49.46, 62.63]	1
bim:RR-gris:ARB	-48.57 [-130.05, 32.92]	0.835	vag:RS-sand:CL	-17.19 [-167.45, 133.07]	1
gris:RR-imp:RS	35.93 [-27.52, 99.37]	0.888	sand:RR-bim:RR	-8.04 [-83.79, 67.70]	1

sand:RS-sand:CL	-79.58 [-221.66, 62.50]	0.897	bim:RR-bim:ARB	7.95 [-67.80, 83.69]	1
imp:RR-bim:RR	-33.46 [-93.71, 26.79]	0.904	gris:CL-bim:ARB	6.81 [-68.94, 82.55]	1
imp:RR-vag:CL	-32.77 [-93.02, 27.48]	0.919	vag:CL-bim:ARB	7.26 [-68.49, 83.00]	1
gris:RR-imp:CL	34.97 [-29.46, 99.40]	0.921	bim:RS-bim:ARB	-7.39 [-79.39, 64.60]	1
vag:RS-imp:RS	41.69 [-35.51, 118.90]	0.924	sand:RR-bim:ARB	-0.10 [-85.10, 84.90]	1
imp:RR-gris:CL	-32.32 [-92.57, 27.93]	0.928	imp:ARB-gris:ARB	-5.16 [-91.91, 81.59]	1
vag:RS-imp:CL	40.74 [-37.28, 118.75]	0.944	sand:CL-gris:ARB	-12.56 [-162.82, 137.70]	1
vag:RR-sand:RR	49.60 [-48.54, 147.75]	0.958	vag:RR-gris:ARB	-7.01 [-109.65, 95.64]	1
vag:RR-bim:ARB	49.51 [-48.64, 147.65]	0.959	sand:CL-imp:ARB	-7.40 [-152.57, 137.76]	1
imp:RR-sand:CL	-69.46 [-209.35, 70.42]	0.965	vag:RR-imp:ARB	-1.85 [-96.88, 93.18]	1
vag:RR-gris:CL	42.70 [-47.55, 132.95]	0.979	sand:CL-bim:CL	10.51 [-131.16, 152.17]	1
sand:RS-bim:ARB	-35.63 [-111.37, 40.12]	0.98	vag:RS-bim:CL	-6.68 [-87.45, 74.08]	1
sand:RR-sand:RS	35.53 [-40.22, 111.28]	0.98	vag:CL-gris:CL	0.45 [-64.74, 65.64]	1
vag:RR-vag:CL	42.25 [-48.00, 132.50]	0.981	bim:RR-gris:CL	1.14 [-64.05, 66.33]	1
gris:RR-gris:RS	30.30 [-34.68, 95.28]	0.982	sand:RR-gris:CL	-6.90 [-82.65, 68.84]	1
sand:RS-bim:RS	-28.24 [-89.03, 32.55]	0.982	gris:RS-imp:CL	4.67 [-52.01, 61.35]	1
vag:RR-bim:RR	41.56 [-48.69, 131.81]	0.984	imp:RS-imp:CL	-0.96 [-55.87, 53.95]	1
imp:RS-gris:CL	-21.74 [-81.49, 38.01]	0.999	vag:RR-sand:CL	5.55 [-149.63, 160.74]	1
bim:RR-imp:CL	21.92 [-38.87, 82.71]	0.999	bim:RR-vag:CL	0.69 [-64.50, 65.88]	1
imp:RR-bim:ARB	-25.51 [-97.05, 46.02]	0.999	sand:RR-vag:CL	-7.36 [-83.10, 68.39]	1
sand:RS-imp:CL	-21.65 [-82.44, 39.14]	0.999	gris:RS-bim:RS	-1.92 [-58.60, 54.76]	1
sand:RR-imp:RR	25.41 [-46.12, 96.95]	0.999	sand:RR-bim:RS	7.29 [-64.70, 79.29]	1
vag:CL-imp:CL	21.23 [-39.56, 82.02]	0.999	imp:RS-gris:RS	-5.63 [-61.19, 49.93]	1
sand:RS-imp:RS	-20.69 [-80.44, 39.05]	1	gris:RR-vag:RS	-5.77 [-90.00, 78.47]	1
imp:CL-gris:CL	-20.78 [-81.57, 40.01]	1			

(B) PA workers, Protein (no Arb.)			(C) Workers vs Queens, Protein		
Comparison	**diff. [95% CI]**	**p adj**	**Comparison**	**diff. [95% CI]**	**p adj**
Species			**Species**		
vag-imp	38.65 [8.36, 68.93]	**0.005**	imp-bim	-29.87 [-60.81, 1.08]	0.0615
imp-bim	-27.90 [-52.78, -3.03]	**0.019**	imp-gris	-16.76 [-47.70, 14.18]	0.401
vag-sand	38.36 [1.93, 74.78]	**0.033**	gris-bim	-13.11 [-46.44, 20.22]	0.615
imp-gris	-22.14 [-47.65, 3.38]	0.123	**Caste**		
sand-bim	-27.61 [-59.68, 4.45]	0.128	W-Q	17.97 [-9.62, 45.56]	0.198
sand-gris	-21.85 [-54.41, 10.72]	0.351	**Species*caste**		
vag-gris	16.51 [-15.37, 48.39]	0.613	bim:W-bim:Q	99.78 [17.34, 182.23]	**0.009**
vag-bim	10.74 [-20.62, 42.11]	0.88	imp:W-bim:W	-47.10 [-88.70, -5.49]	0.018
gris-bim	-5.77 [-32.56, 21.03]	0.976	gris:W-bim:Q	72.58 [-10.21, 155.37]	0.119
sand-imp	0.29 [-30.72, 31.30]	1	imp:Q-bim:Q	67.06 [-26.42, 160.55]	0.297
Location			gris:Q-bim:Q	71.75 [-29.23, 172.72]	0.307
RS-CL	-14.94 [-33.49, 3.61]	0.141	imp:W-bim:Q	52.69 [-28.47, 133.84]	0.408
RR-RS	13.84 [-5.14, 32.81]	0.2	gris:W-bim:W	-27.20 [-71.92, 17.51]	0.481
RR-CL	-1.10 [-21.00, 18.80]	0.991	bim:W-imp:Q	32.72 [-29.60, 95.05]	0.639
Sampling period			bim:W-gris:Q	28.03 [-45.05, 101.12]	0.869
B-A	8.48 [-14.66, 31.63]	0.779	imp:W-gris:Q	-19.06 [-90.69, 52.56]	0.97
D-B	-8.93 [-34.64, 16.78]	0.805	imp:W-imp:Q	-14.38 [-74.98, 46.23]	0.982
C-B	-5.88 [-28.48, 16.71]	0.907	imp:Q-gris:Q	-4.69 [-90.03, 80.65]	1
D-C	-3.05 [-28.90, 22.81]	0.99	gris:W-gris:Q	0.83 [-72.64, 74.30]	1
C-A	2.60 [-20.70, 25.90]	0.992	gris:W-imp:Q	5.52 [-57.26, 68.30]	1
D-A	-0.45 [-26.78, 25.89]	1			

(D) OK workers, Protein					
Comparison	**diff. [95% CI]**	**p adj**	**Comparison**	**diff. [95% CI]**	**p adj**
Species			**Location**		
pen-imp	-25.64 [-70.23, 18.94]	0.252	SUW-CAN	-101.41 [-185.95, -16.87]	0.013
Location			SUW-SBP	-85.96 [-170.50, -1.42]	0.045
WR-CAN	-128.88 [-211.57, -46.20]	**0.001**	WR-SUW	-27.48 [-112.02, 57.07]	0.821
WR-SBP	-113.43 [-196.12, -30.75]	**0.004**	SBP-CAN	-15.45 [-98.13, 67.23]	0.959

(E) PA workers, Lipid (Arboretum included)					
Comparison	**diff. [95% CI]**	**p adj**	**Comparison**	**diff. [95% CI]**	**p adj**
Species			**Location**		
imp-gris	-5.40 [-16.14, 5.34]	0.64	CL-ARB	-5.03 [-17.96, 7.91]	0.747
vag-imp	5.96 [-7.46, 19.38]	0.739	RS-ARB	-4.32 [-16.91, 8.27]	0.811
imp-bim	-4.52 [-14.93, 5.90]	0.756	RR-CL	3.09 [-6.80, 12.98]	0.85
vag-sand	3.60 [-12.89, 20.09]	0.975	RR-RS	2.39 [-7.04, 11.81]	0.914
sand-gris	-3.04 [-17.44, 11.36]	0.978	RR-ARB	-1.93 [-15.02, 11.15]	0.981
sand-imp	2.36 [-11.39, 16.11]	0.99	RS-CL	0.71 [-8.51, 9.92]	0.997
sand-bim	-2.16 [-16.32, 12.00]	0.994	**Sampling period**		
vag-bim	1.44 [-12.39, 15.28]	0.999	C-A	13.29 [3.25, 23.32]	**0.004**

gris-bim	0.88 [-10.38, 12.14]	1	D-C	-8.76 [-19.10, 1.58]	0.129
vag-gris	0.56 [-13.52, 14.64]	1	C-B	7.50 [-2.20, 17.20]	0.191
			B-A	5.79 [-4.69, 16.27]	0.483
			D-A	4.52 [-6.55, 15.60]	0.717
(F) PA workers, Lipid (Arboretum excluded)					
Comparison	**Lipid diff. [95% CI]**	**p adj**	**Comparison**	**Lipid diff. [95% CI]**	**p adj**
PA workers			**Location**		
imp-gris	-6.02 [-17.67, 5.64]	0.616	RR-CL	3.04 [-6.05, 12.13]	0.71
vag-imp	6.64 [-7.19, 20.48]	0.679	RR-RS	2.40 [-6.27, 11.07]	0.791
imp-bim	-4.34 [-15.70, 7.02]	0.832	RS-CL	0.64 [-7.83, 9.11]	0.983
vag-sand	3.60 [-13.04, 20.24]	0.976	Samp. period		
sand-imp	3.04 [-11.12, 17.21]	0.976	C-A	14.24 [3.60, 24.88]	**0.004**
sand-gris	-2.98 [-17.85, 11.90]	0.982	C-B	8.33 [-1.99, 18.65]	0.16
vag-bim	2.31 [-12.02, 16.63]	0.992	D-C	-9.23 [-21.04, 2.58]	0.183
gris-bim	1.68 [-10.56, 13.92]	0.996	B-A	5.91 [-4.66, 16.48]	0.471
sand-bim	-1.30 [-15.94, 13.35]	0.999	D-A	5.01 [-7.02, 17.04]	0.704
vag-gris	0.63 [-13.94, 15.19]	1	D-B	-0.90 [-12.65, 10.84]	0.997
(G) PA workers, P:L (Arboretum included)					
Comparison	**P:L diff. [95% CI]**	**p adj**	**Comparison**	**P:L diff. [95% CI]**	**p adj**
PA workers			gris:RR-imp:ARB	-0.62 [-3.20, 1.96]	1
imp-gris	0.31 [-0.54, 1.15]	0.854	imp:RR-imp:CL	-0.46 [-2.37, 1.45]	1
gris-bim	-0.24 [-1.13, 0.64]	0.944	imp:RS-gris:ARB	0.64 [-2.02, 3.30]	1
vag-gris	0.16 [-0.95, 1.27]	0.995	vag:CL-gris:ARB	-0.67 [-3.48, 2.14]	1
sand-gris	0.16 [-0.97, 1.30]	0.995	sand:RS-bim:CL	-0.53 [-2.74, 1.69]	1
vag-imp	-0.15 [-1.20, 0.91]	0.995	vag:RS-imp:ARB	-0.71 [-3.70, 2.28]	1
sand-imp	-0.15 [-1.23, 0.94]	0.996	vag:RR-sand:RS	0.73 [-2.38, 3.84]	1
imp-bim	0.07 [-0.75, 0.89]	0.999	imp:RR-vag:RS	-0.61 [-3.29, 2.06]	1
vag-bim	-0.08 [-1.17, 1.01]	1	gris:RR-bim:RS	0.47 [-1.75, 2.69]	1
sand-bim	-0.08 [-1.19, 1.04]	1	sand:RS-imp:CL	0.44 [-1.65, 2.54]	1
vag-sand	0.00 [-1.30, 1.30]	1	gris:CL-bim:ARB	0.54 [-2.07, 3.15]	1
Location			imp:RS-sand:CL	0.99 [-3.82, 5.81]	1
RR-RS	-0.17 [-0.91, 0.57]	0.936	imp:CL-gris:ARB	-0.54 [-3.23, 2.15]	1
RR-ARB	-0.21 [-1.24, 0.82]	0.95	gris:RS-gris:CL	-0.41 [-2.53, 1.70]	1
RR-CL	-0.11 [-0.89, 0.67]	0.982	vag:RS-bim:ARB	0.61 [-2.50, 3.71]	1
CL-ARB	-0.10 [-1.12, 0.92]	0.994	vag:RR-sand:CL	0.99 [-4.37, 6.34]	1
RS-CL	0.05 [-0.67, 0.78]	0.997	imp:CL-bim:ARB	0.45 [-2.03, 2.93]	1
RS-ARB	-0.05 [-1.04, 0.95]	0.999	bim:RR-vag:RS	0.51 [-2.30, 3.32]	1
Sampling period			gris:RR-bim:RR	-0.43 [-2.79, 1.94]	1
C-A	-0.87 [-1.66, -0.08]	**0.024**	vag:RR-gris:ARB	0.63 [-2.91, 4.17]	1
C-B	-0.76 [-1.53, 0.00]	**0.05**	vag:RS-gris:RS	0.48 [-2.23, 3.18]	1
D-C	0.61 [-0.20, 1.43]	0.214	sand:RS-imp:ARB	-0.42 [-2.89, 2.05]	1
D-A	-0.26 [-1.13, 0.61]	0.869	sand:RR-gris:RR	-0.45 [-3.17, 2.26]	1
D-B	-0.15 [-1.00, 0.70]	0.966	gris:RS-imp:CL	-0.32 [-2.28, 1.63]	1
B-A	-0.11 [-0.93, 0.72]	0.987	vag:RR-bim:RR	0.51 [-2.60, 3.62]	1

species*location			gris:CL-gris:ARB	-0.45 [-3.26, 2.36]	1
imp:RR-imp:RS	-1.64 [-3.51, 0.23]	0.174	sand:CL-bim:CL	-0.78 [-5.67, 4.11]	1
imp:RS-gris:RS	1.50 [-0.41, 3.42]	0.354	imp:RR-vag:CL	-0.33 [-2.41, 1.75]	1
imp:RS-bim:RS	1.41 [-0.48, 3.31]	0.451	gris:RR-vag:CL	0.37 [-2.00, 2.74]	1
imp:RR-bim:CL	-1.43 [-3.47, 0.62]	0.583	sand:RS-gris:CL	0.35 [-1.90, 2.60]	1
imp:RS-bim:ARB	1.63 [-0.82, 4.08]	0.673	bim:RS-gris:CL	-0.33 [-2.42, 1.77]	1
imp:RS-vag:CL	1.31 [-0.75, 3.37]	0.746	bim:CL-gris:ARB	0.43 [-2.36, 3.21]	1
imp:RS-imp:CL	1.18 [-0.71, 3.07]	0.777	vag:RS-bim:RS	0.39 [-2.30, 3.08]	1
gris:RS-bim:CL	-1.29 [-3.37, 0.79]	0.785	imp:RS-imp:ARB	0.32 [-1.98, 2.62]	1
bim:RS-bim:CL	-1.20 [-3.26, 0.86]	0.861	bim:RR-bim:CL	-0.30 [-2.52, 1.91]	1
imp:RR-imp:ARB	-1.32 [-3.64, 1.00]	0.881	sand:CL-imp:ARB	-0.67 [-5.68, 4.33]	1
sand:RR-imp:RS	-1.40 [-3.85, 1.06]	0.883	imp:RR-sand:CL	-0.65 [-5.47, 4.18]	1
bim:CL-bim:ARB	1.42 [-1.17, 4.00]	0.913	sand:CL-bim:ARB	0.64 [-4.44, 5.72]	1
vag:RR-imp:RR	1.63 [-1.36, 4.62]	0.917	vag:CL-bim:ARB	0.32 [-2.29, 2.93]	1
imp:RR-bim:RR	-1.12 [-3.20, 0.95]	0.923	bim:RS-imp:CL	-0.23 [-2.17, 1.70]	1
imp:RS-gris:CL	1.09 [-0.97, 3.15]	0.937	sand:RR-vag:RS	-0.37 [-3.48, 2.74]	1
gris:RS-imp:ARB	-1.19 [-3.54, 1.17]	0.96	imp:RR-bim:RS	-0.23 [-2.14, 1.69]	1
vag:RR-gris:RS	1.50 [-1.52, 4.51]	0.966	vag:RS-gris:ARB	-0.39 [-3.66, 2.89]	1
vag:CL-bim:CL	-1.10 [-3.32, 1.12]	0.966	sand:RR-gris:CL	-0.31 [-2.92, 2.30]	1
vag:RR-bim:ARB	1.62 [-1.76, 5.01]	0.975	gris:RR-imp:CL	0.24 [-1.98, 2.46]	1
bim:RS-imp:ARB	-1.10 [-3.43, 1.24]	0.98	imp:ARB-gris:ARB	0.32 [-2.67, 3.31]	1
imp:CL-bim:CL	-0.97 [-3.03, 1.10]	0.98	gris:RS-sand:CL	-0.51 [-5.35, 4.33]	1
vag:RR-bim:RS	1.41 [-1.60, 4.41]	0.981	imp:RS-bim:CL	0.21 [-1.81, 2.24]	1
imp:ARB-bim:ARB	1.31 [-1.49, 4.12]	0.981	gris:RR-gris:ARB	-0.30 [-3.21, 2.60]	1
bim:RR-gris:RS	0.99 [-1.13, 3.10]	0.981	bim:RS-bim:ARB	0.22 [-2.27, 2.70]	1

sand:RR-bim:CL	-1.18 [-3.77, 1.40]	0.985	gris:RS-bim:ARB	0.13 [-2.37, 2.63]	1
imp:RR-sand:RS	-0.90 [-2.98, 1.18]	0.992	imp:RR-bim:ARB	-0.01 [-2.48, 2.46]	1
gris:RR-imp:RS	-0.94 [-3.13, 1.25]	0.992	sand:RR-bim:ARB	0.24 [-2.70, 3.17]	1
bim:RR-bim:RS	0.90 [-1.20, 2.99]	0.993	sand:CL-gris:ARB	-0.35 [-5.54, 4.83]	1
bim:RR-bim:ARB	1.12 [-1.50, 3.73]	0.993	sand:RS-gris:ARB	-0.10 [-2.91, 2.71]	1
vag:RR-vag:CL	1.30 [-1.81, 4.42]	0.995	bim:RR-gris:ARB	0.12 [-2.69, 2.93]	1
vag:RR-sand:RR	1.39 [-2.00, 4.77]	0.996	bim:CL-imp:ARB	0.11 [-2.34, 2.55]	1
vag:CL-imp:ARB	-0.99 [-3.46, 1.48]	0.997	bim:RR-imp:ARB	-0.20 [-2.67, 2.27]	1
gris:CL-bim:CL	-0.88 [-3.09, 1.34]	0.997	vag:RR-imp:ARB	0.31 [-2.97, 3.59]	1
vag:RR-imp:CL	1.17 [-1.83, 4.18]	0.998	vag:RR-bim:CL	0.20 [-2.88, 3.29]	1
vag:RS-imp:RS	-1.03 [-3.69, 1.64]	0.998	imp:CL-gris:CL	-0.09 [-2.19, 2.01]	1
sand:RR-imp:ARB	-1.08 [-3.88, 1.73]	0.998	sand:CL-gris:CL	0.10 [-4.80, 5.00]	1
imp:RR-gris:ARB	-1.00 [-3.68, 1.67]	0.999	vag:CL-gris:CL	-0.22 [-2.47, 2.03]	1
imp:CL-imp:ARB	-0.86 [-3.20, 1.47]	0.999	vag:RS-gris:CL	0.06 [-2.75, 2.87]	1
sand:RS-gris:RS	0.77 [-1.35, 2.88]	0.999	gris:RR-gris:CL	0.15 [-2.22, 2.51]	1
sand:RS-imp:RS	-0.74 [-2.80, 1.32]	0.999	sand:CL-imp:CL	0.19 [-4.65, 5.02]	1
bim:RR-vag:CL	0.80 [-1.45, 3.04]	0.999	vag:CL-imp:CL	-0.13 [-2.23, 1.97]	1
vag:RR-gris:CL	1.08 [-2.03, 4.19]	1	vag:RS-imp:CL	0.15 [-2.54, 2.84]	1
sand:RS-bim:ARB	0.89 [-1.72, 3.51]	1	sand:RR-imp:CL	-0.22 [-2.70, 2.27]	1
sand:RR-bim:RR	-0.88 [-3.49, 1.73]	1	vag:CL-sand:CL	-0.32 [-5.22, 4.58]	1
sand:RS-bim:RS	0.68 [-1.42, 2.77]	1	bim:RS-sand:CL	-0.42 [-5.25, 4.41]	1
gris:RS-gris:ARB	-0.86 [-3.57, 1.84]	1	sand:RS-sand:CL	0.26 [-4.64, 5.16]	1
gris:ARB-bim:ARB	0.99 [-2.12, 4.10]	1	vag:RS-sand:CL	-0.03 [-5.22, 5.15]	1
imp:RR-gris:RR	-0.70 [-2.90, 1.51]	1	bim:RR-sand:CL	0.48 [-4.42, 5.38]	1
bim:RR-imp:CL	0.66 [-1.43, 2.76]	1	gris:RR-sand:CL	0.05 [-4.90, 5.01]	1

gris:RR-bim:CL	-0.73 [-3.06, 1.61]	1	sand:RR-sand:CL	-0.40 [-5.48, 4.67]	1
gris:CL-imp:ARB	-0.77 [-3.24, 1.70]	1	bim:RS-vag:CL	-0.10 [-2.20, 1.99]	1
vag:RS-bim:CL	-0.81 [-3.60, 1.97]	1	gris:RS-vag:CL	-0.19 [-2.31, 1.92]	1
vag:RR-gris:RR	0.93 [-2.26, 4.13]	1	vag:RS-vag:CL	0.28 [-2.53, 3.09]	1
bim:RS-gris:ARB	-0.77 [-3.47, 1.92]	1	sand:RR-vag:CL	-0.09 [-2.70, 2.53]	1
vag:RR-vag:RS	1.02 [-2.52, 4.56]	1	gris:RS-bim:RS	-0.09 [-2.04, 1.86]	1
imp:RR-gris:CL	-0.55 [-2.63, 1.53]	1	sand:RR-bim:RS	0.02 [-2.46, 2.50]	1
sand:RS-vag:CL	0.57 [-1.67, 2.82]	1	imp:RR-gris:RS	-0.14 [-2.07, 1.80]	1
bim:RR-gris:CL	0.57 [-1.68, 2.82]	1	sand:RR-gris:RS	0.11 [-2.39, 2.61]	1
gris:RR-bim:ARB	0.69 [-2.02, 3.40]	1	vag:RR-imp:RS	-0.01 [-2.99, 2.97]	1
sand:RR-sand:RS	-0.66 [-3.27, 1.95]	1	vag:RS-sand:RS	-0.29 [-3.10, 2.52]	1
gris:RR-gris:RS	0.56 [-1.68, 2.80]	1	bim:RR-sand:RS	0.22 [-2.03, 2.47]	1
bim:RR-imp:RS	-0.52 [-2.58, 1.54]	1	gris:RR-sand:RS	-0.20 [-2.57, 2.16]	1
sand:RR-gris:ARB	-0.76 [-3.87, 2.35]	1	gris:RR-vag:RS	0.09 [-2.82, 2.99]	1
sand:RR-imp:RR	0.24 [-2.22, 2.71]	1			

(H) PA Workers, P:L-ARB

Comparison	**PL diff. [95% CI]**	**p adj**	**Comparison**	**PL diff. [95% CI]**	**p adj**
Species			**Species*location**		
gris-bim	-0.42 [-1.39, 0.55]	0.754	bim:RR-imp:RS	-0.52 [-2.52, 1.49]	1
imp-gris	0.28 [-0.65, 1.20]	0.923	bim:RR-gris:CL	0.55 [-1.64, 2.74]	1
vag-gris	0.22 [-0.93, 1.38]	0.984	imp:RR-imp:CL	-0.46 [-2.32, 1.40]	1
sand-gris	0.22 [-0.96, 1.41]	0.985	vag:RR-sand:RS	0.73 [-2.30, 3.76]	1
vag-bim	-0.20 [-1.34, 0.94]	0.989	sand:RS-bim:CL	-0.52 [-2.68, 1.64]	1
sand-bim	-0.20 [-1.36, 0.97]	0.99	imp:RR-vag:RS	-0.62 [-3.23, 1.98]	1
imp-bim	-0.14 [-1.05, 0.76]	0.992	gris:RR-bim:RS	0.49 [-1.67, 2.66]	1
vag-imp	-0.06 [-1.15, 1.04]	1	sand:RS-imp:CL	0.44 [-1.60, 2.48]	1
sand-imp	-0.05 [-1.18, 1.07]	1	imp:RS-sand:CL	0.97 [-3.72, 5.66]	1
vag-sand	0.00 [-1.32, 1.32]	1	gris:RS-gris:CL	-0.41 [-2.47, 1.65]	1
Location		p adj	vag:RR-sand:CL	0.97 [-4.23, 6.18]	1
RR-RS	-0.16 [-0.85, 0.53]	0.842	bim:RR-vag:RS	0.50 [-2.23, 3.24]	1
RR-CL	-0.11 [-0.83, 0.61]	0.929	sand:RR-gris:RR	-0.47 [-3.11, 2.17]	1
RS-CL	0.05 [-0.62, 0.72]	0.982	vag:RS-gris:RS	0.46 [-2.17, 3.10]	1

Sampling period		p adj	gris:RR-bim:RR	-0.40 [-2.70, 1.90]	1
C-A	-0.91 [-1.76, -0.07]	**0.029**	vag:RR-bim:RR	0.52 [-2.51, 3.55]	1
C-B	-0.81 [-1.63, 0.01]	0.056	bim:RS-gris:CL	-0.34 [-2.38, 1.70]	1
D-C	0.57 [-0.37, 1.51]	0.399	gris:RR-vag:CL	0.39 [-1.91, 2.69]	1
D-A	-0.34 [-1.30, 0.61]	0.787	imp:RR-vag:CL	-0.33 [-2.35, 1.69]	1
D-B	-0.24 [-1.17, 0.70]	0.912	gris:RS-imp:CL	-0.30 [-2.21, 1.60]	1
B-A	-0.11 [-0.95, 0.73]	0.988	sand:CL-bim:CL	-0.76 [-5.51, 3.99]	1
Species*location	diff [lwr, upr]	p adj	sand:RS-gris:CL	0.34 [-1.85, 2.52]	1
imp:RR-imp:RS	-1.64 [-3.46, 0.19]	0.133	vag:RS-bim:RS	0.40 [-2.22, 3.01]	1
imp:RS-gris:RS	1.48 [-0.38, 3.35]	0.298	imp:RR-sand:CL	-0.66 [-5.36, 4.03]	1
imp:RS-bim:RS	1.41 [-0.43, 3.25]	0.358	bim:RR-bim:CL	-0.30 [-2.46, 1.85]	1
imp:RR-bim:CL	-1.42 [-3.41, 0.56]	0.476	sand:RR-gris:CL	-0.32 [-2.86, 2.22]	1
imp:RS-vag:CL	1.31 [-0.70, 3.31]	0.642	bim:RS-imp:CL	-0.24 [-2.12, 1.65]	1
imp:RS-imp:CL	1.18 [-0.67, 3.02]	0.673	sand:RR-vag:RS	-0.37 [-3.40, 2.65]	1
gris:RS-bim:CL	-1.27 [-3.30, 0.76]	0.703	imp:RR-bim:RS	-0.23 [-2.09, 1.64]	1
bim:RS-bim:CL	-1.20 [-3.21, 0.81]	0.766	gris:RR-imp:CL	0.26 [-1.90, 2.42]	1
sand:RR-imp:RS	-1.39 [-3.78, 1.00]	0.799	vag:CL-gris:CL	-0.24 [-2.43, 1.95]	1
vag:RR-imp:RR	1.64 [-1.27, 4.55]	0.835	gris:RS-sand:CL	-0.51 [-5.22, 4.20]	1
imp:RR-bim:RR	-1.12 [-3.14, 0.90]	0.85	imp:RS-bim:CL	0.21 [-1.76, 2.18]	1
imp:RS-gris:CL	1.07 [-0.94, 3.07]	0.885	vag:RS-vag:CL	0.29 [-2.45, 3.02]	1
vag:CL-bim:CL	-1.09 [-3.25, 1.06]	0.919	vag:RS-sand:RS	-0.28 [-3.02, 2.45]	1
vag:RR-gris:RS	1.48 [-1.46, 4.42]	0.921	sand:RR-imp:RR	0.25 [-2.15, 2.65]	1
vag:RR-bim:RS	1.41 [-1.51, 4.34]	0.943	bim:RR-sand:RS	0.22 [-1.97, 2.40]	1
imp:CL-bim:CL	-0.96 [-2.97, 1.04]	0.946	bim:RR-sand:CL	0.46 [-4.31, 5.23]	1
bim:RR-gris:RS	0.96 [-1.10, 3.02]	0.956	bim:RS-sand:CL	-0.44 [-5.14, 4.26]	1
sand:RR-bim:CL	-1.18 [-3.69, 1.34]	0.956	sand:RR-imp:CL	-0.21 [-2.63, 2.20]	1
imp:RR-sand:RS	-0.91 [-2.93, 1.12]	0.97	gris:RS-vag:CL	-0.18 [-2.24, 1.88]	1
bim:RR-bim:RS	0.90 [-1.14, 2.94]	0.974	sand:RR-sand:CL	-0.42 [-5.36, 4.52]	1

vag:RR-vag:CL	1.31 [-1.72, 4.34]	0.978	imp:RR-gris:RS	-0.16 [-2.04, 1.73]	1
gris:RR-imp:RS	-0.92 [-3.05, 1.21]	0.978	gris:RR-sand:RS	-0.19 [-2.49, 2.12]	1
vag:RR-sand:RR	1.39 [-1.90, 4.69]	0.982	vag:RR-bim:CL	0.21 [-2.79, 3.22]	1
vag:RR-imp:CL	1.18 [-1.75, 4.10]	0.988	vag:CL-sand:CL	-0.33 [-5.10, 4.44]	1
gris:CL-bim:CL	-0.86 [-3.01, 1.30]	0.99	gris:RR-gris:CL	0.15 [-2.15, 2.45]	1
vag:RS-imp:RS	-1.02 [-3.61, 1.58]	0.991	vag:CL-imp:CL	-0.13 [-2.17, 1.91]	1
sand:RS-imp:RS	-0.73 [-2.74, 1.27]	0.996	imp:CL-gris:CL	-0.11 [-2.15, 1.93]	1
sand:RS-gris:RS	0.75 [-1.31, 2.81]	0.996	sand:CL-gris:CL	0.10 [-4.67, 4.86]	1
bim:RR-vag:CL	0.79 [-1.40, 2.98]	0.996	vag:RS-gris:CL	0.05 [-2.68, 2.79]	1
vag:RR-gris:CL	1.07 [-1.96, 4.10]	0.997	sand:CL-imp:CL	0.20 [-4.50, 4.91]	1
sand:RR-bim:RR	-0.87 [-3.42, 1.67]	0.998	vag:RS-imp:CL	0.16 [-2.46, 2.78]	1
imp:RR-gris:RR	-0.72 [-2.86, 1.43]	0.998	sand:RS-sand:CL	0.24 [-4.53, 5.01]	1
sand:RS-bim:RS	0.68 [-1.36, 2.72]	0.998	vag:RS-sand:CL	-0.04 [-5.09, 5.00]	1
bim:RR-imp:CL	0.66 [-1.38, 2.70]	0.999	gris:RR-sand:CL	0.05 [-4.77, 4.88]	1
gris:RR-bim:CL	-0.71 [-2.98, 1.57]	0.999	bim:RS-vag:CL	-0.11 [-2.15, 1.93]	1
vag:RS-bim:CL	-0.80 [-3.51, 1.91]	1	sand:RR-vag:CL	-0.08 [-2.63, 2.46]	1
vag:RR-vag:RS	1.02 [-2.43, 4.46]	1	gris:RS-bim:RS	-0.07 [-1.97, 1.83]	1
vag:RR-gris:RR	0.92 [-2.19, 4.03]	1	sand:RR-bim:RS	0.02 [-2.39, 2.44]	1
imp:RR-gris:CL	-0.57 [-2.59, 1.45]	1	sand:RR-gris:RS	0.09 [-2.34, 2.52]	1
sand:RS-vag:CL	0.57 [-1.61, 2.76]	1	vag:RR-imp:RS	0.00 [-2.90, 2.90]	1
sand:RR-sand:RS	-0.66 [-3.20, 1.89]	1	gris:RR-vag:RS	0.10 [-2.73, 2.93]	1
gris:RR-gris:RS	0.56 [-1.62, 2.74]	1			

Table 11. ANOVA table for nutritional flexibility in B. impatiens for protein collection **(A)**, lipid collection **(B)**, and P:L collection **(C)**.

Variable	Factor	df	F value	Pr(>F)
A				
Protein	Location	3	6.91	**> 0.001**
	Sampling period	3	1.86	0.143
	Residuals	77	NA	NA
B				
Lipid	Location	3	2.09	0.109
	Sampling period	3	3.59	**0.01**
	Residuals	77	NA	NA
C				
P:L ratio	Location	3	4.07	**0.010**
	Sampling period	3	4.06	**0.010**
	Residuals	77	NA	NA

Table 12. Tukey table for nutritional flexibility in B. impatiens. Pairwise differences among sites for pollen protein concentration **(A)**, pairwise differences among sampling period for pollen lipid concentration **(B)**, pairwise differences among sites for pollen P:L ratio **(C)**, and pairwise differences among sampling period for pollen P:L ratio **(D)**.

(A) Protein			**(B) Lipid**		
Comparison	**diff. [95% CI]**	**p adj**	**Comparison**	**diff. [95% CI]**	**p adj**
Site			**Sampling period**		
Colyer Lake-Arboretum	-65.06 [-111.90, -18.23]	0.00	B-A	-4.97 [-26.52, 16.59]	0.93
Rock Springs-Arboretum	-66.75 [-112.94, -20.56]	0.00	C-A	6.21 [-13.95, 26.37]	0.85
Rothrock-Arboretum	-77.17 [-123.68, -30.67]	0.00	D-A	-16.41 [-36.57, 3.75]	0.15
Rock Springs-Colyer Lake	-1.69 [-39.69, 36.32]	1.00	C-B	11.18 [-8.98, 31.34]	0.47
Rothrock-Colyer Lake	-12.11 [-50.49, 26.27]	0.84	D-B	-11.45 [-31.61, 8.71]	0.45
Rothrock-Rock Springs	-10.43 [-48.01, 27.16]	0.89	D-C	-22.63 [-41.29, -3.96]	0.01
(C) P:L			**(D) P:L**		
Comparison	**diff. [95% CI]**	**p adj**	**Comparison**	**diff. [95% CI]**	**p adj**
Site			**Sampling period**		
Colyer Lake-Arboretum	-0.87 [-2.54, 0.79]	0.51	B-A	0.81 [-0.74, 2.37]	0.52
Rock Springs-Arboretum	0.26 [-1.38, 1.90]	0.98	C-A	-0.58 [-2.04, 0.87]	0.72
Rothrock-Arboretum	-1.37 [-3.02, 0.28]	0.14	D-A	1.05 [-0.41, 2.50]	0.24
Rock Springs-Colyer Lake	1.14 [-0.21, 2.48]	0.13	C-B	-1.39 [-2.85, 0.06]	0.07
Rothrock-Colyer Lake	-0.49 [-1.86, 0.87]	0.78	D-B	0.24 [-1.22, 1.69]	0.97
Rothrock-Rock Springs	-1.63 [-2.96, -0.30]	0.01	D-C	1.63 [0.28, 2.98]	0.01

Chapter 3

Variance in heat tolerance in bumble bees correlates with species geographic range and is associated with several environmental and biological factors

This work has been published. Author contributions are listed on page 154 and acknowledgements are listed on page xvi (Chapter 3 acknowledgements).

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Abstract

Globally, insects have been impacted by climate change, with bumble bees in particular showing range shifts and declining species diversity with global warming. This suggests heat tolerance is a likely factor limiting the distribution and success of these bees. Studies have shown high intraspecific variance in bumble bee thermal tolerance, suggesting bees' biological and environmental factors may be impacting heat resilience. Understanding these factors is important for assessing vulnerability and finding environmental solutions to mitigate effects of climate change. In this study, we assess whether geographic range variation in bumble bees in the eastern United States is associated with heat tolerance and further dissect which other biological and environmental factors explain variation in heat sensitivity in these bees. We examine heat tolerance by caste, sex, and rearing condition (wild/lab) across six eastern U.S. bumble bee species, and assess the role of age, reproductive status, body size, and interactive effects of

humidity and temperature on thermal tolerance in *Bombus impatiens*. We found marked differences in heat tolerance by species that correlate with each species' latitudinal range, habitat, and climatic niche, and we found significant variation in thermal sensitivity by caste and sex. Queens had considerably lower heat tolerance than workers and males, with greater tolerance when queens would first be leaving their natal nest, and lower tolerance after ovary activation. Wild bees tended to have higher heat tolerance than lab reared bees, and body size was associated with heat tolerance only in wild-caught foragers. Humidity showed a strong interaction with heat effects, pointing to the need to regulate relative humidity in thermal assays and consider its role in nature. Altogether, we found most tested biological conditions impact thermal tolerance and highlight the stages of these bees that will be most sensitive to future climate change.

Introduction

Across much of the northern hemisphere and South America, where the ~270 bumble bee species are native, numerous flowering agricultural crops and wild flowering species have evolved to be heavily dependent on bumble bees for their pollination (Goulson, 2010). These services, however, are under threat given documented declines in these bees over the last century (Cameron et al., 2011; Goulson et al., 2008a; Soroye et al., 2020). In Europe and North America, 26% of bumble bee species now are listed as threatened or endangered (Cameron & Sadd, 2020), and bumble bee species richness has declined 30% in the Northeastern United States since the late 1800s (Bartomeus et al., 2018). While there are many stressors impacting bee populations (e.g., pathogens, habitat loss, pesticides), climate change is a major factor linked to range shifts and biodiversity loss in bees and other pollinators (Soroye et al., 2020; Vasiliev and Greenwood, 2021), with several studies in bumble bees exemplifying these effects.

Bumble bees are particularly vulnerable to increasing temperatures associated with climate change. Hamblin et al (2017) found bumble bees to be most susceptible to heat among 15 tested bee species in North Carolina, U.S.A., and Pardee et al. (2022) found that bumble bees were less abundant during warmer years compared to 28 other bee genera in a montane region in Colorado, U.S.A. Although bumble bees could benefit from some warming as it might reduce their expenditures towards maintaining endothermy (Jackson et al., 2023), bumble bees have been found to decrease foraging and their colonies to have decreased growth with high heat (Gérard et al., 2022; Hemberger et al., 2023; Vanderplanck et al., 2019). Effects of climate change in bumble bees have been well documented using large-scale geographic and climate modeling approaches. In Europe and North America, climate has been found to be a better predictor than habitat in explaining declines, and regions with greater increase in temperature have experienced greater declines in bumble bee diversity (Soroye et al., 2020, although see Guzman et al., 2021). Bumble bee species have also experienced range contraction in response to climate warming, with southern ranges receding northward without a corresponding shift at the northern edges of their ranges (Kerr et al., 2015). Bumble bees may also be shifting altitudinally with climate, potentially creating isolated populations that are vulnerable to extirpation (Kerr et al., 2015; Pyke et al., 2016). Forecasting models taking into account climatic niches and dispersal abilities of bumble bees predict that many species will fail to disperse into new climatically suitable conditions with projected climate change, leading to declining populations (Sirois-Delisle and Kerr, 2018).

Not all bumble bee species have declined equally in the face of recent climate and land use changes (Cameron et al., 2011; Colla et al., 2012; Grixti et al., 2009). Determining the factors that make species resilient requires a better understanding of the factors that limit species distributions. Bumble bees are cold-adapted with the highest species diversity in cool, temperate-boreal and montane habitats, although a few species are endemic to deserts and tropical lowlands

(Hines, 2008; P.H. Williams et al., 2014). Recent studies point to cold tolerance being a limiting factor on altitudinal and geographic distribution, and cold tolerance is also likely under selection (Jackson et al., 2020; Keaveny et al., 2019, 2023; Maebe et al., 2021; Oyen et al., 2016; Pimsler et al., 2020). However, the global distributions of bumble bee species appear to be further influenced by species-level variation in heat tolerance (Table 13): Martinet et al. (2021a) performed an analysis of thermal tolerance on males of 39 bumble bee species across Europe and North America and found interspecific variation in heat stress tolerance that matches species distributions. Arctic and boreal species had lower resistance to hyperthermic stress than temperate and Mediterranean species. The high heat tolerance in the most southern distributed Mediterranean species *Bombus terrestris* (Linnaeus) is particularly striking given that this species is generally expanding its range. Oyen and Dillon (2018) likewise found that the lower altitude Rocky Mountain species *Bombus huntii* Greene had higher heat tolerance than *Bombus sylvicola* Kirby, which inhabits higher altitudes. Gonzalez et al. (2022) applied thermal tolerance data from other studies noted a relationship between heat tolerance and several climatic variables in 5 North American bumble bee species. Differential heat tolerance in bumble bees may be conferred through differences in molecular heat shock response (Blasco-Lavilla et al., 2021; Kuo et al., 2023; Pimsler et al., 2020), interspecific differences in heat shunting by means of counter-current exchange between the thorax and abdomen (cf. Heinrich and Vogt, 1993; Heinrich, 1976b), or through other physiological adaptations such as shifting metabolism or potential for evaporative cooling (Johnson et al., 2023).

The susceptibility of bumble bees to climate is likely to vary not only across species but by life history and condition within species. Prior studies examining species heat tolerance have shown high variance across individuals within species (Oyen and Dillon, 2018, Martinet et al., 2021a), suggesting that other biological factors interplay with thermal physiology to impact tolerance. Bumble bees, as social species, exhibit variation across individuals in physiology and

behavior depending on caste and stage of their life cycle (Amsalem et al., 2015b). Queen bumble bees for example are known to shift their nutritional reserves in preparation for diapause and upon ovary activation (Amsalem et al., 2015a; Treanore and Amsalem, 2022), and workers in late or queen-less colonies can also lay eggs (Goulson, 2010). There may also be selection for bumble bees in different roles to have different heat tolerances based on differences in thermal exposure. Bumble bees typically nest underground where they tend to be buffered from the elements, although some species tend towards surface nesting (Liczner and Colla, 2019). Males are exposed to environmental conditions more than workers, as workers can escape the heat in nests (Heinrich, 2004). Among workers, foragers are more exposed to heat stress than nurses, which usually reside only in the nest. Queens in general receive little exposure to heat, as they are typically produced in late summer where they initially feed in natal nests, subsequently receiving the most exposure when they leave the nest to mate (~1-2 weeks post emergence, Roseler and Van Honk, 1990) and dig themselves a hibernaculum underground for overwintering. They will emerge again in the cool of spring to forage for provisions for their nest, thereafter, remaining protected in underground colonies (Gardner et al., 2007).

There have been a few studies examining how thermal heat tolerance in bumble bees varies with sex and caste (Table 13). Maebe et al. (2021) found differences between *B. terrestris* workers and queens in critical minimum temperatures (CT_{min}) but saw no difference in critical maximum temperatures (CT_{max}) between castes. Oyen et al. (2016) found no difference in CT_{max} between males and workers within three Rocky Mountain species (*B. huntii*, *B. bifarius* Cresson and *B. sylvicola*) and Gonzalez et al. (2022) found no role of sex in thermal tolerance in Colombian bumble bees. The role of reproductive state has yet to be examined.

Age-related variation in thermal tolerance has been documented in a number of holometabolous insects with many species showing a decline in tolerance with age (Bowler and Terblanche, 2008). Bumble bee workers tend to live from 2-6 weeks and can switch roles from

in-nest and nursing tasks to foraging as they age (Tobback et al., 2011). A study examining the critical thermal limits of *Bombus impatiens* Cresson at 3, 4, and 7 days, found 3-day-old and 7-day-old workers are more heat resistant than 4-day old workers (Oyen and Dillon, 2018). The study, however, did not test the effect of older ages (> 1 week) on thermal tolerance.

Bumble bees are facultative endotherms and can maintain constant body temperatures through a wide range of air temperatures (Church, 1960; Goulson, 2010; Heinrich, 2004). Nevertheless, by Bergmann's rule, the small surface to volume (S/V) ratio of larger bees should reduce their thermoregulatory capabilities for dissipating heat (Heinrich, 2004), while increasing their ability to retain heat in cold conditions. Given evolutionary tradeoffs in body size in insects, however, Bergmann's rule may not always apply (Lozier et al, 2021; Shelomi, 2012). Prior work found that CT_{max} increased with mass in reared workers of *B. huntii* (Oyen et al., 2016), but not in wild workers of *B. bifarius, B. sylvicola* (Oyen et al., 2016), *B. impatiens* (Oyen & Dillion 2018), or *B. terrestris* (Maebe et al. 2021). Martinet et al. (2021a) found only weak explanatory power of body mass on time to heat stupor at 40°C among males of 39 bumble bee species. Recent studies have found a decrease in body size in workers in the last 100+ years in certain bumble bees (Nooten and Rehan, 2020) and other bee populations (Oliveira et al., 2016). Although developmental plasticity could be involved in this, it is possible that climate change may be implicated via natural selection if larger bees are more negatively impacted by heat. More evidence is needed to understand if body size indeed plays a role in heat stress tolerance and long-term shifts in body size in bumble bees.

Nutrition may also impact thermal tolerance (Vanderplanck et al., 2019) and, if so, this can both explain natural variance in heat tolerance and present a solution to mediate effects of thermal stress. There have been mixed results on the effects of starvation in thermal tolerance with some studies finding a role (Blasco-Lavilla et al., 2021; Quinlan et al., 2023b) and another seeing no correlation (Oyen & Dillon 2018).

In this study, we sought to better understand which factors contribute to heat stress and resilience in bumble bees. In particular, we sought to (i) better understand what biological factors (e.g., age, caste, sex, or rearing conditions) contribute to variance in thermal tolerance, (ii) determine points of vulnerability within the life cycle, (iii) better understand whether heat tolerance may be a factor limiting distribution and niche, and (iv) understand whether heat tolerance may impact success of different species in the face of climate change. Towards these goals we examine heat stress tolerance variation across 6 species native to the Northeastern United States: *Bombus impatiens, Bombus bimaculatus* Cresson*, Bombus griseocollis* (De Geer)*, Bombus perplexus* Cresson*, Bombus vagans* Smith and *Bombus sandersoni* Franklin. These species have different but overlapping distributions and habitat preferences (Gratton et al., 2023; P.H. Williams et al., 2014) and vary in their abundance in our study region in Pennsylvania. In particular, *B. impatiens* is increasing in abundance, *B. bimaculatus* and *B. griseocollis* are stable or increasing, and *B. perplexus, B. vagans*, and *B. sandersoni* are more rare and potentially decreasing (Colla et al., 2012; Grixti et al., 2009; Jacobson et al., 2018). In terms of habitat, *B. impatiens* and *B. griseocollis* are more abundant in open valleys, *B. sandersoni* and *B. vagans* in the ridges, and *B. perplexus* and *B. bimaculatus* in ecotonal regions (Gratton et al., 2023; P.H. Williams et al., 2014). To understand how aspects of life history within species impact thermal tolerance and vulnerability, we examine the role of sex and caste across species, and of age, body size, and reproductive state of queens on thermal tolerance in *B. impatiens*. As an indirect test of potential impacts of environmental factors like nutrition, we compare wild foraging workers to lab reared bees. Finally, we examine the impact of humidity levels on resulting heat stress tolerance. Humidity and temperature interactions may be important as high humidity can reduce the potential for evaporative cooling in insects as it reduces the hygric gradient between the bee and environment (Church, 1960; Prange, 1996). While evaporative cooling is thought to not be a major factor in most bees (Johnson et al. 2023, Heinrich 2004), in bumble bees, high humidity

has been found to reduce cooling during flight (Church, 1960). Altogether, our data reveal that many biological factors impact thermal tolerance in bumble bees, providing a baseline for understanding points of vulnerability and for guiding solutions to better manage bumble bees under climate change.

Methods

Bee specimen sources

Bees for our thermal trials were sourced either directly from the wild (collected as foraging queens, workers, or males), drawn from lab-reared colonies founded by wild-caught queens, or from commercially purchased colonies (thus from lab-reared queens). While we sought to balance replicates in the design as much as possible with our sampling effort, sample size of field-collected bees was somewhat uneven across groups due to limited field availability. A total of 379 bees were tested for these assays with sample sizes and species information contained in Appendix: Supplemental Table 1, with a more detailed table in Scholarsphere (https://doi.org/10.26207/gqy8-e215), and apparent in Figure 22a.

Wild bees:

A bee is defined as wild if thermal tolerance was tested within 24 hours of its collection from nature. The Northeast U.S., and Pennsylvania in particular, is considered to have a humid continental climate (“Pennsylvania State Climatologist,” n.d.), characterized by widely varying temperatures between summer and winter. Wild-caught bees for our study were collected from

central Pennsylvania's Ridge and Valley Province and Allegheny Plateau which have greater temperature extremes than the Southeastern Coastal Plain and Piedmont Plateau ("Pennsylvania State Climatologist," n.d.). Bumble bee field collections were conducted from June 29th to November 8th, 2022, and from July 25th to July 26th, 2023, across 11 sites in Pennsylvania (Appendix: Supplemental figure 1), each within a 1.5-hour drive from the research lab at Penn State's University Park campus. Bees were collected by hand netting in an opportunistic manner, mostly while foraging on flowers, and transported in vials from the field at ~7°C in a cooler containing ice packs to slow down their activity during transport. Once in the lab, bees were transferred into plastic cages, provisioned *ad libitum* with pollen patties (pollen collected from honey bees; sourced from Swarmbustin' Honey Bee Farm, Westgrove, PA) and artificial nectar used for lab colonies (see below), and placed in a walk-in environmental chamber set to 28°C, ~65% relative humidity (RH) for 12 - 16 hours prior to trials (cf. Martinet et al., 2015a).

Lab-reared bees from wild queens:

We identified bees as lab-reared if they were drawn for thermal tolerance experiments from lab reared colonies. For lab-reared bees from wild queens, spring foundresses of each of the species/species-groups were collected from the field while either foraging or nest searching (April 24th - May 17th, 2022) and used to rear bumble bee colonies in the lab, resulting in 5 *B. impatiens*, 4 *B. griseocollis*, 4 *B. bimaculatus,* 2 *B. perplexus*, and 3 *B. vagans/sandersoni* source colonies. For this, wild queens were chilled and brought back to the lab, kept in an incubator at 28°C, ~65% RH, and provisioned with a bee pollen patty blended with Biogluc proprietary nectar (Biobest, Canton, MI) or a lab-made sugar solution *ad libitum* throughout the colony development. The lab-made solution was composed of 50% sucrose and 50% invert sugar with

amino acid supplement (Amino-B Booster, Honey-B-Healthy Inc., Cumberland, MD) to provide bees with essential amino acids.

Lab-reared bees from commercial colonies:

For some of our experiments, we assayed workers, males, and newly emerged queens (we use the term "queen" here to include both mated or unmated and those that have or have not started a nest) produced from commercial, research-grade *B. impatiens* colonies (sourced from Biobest, Canton, MI). Commercial colonies were kept under the same conditions as lab-reared wild queen colonies.

Time to Heat Stupor assays: Cross-species comparisons

Time to Heat Stupor (THS) protocol:

We used time to heat stupor (THS) as a measure of thermal stress across all bees (Martinet et al., 2021a). Symptoms of heat stupor include a loss of neuromuscular function, which is characterized by uncoordinated movements, the inability right itself when flipped on its back, twitching in the extremities (e.g., tarsi), and heat coma (cf. Martinet et al., 2021a). We used THS instead of CT_{max} (critical maximum temperature, the temperature at which bees reach heat stupor following an incremental temperature ramping rate from ambient temperature (Oyen and Dillon, 2018), as we sought to test thermal endurance without the confounding factor of ramping rate. We felt THS would be more akin to the temporal response upon entering a hot environment from a cool nest. Moreover, THS has been useful for discriminating bumble bee species-specific tolerances in the past (Martinet et al., 2021a; Zambra et al., 2020). Static (THS) and dynamic (CT_{max}) assays of heat tolerance have been found to be comparable and thus reliable methods of

assessing heat tolerance in *Drosophila* (Jørgensen et al., 2019) and thus these methods should yield similar results in bumble bees, however, we recognize that the method applied will likely affect magnitudes and variance of response. Trials were limited to 3 hours to avoid confounding effects of starvation on THS. We thus consider bees reaching 3 hours as "heat resistant." In our preliminary THS trials, we used 40°C as our critical temperature. This temperature has been used in previous studies to induce heat stupor across bumble bee species (Martinet et al., 2021a; Zambra et al., 2020) and is above the threshold temperature commonly used to define a heat wave (Xu et al., 2016). However, most bees in our preliminary study were reaching three hours without entering heat stupor, so we decided to use a higher temperature of 43°C that would capture the full range of responses to heat stress within the trial period.

We used a water bath (Benchmark, MyBath 4L, model H2004) for these trials, to retain consistent heat and humidity (constant at 90% RH) across periodic (5 minute) THS checks. Bees were taken from their holding container/colony and transferred to preheated (43°C ± 0.5°C) cylindrical glass vials (volume = 35.35 cm^3) (Genesee Scientific, Flystuff, 32-117BF) placed in a rack in a water bath (Benchmark, MyBath 4L, model H2004) (90% RH). Glass vials were weighed down with hex nuts (29.5 grams) glued to the bottom of the vials and were cotton-stoppered to allow for gas exchange and prevent CO_2 build-up from respiration. Bees were checked every 5 minutes for symptoms of heat stupor by briefly lifting them from the water bath and flipping or shaking the vial as needed to assess if they could remain upright.

Cross-species analysis:

We performed thermal tolerance assays on collected workers, males, and queens of wild and lab-reared *B. impatiens* (n=129 bees tested), *B. bimaculatus* (n=45), *B. griseocollis* (n=39), *B. perplexus* (n=17), and the *B. vagans*/*B. sandersoni* (VS, n=28) complex (Appendix: Supplemental

Table 1). *B. vagans* and *B. sandersoni* are less common, are hard to tell apart morphologically, and occupy the same ecological niche in Pennsylvania higher elevation forests (Gratton et al., 2023), thus these were pooled to improve sample sizes. We identified *B. sandersoni* and *B. vagans* after running THS trials and found 31.6% of wild sampled bees were *B. sandersoni* (36.4% of workers, 37.5% of males) and all lab-reared individuals were *B. sandersoni* (n=4 colonies). If the analyses described below are run with these separately the two species do not differ in THS (*B. vagans* (56.92 ± 16.78) and *B. sandersoni* (56.33 ± 29.72), p=0.95) thus further justifying pooling them.

All statistical analyses were carried out in R version 4.2.1. We conducted an analysis of variance (ANOVA), both with and without interactions, to compare the effects of species, sex/caste (i.e., workers, males, and queens), and bee origin (lab-reared or wild) on THS, followed by a Tukey's HSD test for multiple comparisons. For these two models (additive and interactive) we also calculated Akaike's Information Criterion, corrected for small sample size (AICc), using the AICcmodavg package (Mazerolle, 2017). While these models reveal the main effects, we also conducted separate analyses for visualization purposes on subsets of the data. In particular, we pooled wild and lab-reared workers within each species and compared THS among species. We also compared lab-reared *B. impatiens* workers, males, and queens, for which we had the most individuals of each sex/caste. We chose to exclude wild bees from this analysis due to potential confounding effects of origin on THS.

To assess species niche (i.e., latitude, climate, habitat type) relative to heat tolerance results, we used historical, georeferenced records of each *Bombus* species from GBIF (gbif.org). For latitude, we filtered specimen records from the United States and Canada and only used specimen occurrence records east of 100.00° longitude to focus on eastern North America. From this, the 1% tails of the distribution were excluded to focus on the core range of each species. Within each of these ranges, we obtained average summer temperatures for the months when

workers are primarily foraging - June, July, and August - aggregated over 30 years (1991 – 2020). Climate data was extracted from PRISM (2023) using the prism package (Hart and Bell, 2015). We used separate ANOVA models followed by Tukey's HSD tests to assess both species differences in mean latitudinal distributions and summer mean temperatures. As PRISM data does not include climatic data for eastern Canada, Canadian specimens were not included in the temperature analysis. We also compare our results to data on altitudinal and habitat niche (forested hills, open valleys, and the transitional habitat between these) of each of these species in Pennsylvania (Gratton et al., 2023).

Time to Heat Stupor assays: B. impatiens assays of age, reproductive state, and body size

We assessed the effect of colony origin, age, reproductive state, body size, and humidity on thermal tolerance in *B. impatiens*. We focused on just *B. impatiens* because it had more robust sample sizes for these comparisons.

Colony:

We tested the effect of colony origin on THS in workers from 5 commercial colonies (n=3-5 bees /colony) and 3 colonies reared from wild queens (n=13-16 bees /colony; Appendix: Supplemental Table 1), using a two-way ANOVA followed by a Tukey's HSD test, testing the effect of both colony and wild vs. commercial origins. There was no significant variation in thermal tolerance with colony and thus we did not include colony identity in future models.

Age:

We compared THS among *B. impatiens* queens of different ages, drawn from three different commercial colonies: 4-day-old (n = 17), 7-day-old (n = 4), 10-12-day-old (n = 9), and 12-32-days-old (n = 17) (Appendix: Supplemental Table 2). For the first three age groups, callow queens were removed from their natal colonies daily, and individuals from the same colony were kept in a container together and provisioned with artificial nectar and pollen *ad libitum* until they reached the desired age. The 12-32-day old cohort was removed from natal colonies at 2-20 days after adult eclosion and kept together prior to sampling. We also compared THS among 2 different age groups of *B. impatiens* workers. Seven-day-old (n = 12) and 14-day old workers (n = 13) workers were sampled from 3 lab colonies reared from wild-caught queens. Callow workers were removed from their natal colonies and placed in mini-colonies (a few individuals together per container) provisioned with artificial nectar and pollen *ad libitum* until they reached the desired age. The effect of age on mean THS for both queens and workers were tested separately using ANOVAs followed by Tukey's HSD tests.

Ovarian activation in queens:

We sought to test whether ovarian and physiological state may impact heat tolerance in *B. impatiens* queens. Carbon dioxide (CO_2) narcosis in bumble bees is known to induce a post-diapause reproductive state in bumble bees and trigger them to initiate egg production (Amsalem and Grozinger, 2017). We thus tested bees with and without CO_2 narcosis to test effects of diapause, and bees at 2 days post narcosis, which is prior to or during early ovarian activation/egg development for CO_2 treated bees, and 7 days post-narcosis, when ovarian activation/egg development is likely already completed for CO_2 treated bees (Amsalem & Grozinger, 2017), to examine effects of ovarian activation state. We administered CO_2 to 17 *B. impatiens* queens

reared from two commercial colonies that were between 12- and 21- days-old age by placing bees in plastic boxes that were sealed except for a small hole in the top where we administered CO_2 through a hose at low pressure for 1 minute, which is enough time for bees to enter CO_2 narcosis. The hole was immediately taped over, sealing in the gas where the bees remained for 30 minutes. They were then placed into individual ventilated containers, where they emerged from their narcosis, and were provisioned with pollen patties and artificial nectar *ad libitum*. Another 17 *B. impatiens* queens of the same age group that did not undergo CO_2 narcosis were used as controls. We divided treated and untreated bees into two subgroups in a fully crossed design: 2-day-post-CO_2 narcosis/ 2-day-control (n=8 each) and 7-day-post-CO_2 narcosis/ 7-day-control (n=9 each) (Appendix: Supplemental Table 2). After queens went through THS trials, queens were dissected, and ovary activation was measured by averaging the length of the largest terminal oocytes from each ovary, plus the largest remaining oocyte from one side (cf. Amsalem & Grozinger, 2017). We used ANOVAs followed by Tukey HSD tests to assess whether there are differences in mean THS between CO_2 narcosis treatments and ovary activation (average terminal oocyte length). To further test the effects of ovarian activation on heat stress response, we performed a linear regression model comparing THS against average terminal oocyte length across all samples. Finally, some queens had started laying eggs, so we performed ANOVAs to test the effects of presence/absence of brood on mean THS and examined whether bees with brood indeed had more ovary activation.

Body size:

To test the effect of body size on heat tolerance, intertegular distances (ITD) were measured in 71 *B. impatiens* workers that underwent THS trials. ITD is a commonly used metric to assess body size in bumble bees and other bees and is considered a reliable metric of body size

(Cane, 1987; Hagen and Dupont, 2013). ITD was measured with a dissection microscope ocular reticle and converted to millimeters for analysis. We did separate analyses for workers reared from wild-caught queens (n = 55) and for wild workers (n = 17). For each group of workers (lab-reared and wild) we used a simple linear regression model to test the effect of ITD on THS. The data for lab-reared workers appeared somewhat curvilinear, so we also ran a second-order polynomial regression on this data. We then compared this model to our linear regression for lab-reared workers using AICc. We retained the linear regression model since it had the lower AICc (172.7 compared to 176.0.)

Humidity and temperature interactions in THS

To test the interaction between temperature and humidity we first conducted an experiment on commercial *B. impatiens* workers (n=4 colonies; 3 replicates per colony/condition; n=12 bees/condition) at 3 different humidity conditions: 20%, 50%, and 70% RH (range ± 4% during trials). These humidities were chosen to span dry, average, and higher humidity conditions. Bees were taken from their natal colony, placed into separate dry incubators (Thermo Scientific, 371 L) set at 43°C (± 2 °C during trials), and checked every 5 minutes for heat stupor.

As the temperature fluctuated quite a bit during the first experiment, likely due to our regular opening of the door, we ran a second experiment using a single device for all specimens, seeking to control environmental conditions more. Given the results from the first experiment, we focused only on the highest and lowest humidities (20 and 70%). For this we drew 10 workers randomly for each condition [20% (17-29%) and 70% (67-77%)] from a single commercial parent colony that was generating only workers, and we also drew 10 queens (aged 10- to 21-days-old) from a different commercial colony that was in a queen producing stage. Bees were moved into a small (23.5cm x 27.5cm x 36 cm) incubator (Vevor, model: XHC-25) that was set

to 43°C (actual temperature: 42.9-43.5°C at 20% and 42.4-42.7°C at 70%), set within a walk-in incubator at similar humidity to the treatment and at 28°C, and assessed for THS. We compare these data with THS results from *B. impatiens* workers (n=69) run at a stable 43°C and 90% RH in a water bath. Note that only for humidity trials did we use incubators; water baths were used for all other trials.

For the humidity trial analysis, given that much of our data was truncated to 3-hours due to the great number of heat resistant bees at low humidity, we ran a GLM binomial regression to assess the proportion of bees that entered heat stupor as a function of humidity (20% vs 70% RH) and caste (workers and queens). We also ran an ANOVA followed by Tukey tests on both humidity datasets separately, testing the effects of humidity treatment for the first experiment and both humidity and caste for the second.

Results

Cross-species analysis (species, caste, origin) of heat tolerance

Species:

We found a strong effect of species ($p < 0.001$) on mean THS in our full model (Table 14). Overall means in THS minutes by species placed *B. griseocollis* (96.9 min ± 44.4 [sd]) as the most heat tolerant, followed by *B. impatiens* (80.8 ± 30.1), *B. perplexus* (71.8 ± 40.0)*, and B. bimaculatus* (71.1 ± 35.2), which were not statistically different from one another, and *B. vagans/sandersoni* (56.6 ± 24.2) as the least heat tolerant. Pairwise comparisons among species (Tukey's HSD Test) (Table 15) revealed *B. griseocollis* to have a higher THS than *B. impatiens*,

B. perplexus, *B. bimaculatus*, and *B. vagans/sandersoni*, while *B. impatiens* have a higher mean THS than *B. vagans/sandersoni*. The interactive model had a slightly lower AICc (2477) compared to the additive model (2481), however, we report statistics from both models in Table 14. A subset model run only on workers likewise found the same patterns of significance, except that *B. perplexus* was not significantly different from *B. griseocollis* ($p = 0.161$): *B. griseocollis* (117.9 min ± 37.2 [sd]) > *B. impatiens* (87.5 ± 29.4) = *B. perplexus* (78.8 ± 53.4) = *B. bimaculatus* (77.7 ± 35.2) > *B. vagans/sandersoni*) (55.9 ± 21.2) (Figure 22b, Table 15). This THS order tracks the mean latitude, climatic distribution, and habitat preferences for these different species based on species distributions obtained from GBIF (Figure 22b). Species varied significantly in median latitude ($F_{4,86440} = 2844$, $p < 0.001$) with *B. impatiens* (40.78*)* < B. *bimaculatus* (41.0) < *B. griseocollis* (41.17) < *B. perplexus* (41.81) < *B. vagans/sandersoni* (43.65), while the southern and northern latitudinal limits, respectively, show *B. impatiens* (28.28,44.93) < *B. griseocollis* (30.84,46.98) </> B*. bimaculatus* (33.25, 46.87) </> *B. perplexus* (35.57,49.69) < *B. vagans/sandersoni* (35.75, 48.78). Similarly, when these georeferenced datapoints are used to extract 30-year normal June-August temperatures, we see a highly significant difference in mean temperatures by species ($F_{4,83684} = 4114$, $p < 0.001$) and between each species pair (Tukey $p < 0.001$ for all comparisons) where *B. griseocollis* (22.32°C) > *B. impatiens* (22.21°C) > B*. bimaculatus* (21.54°C) > *B. perplexus* (20.95°C) > B. *vagans/sandersoni* (19.76°C) (Figure 22b). This matches well with the recognized differences in distribution in Pennsylvania, where *B. impatiens* and *B. griseocollis* are more abundant in open valleys, *B. vagans/sandersoni* are more abundant in hilled forests and *B. bimaculatus* and *B. perplexus* are most abundant at the interface of these habitat zones.

Caste differences:

THS was different by sex/caste ($p < 0.001$) (Table 14) with the greatest difference in queens from workers and males (Table 15, $p < 0.001$) and a weakly significant difference between workers and males ($p = 0.044$). Workers had the highest THS across species (mean = 86.37 min ± 34.91 [sd]) followed closely by males (75.50 ± 29.57), and then by queens (44.35 ± 25.68) (Figure 22a). There was a marginally significant interaction between sex/caste and species ($p = 0.066$). *B. griseocollis* had a particularly marked difference between workers (mean THS = 117.86 min ± 37.17) and males (72.33 ± 36.59) ($p < 0.001$).

When looking at sex/caste difference just within *B. impatiens*, we found a significant difference in mean THS ($F_{2,93} = 20.67$, $p < 0.001$), with higher THS in workers (mean 82.54 min ± 25.31 [sd]) than queens (43.06 ± 16.46) ($p < 0.001$) and males (76.5 ± 17.49) and queens ($p = 0.012$), but not between workers and males ($p=0.716$) (Figure 22c; Table 15).

Origin of bees:

We found a significant difference between lab-reared and wild bees overall ($p < 0.001$), an interactive effect of species and origin (wild- vs lab-reared) ($p=0.002$) (Table 14), and a trend for interactions of origin with sex/caste and species ($p = 0.062$). Across species, wild-caught workers had higher THS on average than lab reared workers with the exception of *B. griseocollis,* where the opposite pattern was observed (Figure 22a).

With our analyses in *B. impatiens* workers, there was no significant colony effect (n=8 colonies) on mean THS ($F_{6,56} = 1.85$, $p = 0.105$) (Appendix: Supplemental figure 2). There was no

difference between colonies obtained commercially (mean = 80.68 min ± 24.17 [sd]) from those reared from local queens (82.73 min ± 25.90) ($F_{1,56} = 0.10$, $p = 0.749$).

Effect of age, physiology and body size in Bombus impatiens

Age trials:

The ANOVA showed a significant effect of age on mean THS ($F_{3,43}=16.35$, $p < 0.001$) in queens (Figure 23). Queens aged 12-32 days (mean = 43.82 min ± 16.63), 10-12 days (75.56 ± 32.05), and 7 days (58.75 ± 34.25) had higher THS than 4-day-old queens (21.18 ± 3.32), and the 10-12-day-old queens had higher mean THS than 12-32-day-old queens (Table 1). The 7- and 12-32-day-old queens showed similar THS to spring queens sampled from the wild (Figure 23 and Figure 23), while 4-day-old queens had lower THS, and 10-12-day-old queens had higher THS than wild-caught spring queens. We found no significant difference in mean THS between 7-day-old (85.42 ± 18.15) and 14-day-old (90.38 ± 25.20) workers (Figure 23).

Ovarian activation in queens:

CO_2 narcosis did not result in a significant effect on mean THS ($F_{3,30} =1.11$, $p=0.36$; Figure 24a). However, CO_2 narcosis was effective at initiating ovary activation; average terminal oocyte length was significantly correlated with narcosis treatment ($F_{3,30} = 6.02$, $p = 0.002$) (Figure 24b), with 7-day-post-CO_2 narcosis bees having higher ovary activation than all other treatment groups, which were not significantly different from each other. Overall, degree of ovary activation showed a trend towards a correlation with THS ($F_{1,32} = 3.74$, $p = 0.062$) (Figure 24c), whereby greater ovary activation was associated with decreased THS. Queens that were egg-

laying had higher rates of ovary activation ($F_{1,32} = 10.7$, $p = 0.026$), and showed lower mean THS ($F_{1,32} = 9.06$, $p = 0.005$) (Figure 24d and 24e).

Body size:

We found larger body size was associated with decreased THS for wild *B. impatiens* workers (R-squared = 0.26, $F_{1,15} = 5.29$, $p = 0.036$) but there was no effect of body size on lab-reared workers (Figure 25). On average, wild workers had a larger body size than lab-reared workers ($p=<0.001$).

***Evaluating the interaction of humidity and temperature on THS in* B. impatiens**

In our initial humidity experiment evaluating the effect of humidity (20, 50, 70% RH) on the THS of worker *B. impatiens* bees, we found significant differences in mean THS between 20% and 70% RH ($p < 0.001$, 95% C.I. = [-140.77, -72.56]), 50% and 70% RH ($p < 0.001$, 95% C.I. = [-126.19, -57.98]), but not between 20% and 50% RH (Figure 26a). Overall, heat tolerance declined with increasing humidity, with more dramatic declines around 70%. Many bees in the 20% and 50% RH trials were heat resistant, which may explain in part the non-significant results between these two groups.

In the second experiment, bees at 20% RH were more heat resistant than bees exposed to 70% RH ($p < 0.001$, 95% C.I. = [-0.81, -0.29]), with no significant effect of caste (queen vs. workers). Humidity had an effect on heat resistance in both workers ($p = 0.004$, 95% C.I. = [-0.98, -0.22]) and queens ($p = 0.018$, 95% C.I. = [-0.90, -0.10]). All three humidity levels (20, 70, and 90%) support a highly significant effect of humidity for both workers ($F_{2,85} = 69.39$, $p < 0.001$) and queens ($F_{2,35} = 147$, $p < 0.001$) (Figure 28b,c). Treatments 20% and 90%, and 70%

and 90% for both queens and workers differed in THS ($p < 0.001$) and THS at 20% and 70% were also different for queens ($p=0.045$) and workers ($p < 0.001$), with THS being greater at lower humidity.

Discussion

Prolonged hyperthermic stress in bumble bees can have lethal as well as sublethal effects on bee health, such as limiting forage time and flight ability (Hemberger et al., 2023), and impact colony growth and reproduction (Vanderplanck et al., 2019). The frequency and duration of heatwaves across the globe are predicted to increase due to climate change (Domeisen et al., 2023), and thus many bumble bee populations are more likely to experience hyperthermic stress in the future. To better understand how these bees will respond to this change, we have provided data towards understanding how different species respond to heat stress and which physiological, morphological, and life history factors make them more vulnerable. We found that species, caste and sex, origin, age, ovary activation, body size, and relative humidity all play a role in how the bumble bee species in our study region respond to heat stress, but to different extents. Most notably, we found that bumble bee species vary in heat tolerance in a way that matches their habitat distributions, suggesting that heat sensitivity likely impacts their range. We find that queens are most vulnerable, but that their vulnerability is dependent on age and reproductive status. Our field to lab comparisons suggest that some of the exceptional variability we find in heat tolerance in the field may be due to physiological status or environmental conditions. Our data further highlight the strong effect humidity plays in thermal resilience and the need to consider humidity and temperature interactions in future work. Our results identify points of vulnerability in these bees to consider when addressing decisions and policy regarding bumble bee conservation, especially in the context of climate change.

Thermal tolerance of species tracks their distribution

Martinet et al. (2015, 2021a), in comparing bumble bees across the globe, found differences in heat tolerance that reflected their respective ecoregions. Other work in montane regions have found that bumble bee altitudinal ranges are correlated more with cold tolerance than with heat tolerance (Pimsler et al., 2020). Large scale climate modeling lends support to heat vulnerability driving shifting ranges, range contraction, and species loss, suggesting natural variation in heat tolerance may be a reason that some species are particularly vulnerable (Kerr et al., 2015; Sirois-Delisle and Kerr, 2018; Soroye et al., 2020). We find a clear trend in inferred heat tolerance that matches the temperature of zones these species occupy. Species with higher mean THS have more southerly median latitudinal distributions, warmer climatic distributions, and prefer valleys to forested and higher elevation habitats. Specifically, we found that *B. griseocollis* and *B. impatiens* were more heat tolerant and occur in the warmer latitudes/climates and in Pennsylvania valleys, while the higher latitude forest-restricted species *B. vagans/sandersoni* were the least heat tolerant, and *B. bimaculatus* and *B. perplexus*, which have been found in edge habitat between these zones and were intermediate in their climatic and distributional means had more intermediate heat tolerance. Bumble bees may also vary in nesting position relative to the ground surface, which can impact the ability to escape heat and the overall exposure to ambient temperatures. For the species studied here, *B. impatiens* is a primarily underground nester, *B. bimaculatus*, *B. vagans*, and *B. perplexus* are thought to be mostly underground nesters with some surface nesting, and *B. griseocollis* is primarily a surface nester (Plath, 1922, 1927, 1934, Husband, 1977), thus *B. griseocollis* would have nests that are less buffered from heat waves. *B. griseocollis* did show slightly higher heat tolerance than *B. impatiens*, which was not well matched by differences in the thermal occupancy between them.

These results suggest that some species in a community are more vulnerable to heat stress than others, and that the increased frequency and duration of heat events under climate change may disproportionately impact bumble bee species with cooler climatic distributions that typically inhabit forests and more northerly latitudes. For example, the relatively high heat tolerance of *B. impatiens* and *B. griseocollis* may be one reason these species' populations are increasing proportionally in the Eastern U.S. and Midwest in the last several decades (Averill et al., 2021; Jacobson et al., 2018; Novotny et al., 2021) compared to the species that occupy cooler areas. *B. vagans* and *B. sandersoni* may be especially impacted since they not only have cooler climatic distributions, but have suffered recent population declines in parts of their ranges (Colla et al., 2012; Grixti et al., 2009; Jacobson et al., 2018). These results can help inform conservation management decisions by prioritizing species that are particularly sensitive to extreme heat events and are already of conservation concern.

The environment influences variance in thermal tolerance

We found that wild bees have higher tolerance to hyperthermic stress than bees reared in the lab (with the exception of *B. griseocollis*), indicating that environmental conditions may be involved in heat stress resilience. In contrast, Gonzalez et al. (2022) found lab-reared bumble bees to have slightly higher thermal tolerance. Although it would require further testing, lab-reared bees were fed honey bee-collected pollen which did not allow the bees to seek out their nutritional optima. For example, honey bee pollen is lower in protein:lipid ratios compared to what bumble bees obtain when foraging in the wild (Vaudo et al., 2018). This could have reduced food quality and have negative effects on heat resilience at the colony (Vanderplanck et al., 2019) and individual (Quinlan et al., 2023b) level. Other factors besides nutrition may also vary between field and lab, such as pathogen levels, optimal hydration, and ability to attain optimal

nest conditions: these factors need further study to fully understand their impact on thermal tolerance. Furthermore, lab reared bees may have included nurse bees, while wild-collected bees are only foragers. While wild bees will have experienced greater temperature fluctuations during their lifetimes than lab-reared bees, recent studies demonstrate that prior experience of thermal stress does not result in increased thermal tolerance through priming (Quinlan et al., 2023b; Sepúlveda and Goulson, 2023).

Sex and caste impact thermal tolerance

Across all species, and when analyzing *B. impatiens* alone, queens had lower THS than workers and males. The relatively high sensitivity of queens to hyperthermic stress in the lab appear to make them particularly vulnerable to extreme heat compared to workers and males. This effect, however, was only observed in our water bath trials where relative humidity was 90%. At 70% RH, queens were not different than workers, indicating an interaction between caste and humidity.

In our assays, males were slightly less heat tolerant than workers, although the significance was marginal, and this showed a trend towards being dependent on species. Prior work found no difference in CT_{max} between males and workers in three species in the Rocky Mountains (Oyen et al., 2016) and no differences in CT_{max} between worker and males among 4 *Bombus* species native to Columbia (Gonzalez et al., 2022). There is temporal overlap between male and queen emergence, both leaving the nest in the heat of late summer, putting them at similar risk of encountering the same extreme heat events. While males were less sensitive to heat stress (had higher THS) than queens, sperm viability has been documented to decline in male bumble bees exposed to elevated temperatures that fall far below the temperatures tested here

(Campion et al., 2023; Martinet et al., 2021b), thus cross-generational effects must also be considered in their heat adaptation and vulnerability.

Age effects on thermal tolerance depend on caste

Our *B. impatiens* trials show that queens differ in their thermal tolerance by age. Four-day-old queens are the most vulnerable to heat stress, followed by a steady increase in THS as they age until around 12-days where their levels reached the higher levels typically observed for workers, after which they decline to the levels we typically observed in wild queens/queens. This suggests that queens change physiologically as they age, which impacts their response to heat stress. Oyen and Dillon (2019) similarly found that 4-day-old workers were less heat tolerant than 3 or 7-day-old workers, suggesting this may be a vulnerable stage. The peak of heat tolerance in queens at 12 days is intriguing in its biological correlates: queens are thought to leave the natal nest to mate and find a hibernaculum at 7-12-days-old (Tasei et al., 1998; Treanore et al., 2021) and then likely enter cooler substrates for diapause thereafter. Thus, thermal tolerance may align with the period when queens are most exposed to thermal stress.

We have also expanded on data by Oyen and Dillion (2018) by examining the effects of age in older worker bees. We found no effect of age between 7- and 14-day olds on heat tolerance, despite 14-day olds being near the point of mortality (only one 21-day-old worker survived and was tested, but it had a similar thermal tolerance to 7- and 14-day olds).

Ovary activation state impacts thermal tolerance

One factor that may make queens more vulnerable to heat is their investment in reproduction. Queens increase ovary activation 1-2 weeks after diapause or CO_2 narcosis (Amsalem et al., 2015a). Ovary activation in queens is associated with physiological changes

such as increases in juvenile hormone (JH), ecdysteroids, and body fat metabolism (Amsalem et al., 2015a; Bloch et al., 2000). These hormones have been shown both to respond to temperature (González-Tokman et al., 2020) and regulate thermal tolerance stress responses in insects (e.g., in *D. melanogaster,* Gruntenko and Rauschenbach, 2008). Our data did not support that shifting to a physiological state akin to diapause influences thermal tolerance, as THS was not significantly different between 2-day and 7-day post CO_2 narcosis-treated queens and controls. It did, however, support that shifts in ovarian activation and brood production post-narcosis impact thermal tolerance.

Body size effects are context-dependent

Body size in principle, given the surface to mass ratio and ability to dissipate heat, should relate to thermal tolerance, with smaller bees better able to handle the heat. Prior research in bumble bees have found mixed effects of body size on thermal tolerance, from no effect of body size to effects in some species (Table 13). We found larger body size was associated with lower THS for wild *B. impatiens* workers but no effect of body size on lab-reared workers. However, since we randomly selected workers from lab colonies for our trials, we likely picked some bees that were nurse bees that are restricted to in-nest activities and some that are "foragers", while only foragers were sampled in the wild. Also, in line with nurse bees being generally smaller than foragers (e.g., Holland et al., 2021), wild workers were larger on average than lab-reared workers and the smallest individuals were from lab-reared individuals. Our findings could reflect interactions of size with ovarian activation/physiology of workers with different tasks (e.g., foragers and nurses) and nutritional state. Foragers may also be healthier bees, thus allowing better assessment of effects of body size without confounding factors (nutrition and division of labor). These correlations suggest that declines in body size of collected bumble bees over time

(Gerard et al., 2020, Nooten and Rehan, 2020) should consider potential roles of climate in this shift.

Humidity impacts thermal tolerance

Prior studies on bumble bee thermal tolerance have focused on temperature without mentioning potentially confounding effects of humidity (Gonzalez et al., 2022; Hamblin et al., 2017; Maebe et al., 2021; Martinet et al., 2021a; Oyen et al., 2016; Oyen and Dillon, 2018; Pimsler et al., 2020). We found that humidity can have strong effects on thermal tolerance and thus that humidity needs to be carefully controlled in these thermal experiments. Heat tolerance declined with increasing humidity (20-70-90% RH) for both workers and queens, with the effects being most notable at the highest humidity levels. A likely possibility for this association is that as air becomes more saturated at higher humidity levels, bees are less able to use evaporative cooling to bring down their body temperature. While convective cooling is thought to be the primary means by which bumble bees cool themselves, such as through shunting of hemolymph to dissipate heat through the abdomen (Heinrich, 2004), evaporative cooling is another means by which some insects survive otherwise lethal temperatures (Prange, 1995). Evaporation occurs through tracheal ventilation and in bees cooling through mouth regurgitation of nectar is purported (Heinrich, 2004; Prange, 1996). Evaporative cooling has been observed to be more efficient in dry air than in saturated air in a number of insects, including bumble bees (Church, 1960; Prange, 1996). Bumble bee wet body mass has also been found to be more related to heat tolerance than dry mass, suggesting hydration may be important to thermal tolerance in these bees (Martinet et al., 2021a). The interaction between humidity and caste, whereby the reduced thermal tolerance of queens was supported only at higher humidity, may suggest that queens rely

on evaporative cooling more than workers. Given these strong effects, there is a need for research on the physiological effects of humidity on thermal tolerance.

Conclusion

Our data support heat tolerance as a factor impacting where species can occur, both in terms of geographic ranges and habitat preferences, suggesting these bees are limited in part by heat sensitivity and this may play a role in their declines. We also show that queens are more vulnerable to heat stress than workers and males and especially when they are newly emerged, have active ovaries, and are producing brood. This supports a role of physiological and developmental state in influencing thermal response but also points to a bottleneck of climate vulnerability in the founding queen. The observed effect of body size in wild foragers suggests that selection on body size should be considered in the context of climate warming. Finally, we show that humidity has a strong effect on thermal tolerance suggesting that humidity and temperature need to be considered together in assessing climate impacts. Future research on thermal impacts in these bees should assess if adaptation is driving distant populations of the same species towards variable responses to heat stress, mechanisms behind how humidity and differences in physiology influence thermal tolerance, and the role of landscape health, such as pathogens and nutrition, in thermal resilience. Our work will be foundational to these future studies and will contribute to devising conservation strategies for bumble bees that continue to experience more extreme temperatures.

Figures

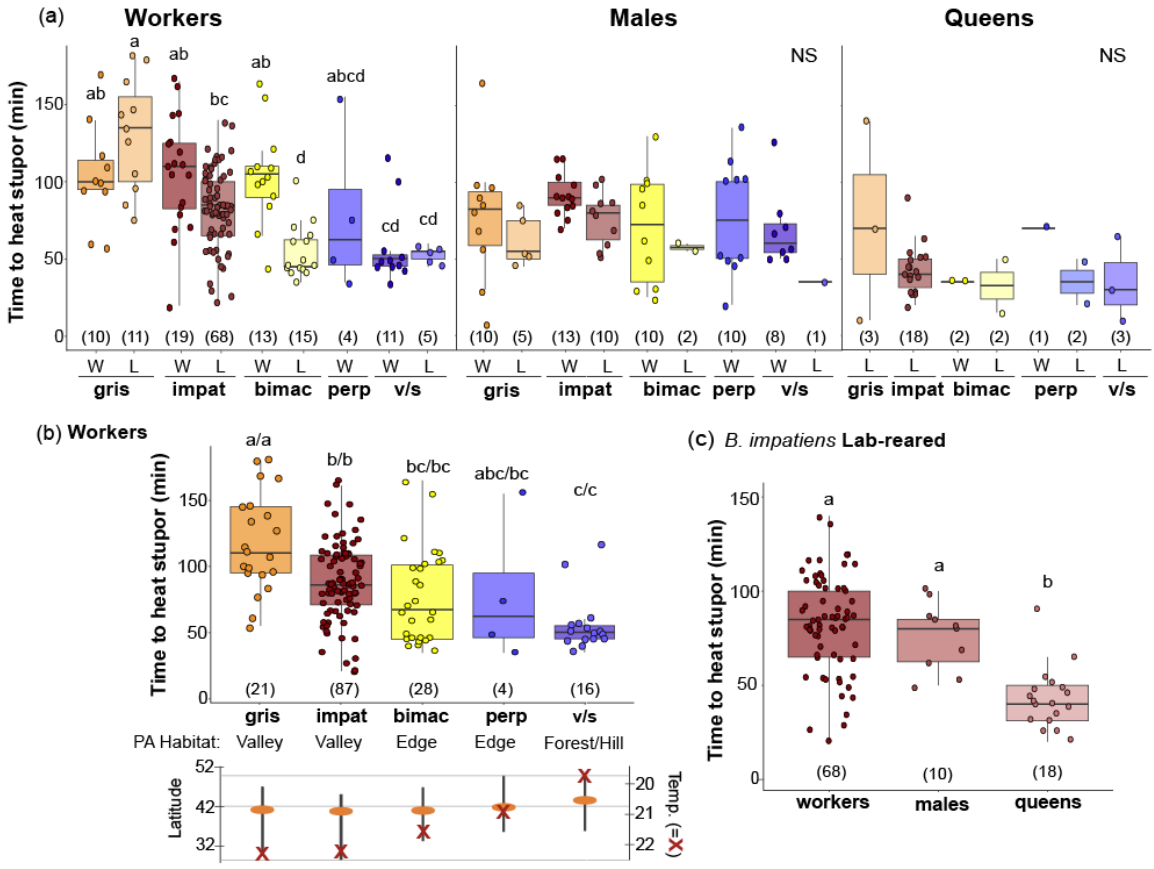


Figure 22. Thermal tolerances (Time to heat stupor, THS) of different bumble bee species, castes, and in wild vs. lab-reared bees. **(a)** Interspecific differences in THS across all variables, highlighting differences by species, sex/caste, and between wild (W) vs. lab-reared (L) bees for each caste-species combination. For ease of viewing, significance letters in 1a represent pairwise differences among species/origin (Tukey HSD) within each caste. **(b)** Interspecific differences among workers (wild and lab-reared bees pooled) highlighting significance values and how THS of species directly relates to their latitudinal averages of GBIF records, mean temperature (°C) across this range for June, July, and August (indicated with Xs), and habitat preferences across a

forested hill to open and developed valley landscape in Pennsylvania (Gratton et al., 2023). Significance letters on the left of the slash represent pairwise differences (Tukey HSD) between workers of different species and letters on the right of the slash represent pairwise differences (Tukey HSD) between species from the full model, which includes workers, males, and queens in the analysis. **(c)** Sex/caste differences in lab-reared *B. impatiens*, which had the best sample sizes to observe caste differences, and their significance. Sample sizes for each condition are indicated in parentheses. gris = *B. griseocollis*, imp = *B. impatiens*, bimac = *B. bimaculatus*, perp = *B. perplexus*, v/s = *B. vagans/sandersoni*.

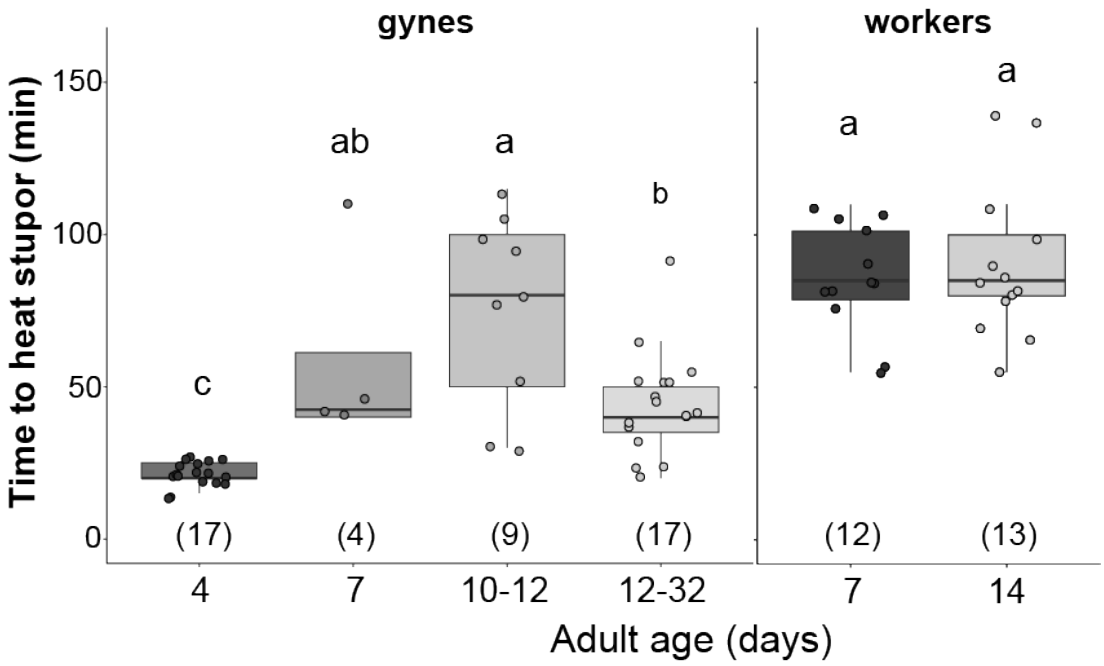


Figure 23. Differences in mean Time to Heat Stupor relative to age in *Bombus impatiens* queens and workers. Individuals are age-staged from commercially reared *B. impatiens* colonies. Significance letters represent differences within each caste (queens or workers). Numbers in parentheses are sample sizes. min = minutes.

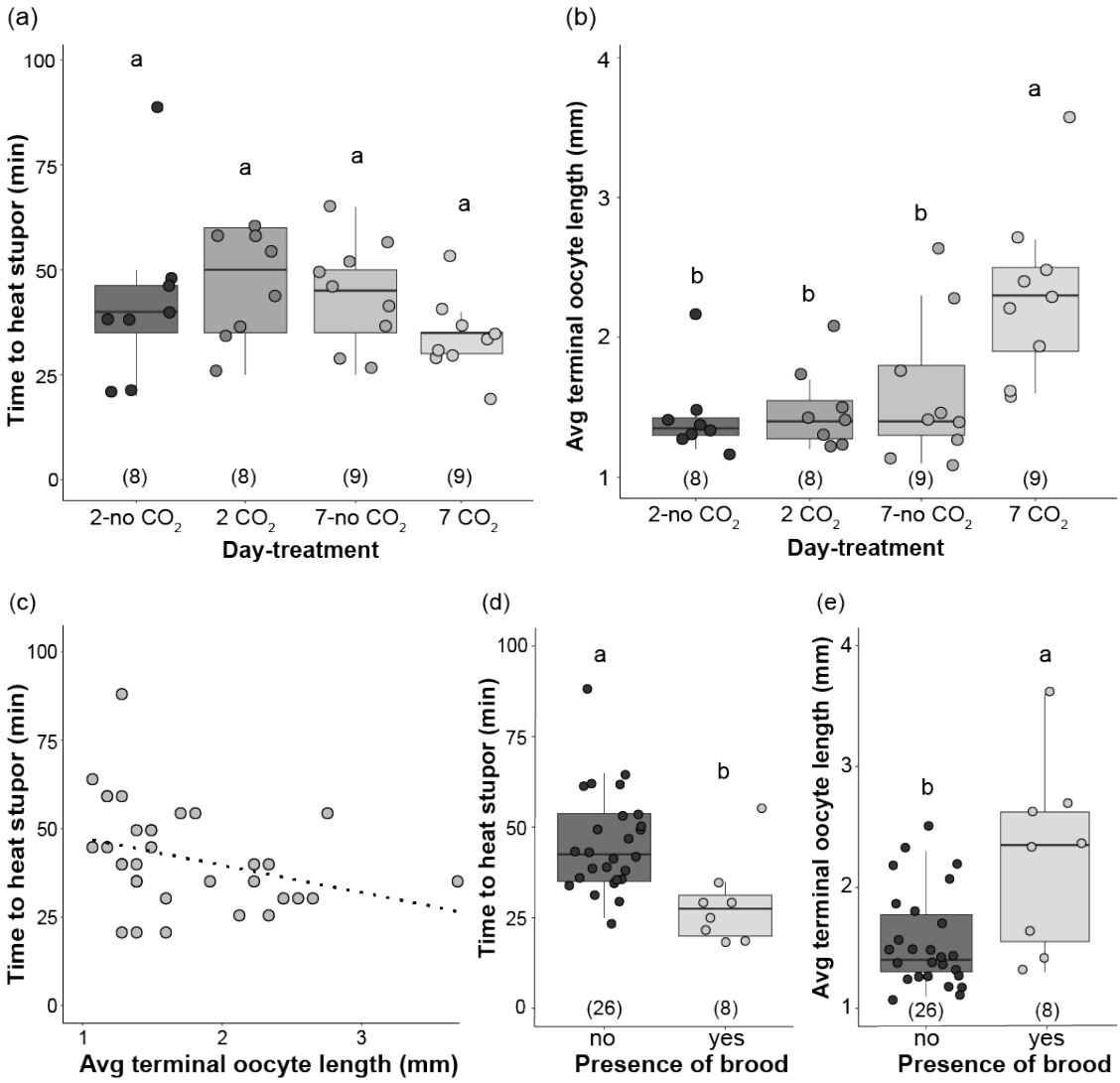


Figure 24. Effect of queen physiology (diapause condition and ovary activation) on Time to Heat Stupor (THS). **(a)** Effect of CO_2 narcosis treatment and age on mean THS in commercially reared *Bombus impatiens* queens. CO_2 narcosis treated bees represent a physiological state akin to having gone through diapause (spring queens) while those without it represent the state of pre-diapause fall queens. 2-day treatments represent an age typically too early for ovaries to have developed and 7 days should be sufficient to allow ovary activation for diapaused queens. **(b)** Average terminal oocyte length (a measure of degree of ovary activation) by CO_2 treatment,

showing that 7-day, CO_2 treated queens had greater ovarian activation. **(c)** THS regressed against ovary activation across all CO_2 treated bees. CO_2 was administered to queens aged 12-21 days (adjusted $R^2 = 0.077$, $p = 0.062$). **(d)** THS regressed against presence/absence of brood, showing that queens with brood have lower THS than queens without brood. **(e)** Average terminal oocyte length regressed against presence/absence of brood, showing that presence of brood is positively associated with ovary activation. Sample sizes for each condition are indicated in parentheses.

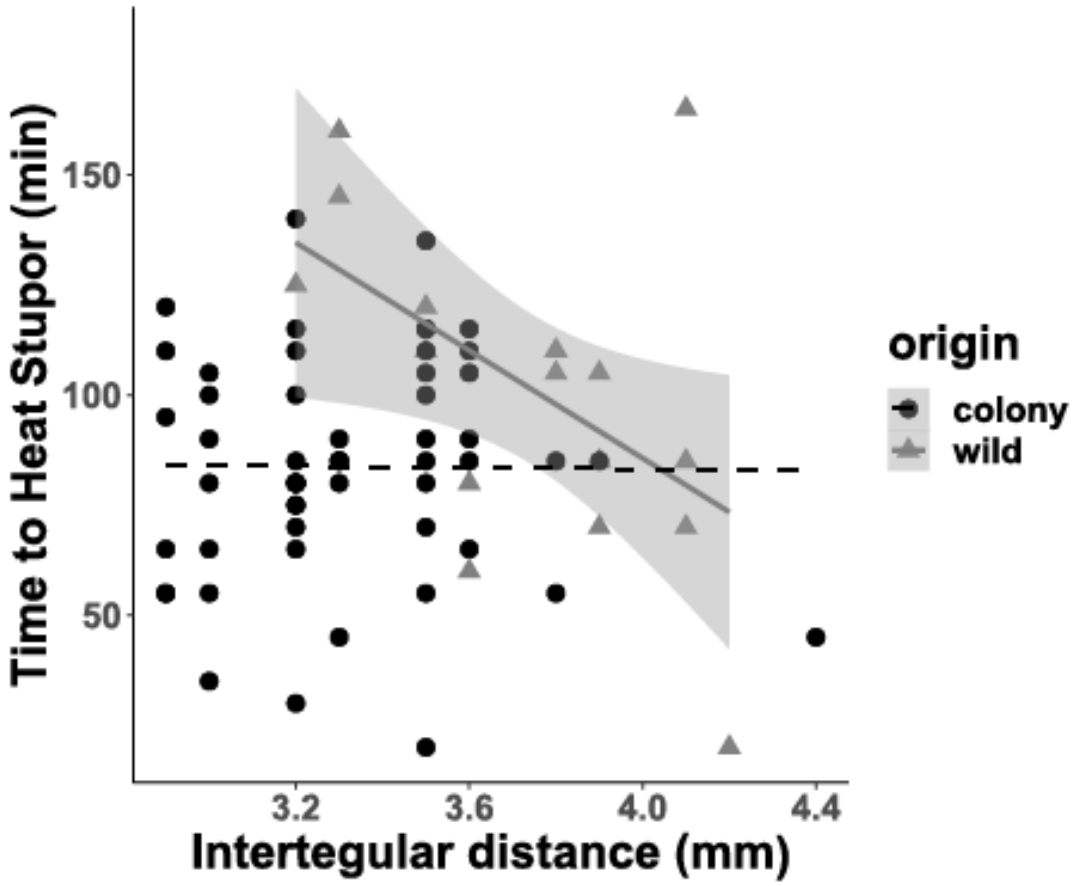


Figure 25. Time to Heat Stupor regressed against body size (intertegular distance in millimeters) for wild (triangles) and lab-reared (circles) *Bombus impatiens* workers. THS was correlated with body size in wild workers (adjusted $R^2 = 0.26$, $p = 0.0363$) but not in lab-reared workers (adjusted $R^2 = -0.0188$, $p = 0.95$).

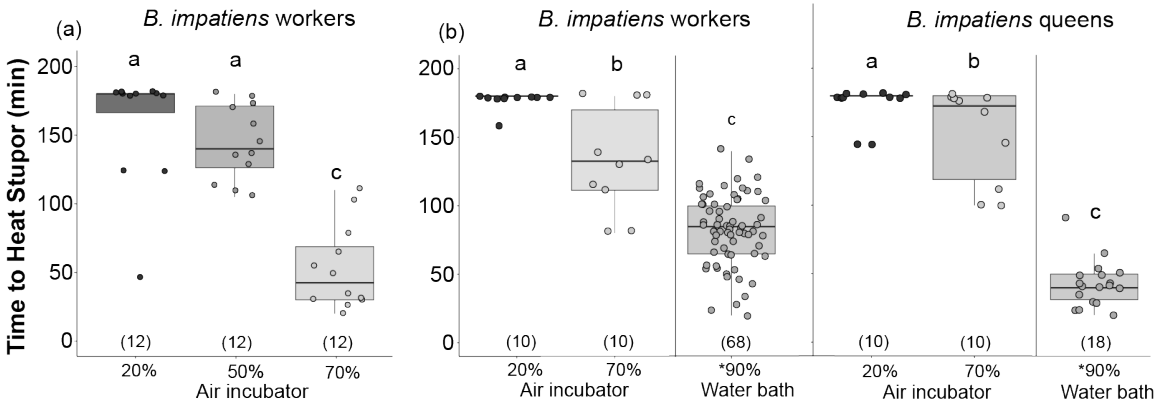


Figure 26. Time to Heat Stupor (THS) relative to humidity for commercially reared *Bombus impatiens* workers and queens. (a) Experiment 1 with workers. (b) Experiment 2 with both workers and queens. Significance from Tukey tests is indicated. Sample sizes for each condition are indicated in parentheses. *The 90% condition involved water baths while the remainder of the treatments were performed in air incubators. Differences in results by experiment are likely due to the way temperature was calibrated for each experiment which resulted in slightly higher temperatures for Experiment 1.

Tables

Table 13. Summary of the literature on bumble bee heat tolerance for the parameters examined here. Provided are the parameters studied, whether or not there was an effect ("Eff?") on heat tolerance, the bumble bee species studied, how the parameters relate to heat tolerance, and the citation of the study.

Parameter	Eff.?	*Bombus* spp.	Relationship	Reference
Species	Yes	39 spp. From 3 continents	Higher THS in species from warmer habitats	Martinet et al., 2021a
	Yes	*huntii, bifarius, sylvicola*	CT_{max} higher in *B. huntii* than *B. sylvicola*	Oyen et al. 2016
	No	*hortulanus, funebris, pauloensis, rubicundus*	CT_{max} did not differ among these high-elevation Colombian species	Gonzalez et al., 2022
	Yes	5 spp. from Europe	THS was higher in Boreo-alpine than arctic species	Martinet et al., 2015a
	Yes	10 spp. from Belgium	THS was higher in *B. lucorum* and *B. terrestris* than other species	Zambra et al., 2020
	Yes	5 N. American spp.	CT_{max} relates to several climate variables	Gonzalez et al., 2022
Latitude	No	*vosnesenskii*	CT_{max} invariant from queens originating at different latitudes	Pimsler et al., 2020
Altitude	Yes	*huntii, bifarius, sylvicola*	Low altitude species had higher CT_{max}	Oyen et al. 2016
	No	*hortulanus, funebris, pauloensis, runicundus*	CT_{max} did not differ between low- and high-elevation species	Gonzalez et al., 2022
Body Size/mass	No	*impatiens*	CT_{max} not different by body mass in lab-reared workers	Oyen & Dillon, 2018
	Yes	*huntii, bifarius, sylvicola*	CT_{max} increased with body mass in *B. huntii*	Oyen et al. 2016
	No	*B. terrestris (3 subspp.)*	CT_{max} not correlated with body size in lab-reared workers or queens	Maebe et al., 2021
	No	*hortulanus, funebris, pauloensis, rubicundus*	CT_{max} not correlated with ITD comparing all sexes and species	Gonzalez et al., 2022
	Yes	39 spp. From 3 continents	Body mass weakly explained variance in THS in wild males	Martinet et al., 2021a
	No	10 spp. from Belgium	THS was not related to body size of wild bees (ITD)	Zambra et al., 2020
	No	*B. terrestris audax*	CT_{max} did not relate to body mass in lab-reared workers	Sepúlveda & Goulson, 2023

Caste/Sex	No	*terrestris (3 subspp.)*	CT_{max} not correlated with caste (workers vs queens)	Maebe et al., 2021
	No	*huntii, bifarius, sylvicola*	CT_{max} did not differ between males and workers	Oyen et al. 2016
	No	*hortulanus, funebris, pauloensis, rubicundus*	CT_{max} did not differ between males and workers	Gonzalez et al., 2022
Age	Yes	*impatiens*	CT_{max} was lower in 4-day-old than 3- and 7-day-old worker bees	Oyen & Dillon, 2018
Wild vs. Lab	Yes	*pauloensis*	CT_{max} was higher and thermal breadth greater for lab-reared bees	Gonzalez et al., 2022

Table 14. ANOVA tables for significance in THS across conditions. (a) Statistics for the additive MLRM on THS across species, sex/caste, and origin. (b) Statistics for the interactive multivariate model on THS across species, sex/caste, origin, and their interactions.

Variable	**df**	**F value**	**Pr(>F)**
species	4	8.38	**<0.001*** **
origin	1	21.18	**<0.001*** **
sex/caste	2	21.6	**<0.001*** **
Residuals	248		
species	4	9.24	**<0.001*** **
origin	1	23.36	**<0.001*** **
sex/caste	2	23.82	**<0.001*** **
species*origin	4	4.56	**0.001** **
species*sex/caste	8	1.88	0.066·
origin*sex/caste	2	0.924	0.398
species*origin*sex/caste	3	2.476	0.062·
Residuals	231		

Table 15. Results from the post hoc Tukey analyses by species (all caste/sex and workers only), sex/caste (for all species and B. impatiens only), and queens of different ages. min=minutes; gris = B. griseocollis, bimac = B. bimaculatus, imp = B. impatiens, vs = B. sandersoni + B. vagans, perp = B. perplexus. 10 day is 10-12 day.

Comparison	**THS diff. [95% CI]**	**p-adjusted**	**THS diff. [95% CI]**	**p-adjusted**
All caste/sex			**Workers only**	
gris-bimac	25.79 [7.56,44.01]	**0.001**	40.18 [14.98,65.37]	**<0.001**
imp-gris	-16.10 [-31.26,-0.94]	**0.031**	-30.33 [-51.55, -9.11]	**0.001**
perp-gris	-25.16 [-49.24,-1.07]	**0.036**	-39.11 [-86.72,8.51]	0.161
vs-gris	-40.32 [-60.85,-19.79]	**<0.0001**	-61.92[-90.88,-32.96]	**<0.001**
imp-bimac	9.69 [-4.80,24.17]	0.355	9.85 [-9.11,28.81]	0.607
perp-imp	-9.06 [-30.45,12.34]	0.772	-8.78 [-53.41,35.85]	0.983
vs-imp	-24.21 [-41.50,-6.92]	**0.001**	-31.59 [-55.33,-7.85]	**0.003**
bimac-perp	0.63 [-23.04,24.30]	1.0	1.07 [-45.58,47.72]	1.0
vs-bimac	-14.53 [-34.56,5.51]	0.273	-21.74 [-49.09,5.61]	0.190
vs-perp	-15.16 [-40.64,10.33]	0.477	-22.81 [-71.60,25.98]	0.697
Caste-All spp.			**Caste-*B. impatiens***	
queen-male	-30.90 [-46.27,-15.52]	**<0.001**	-33.44[-55.29,-11.60]	**<0.001**
worker-male	10.50 [0.22,20.78]	**0.044**	6.14 [-12.61,24.90]	0.716
worker-queen	41.40 [27.41,55.38]	**<0.001**	39.59 [24.91,54.27]	**0.001**
Queen Age				
7-4day	37.57 [8.62,66.53]	**0.006**		
10-4day	54.38 [32.90,75.86]	**<0.001**		
12+-4day	22.64 [4.78,40.52]	**0.008**		
10-7day	16.80 [-14.50,48.11]	0.485		
7-12+day	14.93 [-43.88,14.03]	0.520		
10-12+day	31.73 [-53.21,-10.25]	**0.002**		

Conclusion

Understanding the underlying causes for the differential success among bumble bees can help support diverse and healthy bumble bee populations by informing conservation efforts and allowing for targeted monitoring of vulnerable species. In this thesis I assess variance in Pennsylvania bumble bee distribution, phenology, habitat, and floral visitation patterns (Chapter 1), pollen macronutrient preferences (Chapter 2), and thermal tolerance (Chapter 3) to better assess how these factors may contribute to their differential response to anthropogenic change. This research complements previous studies on phenology and habitat preferences in Pennsylvania bumble bee species (Gratton et al., 2023; P.H. Williams et al., 2014), providing more extensive and controlled data to understand species differences. It expands on previous thermal tolerance studies (Maebe et al., 2021; Martinet et al., 2021; Oyen & Dillon, 2018) by examining the degree to which several life history and environmental conditions respectively contribute to thermal tolerance and whether species within a geographic region vary in heat tolerance in ways that match and may explain their distributional differences. It tests theories developed in previous studies examining bee pollen macronutrient preferences (Vaudo et al., 2016; Vaudo et al., 2017; Vaudo et al., 2018; Vaudo et al., 2020) by assessing whether the higher protein requirements of *B. impatiens* apply to other bumble bee species and how site factors influence this. These studies also refine what is known about plant-pollinator visitation patterns in bumble bee species with a focus on how these bees differ in floral resource use when occupying the same location. My aim was to inform factors influencing the vulnerability of bumble bee species native to the eastern United States towards better solutions to support bumble bees globally.

In **Chapter One** I found that species differ in their habitat choice across Pennsylvania. *B. impatiens* was the most abundant in all habitat types (valley, forest, and edge) making up more than 50% of all bees in each habitat type, indicating this species is a habitat generalist as supported by previous work. *B. griseocollis* had the most representation in open valleys, *B. bimaculatus* in edge and forested sites, and *B. vagans, B. sandersoni, B. perplexus* were largely confined to edge and forest sites. Local phenology patterns among species reveal that species also vary in their phenological strategies. *B. griseocollis* and *B. bimaculatus* had early worker and male phenology and shorter seasonality, a factor that ultimately corresponds to smaller colony sizes. *B. impatiens* has an especially long seasonality, a factor that relates to later production of reproductives and larger colony sizes. For the first time, phenology data from co-occuring co-mimics *B. sandersoni*, *B. perplexus* and *B vagans* are reliably compared, revealing that *B. vagans* has a later phenology relative to *B. sandersoni* and *B. perplexus*, leading to proportional shifts in which of these co-occurring species is more abundant in different parts of the season and supporting that these species phenologically partition their local resources. These species also show some notable differences in their floral preferences. *B. impatiens* and *B. bimaculatus* had the most similar plant visitation (e.g. > 50% of visits to Fabaceae species), *B. vagans* had unusually high visitation to nonnative Asteraceae (40%), particularly on *Centaurea stoebe* (spotted Knapweed), and *B. griseocollis* specialized on milkweed, consistent with previous studies (Villalona et al., 2020). Overall, these differences show how each of these species occupies distinct temporal, floral and habitat niches that will impact how they respond to change.

In **Chapter Two** I found that species showed similar protein and lipid preferences across sites, all achieving ratios close to the 4:1 protein:lipid ratios previously found for *B. impatiens* (Vaudo et al., 2018), however, there were some minor observable differences in these ratios by species. While there were no differences in lipid collection between species, proteins showed a more notable difference across treatments. Thus, it is likely that these bees are prioritizing

regulation of protein intake more than lipid intake. Nevertheless, my results show that using field collected pollens to infer this may interfere with abilities to detect species specific preferences. The data support that bees will increase protein intake when given the opportunity. Bees attained higher amounts of protein than the sites provided on average, suggesting they seek out higher protein plants. Not all sites, however, offered as much available protein and as such these bees collected higher protein at the Arboretum at Penn State which had the highest floral resource availability. *B. impatiens* collected higher protein in Oklahoma compared to Pennsylvania, and what drove these higher protein values seemed to be this bee's constancy on the legume *Chamaecrista fisciculata* (partridge pea) even when this plant made up 50% or less of the available flowering plant species. *B. vagans* collected higher protein when the invasive thistle, *Carduus acanthoides* (plumeless thistle), was abundant with *B. vagans* showing floral constancy on this species. The species in decline, *B. pensylvanicus*, did not show any difference in pollen macronutrient collection from sympatric *B. impatiens*, suggesting it likely has similar nutritional needs, although more controlled assays in lab settings would be needed to assess inherent species differences.

Overall, these data suggest that efforts to support bumble bees should focus on ensuring that a diversity of highly visited plants are planted in restoration areas, including a large proportion of plants with high protein pollen. It is important that this is maintained throughout the growing season, when possible, including in early spring when foundresses are emerging. Considering that some bumble bees show a propensity to collect higher protein when the available protein in the landscape is higher, a substantial proportion of plants should be those with the highest protein levels. Many of the plants visited by these bumble bees are non-native. The role of non-native plants in these pollinator networks should continue to be investigated as well as which native species can be instead used to match the nutritional needs of bumble bees and other native pollinators within restoration areas. Partridge pea (Fabaceae) is native where it was found,

had an 82 – 100% visitation rate from bumble bees, and has very high pollen protein levels and P:L ratios (as was corroborated from plant visitation and protein data in this study). Wild lupine species (Fabaceae), which have been on the decline in Pennsylvania, and have high P:L ratios, should be considered as an alternative to some of the nonnative Fabaceae that were so commonly visited in my field studies (i.e., *S. varia, T. repens*). As nonnative thistles seem to be an important resource for *B. vagans*, a species that has shown declines in parts of its range, understanding the impact of their reduction or potential for use of natives instead will be of value. Of course, plants that are an important nectar resource should be included in these planting schemes, as nectar is the main source of carbohydrates for bees, and tongue length of bumble bee species should be considered when choosing nectar plants, as bees with longer tongues prefer to forage on plants with deeper corollas. Finally, ensuring high plant diversity will allow these bees to partition resources, ensuring every bee can meet their optimal nutritional needs.

Data from **Chapter 3** support heat tolerance as a factor impacting where species can occur, both in terms of geographic ranges and habitat preferences, suggesting these bees are limited in part by heat sensitivity and this may play a role in their declines. I also show that queens are more vulnerable to heat stress than workers and males, especially when they are newly emerged, have active ovaries, and are producing brood. This supports a role of physiological and developmental state in influencing thermal response but also points to a bottleneck of climate vulnerability in the founding queen. The observed effect of body size in wild foragers suggests that selection on body size should be considered in the context of climate warming. Finally, I show that humidity has a strong effect on thermal tolerance suggesting that humidity and temperature need to be considered together in assessing climate impacts.

Understanding how stressors impact bees is context dependent, as life history traits and distribution appear to have an influence on stress resiliency. I have shown that nine species of North American bumble bees differ in their distribution, geographic range, phenology,

abundance, habitat preference, climatic niche, thermal tolerance, and dietary breadth. A summary of species-specific management considerations based on these factors is outlined in Table 16. *B. impatiens* may be so successful and less in need of focused conservation because of its high heat tolerance, large colony size, long seasonality, and variable habitat needs. *B. vagans*, on the other hand, has strict habitat needs (forest) and is less heat tolerant, and therefore management considerations should include cool, forested habitat for this species and recognize temperature as a vulnerability. *B. griseocollis*, with its heat tolerance, surface nesting in open valleys, and specialization on milkweed, will be best supported in open grassy habitats where milkweed is abundant. Many of these factors remain unknown for many bumble bees thus requiring further research to guide management considerations.

These factors and stressors likely interact in novel ways in influencing the success of different bees. For instance, bees with dissimilar phenology will likely be impacted differently in altered landscapes where floral resources have been unevenly distributed, allowing some species access to preferred resources, but not others. Additionally, phenology, niche, and climatic warming may interact in impacting bees. Species that have an early colony reproductive phase produce queens that must diapause through the summer months. One such species is *B. bimaculatus:* as the climate continues to warm, *B. bimaculatus* queens could be exposed to unusually warm temperatures which have been shown to increase the metabolic rate of diapausing queens, negatively affecting their survival and post-diapause performance as they burn through fat reserves (Vesterlund et al., 2014), leading to changes in their distribution as parts of their historic range become inhospitable. Moreover, climate change has negative effects on range size in species that are unable to track warming (Sirois-Delisle & Kerr, 2018) and this may have a more severe impact on bees with limited distributions, less heat tolerance, and smaller populations such as *B. perplexus* and *B. sandersoni*. Finally, nutritional stress has been shown to impact thermal tolerance in bumble bees (Vanderplanck et al., 2019), and thus adequate floral

resources will likely play an important role in limiting the impacts of climatic warming. It is therefore recommended these factors are not studied in isolation but are used together in models to assess species conservation status and predict future vulnerability.

Further research should assess if adaptation is driving distant populations of the same species towards variable responses to heat stress and if these populations have different phenology that tracks the climate where they live. Comprehensive studies are needed to investigate the role of landscape health, such as nutrition, pathogens, pesticide use on thermal tolerance, the mechanisms behind how humidity and differences in physiology influence thermal tolerance, and how thermal tolerance influences rates of decline in vulnerable bees. Future work should investigate heat stress tolerance in the remaining North American bumble bees that have not been studied to date, although researchers in this field should come to an agreement on a single, standardized method that is easily repeatable so that different teams are able to compare results based on the same data metrics. It will also be important to understand differential responses in heat stress on foraging activity, as well as pollen and nectar collection rates, and how heat stress influences colony metrics such as worker size, development time, emergence rate, survival, colony growth rate, and sex ratios.

To gain a better understanding of species' macronutrient needs, laboratory assays that assess preferences in a controlled manner across species are needed. Field assays with more controlled habitat quality could be used to understand if landscapes are limiting these bees, and lab assays on the impacts of reduced protein could improve understanding whether these slight shifts matter to bee health and fitness outcomes. Furthermore, given these results, data is needed on whether planting higher protein plants supports more diverse bumble bee communities. It will also be important to study if pollen macronutrient preferences shift as species reach the limits of their climatic niche likely as a means to mitigate thermal stress. While my data supports similar protein availability seasonally, other studies suggest late season plant communities may have

reduced pollen protein concentrations (Quinlan et al., 2021; Quinlan & Grozinger, 2023). Longer term studies expanding into the fall should investigate how these preferences shift as the macronutrient availability changes throughout the growing season. Long term studies on colony health outcomes in response to different levels of protein, lipid, and P:L ratios can reveal the optimum nutrition for different species. Finally, more foraging data and pollen identification data is needed to discern what plants bumble bees are collecting pollen from (as opposed to nectar only) and to what level bees are partitioning pollen resources. These data will properly ensure that the appropriate floral resources are being provided when restoring and maintaining landscapes to support bee communities. Overall, this work will be foundational to future studies and will contribute to devising conservation strategies for bumble bees as they continue to be impacted anthropogenic stressors.

Tables

Table 16. Summary of species-specific management considerations based on conservation status (Status), habitat, seasonality, abundance, location nest site habitat (Col. Location), thermal tolerance (Thermal Tol.), average P:L, average protein (both from chapter 2 data), floral visitation preferences (Floral Use Notes). Management considerations discuss the type(s) of habitat that best supports each species. Thermal tolerance is based on mean time before heat stupor (in minutes) at 43˚C (data from Chapter 3). ? = data deficient.

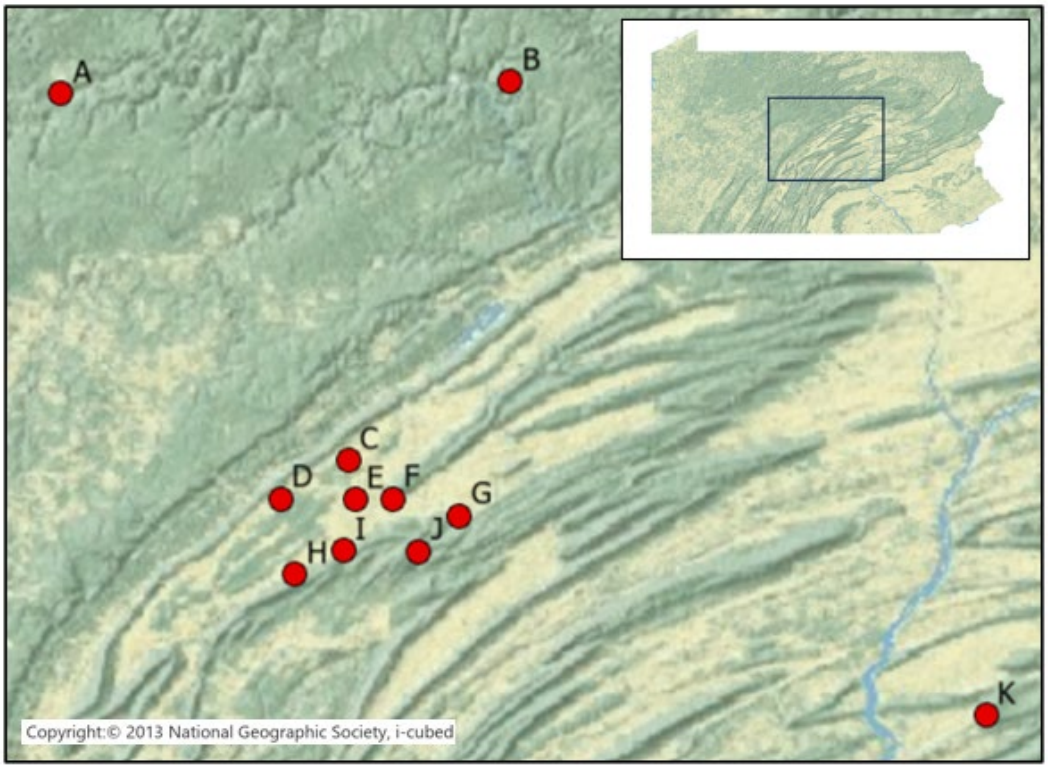
	B. impatiens	***B. bimaculatus***	***B. griseocollis***	***B. vagans***	***B. sandersoni***
Status (PA)	Stable/Increasing	Stable	Stable	Stable/Decreasing	Stable?
Habitat	Variable	Edge	Open Valley	Forested	Forested
Seasonality	Long	Short	Short	Long	Moderate
Abundance	High	Mod - High	Mod	Low	Low - Mod
Col. Location	Underground	Mostly underground	Mixed, mostly surface	Mixed, mostly underground	Mixed, mostly underground
Colony Size	Large	Small - Medium	Small	Small - Medium	Small - Medium
Thermal Tol.	High (81 min)	Moderate (71 min)	High (97 min)	Low (57 min)	Low (57 min)
Average P:L	4.78 ± 1.96 (PA) 5.96 ± 2.29 (OK)	4.71 ± 2.04	4.47 ± 1.59	4.63 ± 1.66	4.63 ± 2.13
Average Protein	295.51 ± 55.88 (PA) 430.87 ± 108.28 (OK)	312.08 ± 50.89	314.50 ± 57.24	324.2 ± 60.8	285.85 ± 58.03
Floral Use Notes	Most often on legumes – clover, crown vetch, partridge pea. Also, asters and mints	Most often on legumes - clover, crown vetch. Also, mints and penstemon	Specializes on milkweed plants (nectar only). Legumes (clover, crown vetch), mints, and asters	Preference for thistle and spotted knapweed	Legumes, mints, and Mountain laurel
Management Consideration	Will likely persist in most conditions	Will be more common in heterogenous habitat that is not too hot	Will be supported more in open grassy habitats with tussocks for nesting and milkweed but also found in/near forested areas (esp. when milkweed present)	Will be supported in areas with forest and trees and in cooler habitats	Will be supported in areas with forest and trees and in cooler habitats
	B. perplexus	***B. fervidus***	***B. pensylvanicus***	***B. terricola***	***B. ternarius***
Status (PA)	Stable/	Threatened?	Threatened/	Threatened	Stable?

	Decreasing		Extirpated?		
Habitat	Edge - Forest	Open Valley	Open Valley	Forested	Edge - Forest
Seasonality	Moderate	Moderate, late	Moderate, late	?	?
Abundance	Low	Very low	Very rare	Rare	Rare
Col. Location	Mixed, mostly underground	Mixed, mostly surface	Mixed, mostly surface	Underground	Mixed, mostly underground
Colony Size	Small - Medium	Medium	Medium	Medium	Medium
Thermal Tol.	Moderate – (72 min)	?	?	?	?
Average P:L	?	?	5.57 ± 2.72	?	?
Average Protein	?	?	405.23 ± 73.92	?	?
Floral Use Notes	Thistles, mints,	Long tongued	Long tongued	?	?
Management. Consideration	Will be supported in areas with forest/edge habitat and in cooler habitats	Will be supported more in open grassy habitats with tussocks for nesting, but also found in/near forested areas	Will be supported more in open grassy habitats with tussocks for nesting	As a more boreal species, will be supported by cooler and more heterogeneous habitat	As a more boreal species, will be supported by cooler and more heterogeneous habitat

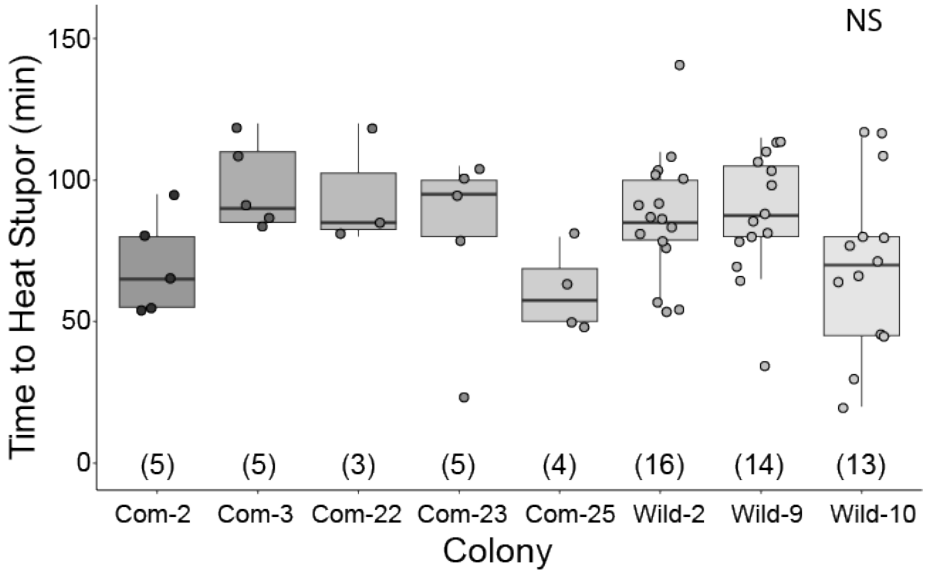
Appendix:

Chapter 3 Supplemental Materials

Figures



Supplemental figure 1. Map of collection sites for wild bees used in THS assays. Elk Country Visitor Center **(A)**, Hyner View State Park **(B)**, Bernel Road Park **(C)**, Circleville Park **(D)**, Penn State - University Park Campus **(E)**, Tom Tudek Memorial Park **(F)**, Colyer Lake **(G)**, The Russell E. Larson Agricultural Research Center at Rock Springs **(H)**, Pine Grove Mills **(I)**, Bear Meadows Natural Area **(J)**, Weiser State Forest **(K)**.



Supplemental figure 2. Comparisons of Time to Heat stupor in Bombus impatiens workers between colonies, including commercial colonies (n=5) and colonies (Com-) reared from wild-caught (Wild-) queens (n=3). There was no significant colony effect, although there were some differences in means that may have been significant with more sample size per colony. Differences were unrelated to whether bees came from commercial or wild-lab-reared colonies thus these bees are treated the same in analyses.

Tables

Supplemental table 1. All bees used in our cross-species analysis. Type refers to whether the bee was captured from the wild or reared from a colony in the lab. Each colony number represents a unique colony for the associated species. Source indicates the site that bees were obtained from (in the case of wild bees used directly in my thermal assays and wild queens used for rearing) or whether they were purchased commercially. A separate THS analysis was conducted on just workers for these species (Figure 22b, Table 14). A separate analysis was also conducted on queens, workers, and males in B. impatiens to assess the effect of caste/sex on THS (Figure 22c, Sup. Table 14). Q = queen, W = worker, M = male, bimac = B. bimaculatus, gris = B. griseocollis, imp = B. impatiens, perp = B. perplexus, v/s = B. vagans/sandersoni. *Indicates that the natal colony for these bees is unknown.

Species	Type	Source	Q	W	M
bimac	col.4	Circleville Park	1		
bimac	col.2	Circleville Park		5	
bimac	col.10	Coyler Lake		5	2
bimac	col.11	Coyler Lake		5	
bimac	col.8	Tom Tudek Memorial Park	1		
bimac	wild	Bear Meadows Natural Area		4	1
bimac	wild	Benezette Elk Center	1		
bimac	wild	Bernel Road Park		2	1
bimac	wild	Circleville Park		1	
bimac	wild	Coyler Lake		1	2
bimac	wild	Penn State (UP)		2	4
bimac	wild	Pine Grove Mills	1	1	2
bimac	wild	Weiser State Forest		2	
gris	col.4	Tom Tudek Memorial Park		2	
gris	col.5	Tom Tudek Memorial Park	1	9	5
gris	col.6	Tom Tudek Memorial Park	1		
gris	col.7	Tom Tudek Memorial Park	1		
gris	wild	Bear Meadows Natural Area			4
gris	wild	Bernel Road Park		1	5
gris	wild	Coyler Lake		1	1
gris	wild	Rock Springs		3	
gris	wild	Hyner View State Park		4	
gris	wild	Penn State (UP)		1	

imp	col.5	Penn State (UP)	1		
imp	col.2	Penn State (UP)		16	
imp	col.9	Fairbrook Park		14	
imp	col.10	Hyner View State Park		12	5
imp	col*	wild		4	
imp	col.1	Commercial			5
imp	col.2	Commercial		5	
imp	col.3	Commercial		5	
imp	col.20	Commercial			
imp	col.22	Commercial	7	3	
imp	col.23	Commercial	10	5	
imp	col.25	Commercial		4	
imp	wild	Bear Meadows Natural Area		5	6
imp	wild	Circleville Park		1	
imp	wild	Hyner View State Park		5	
imp	wild	Penn State (UP)		6	5
imp	wild	Tom Tudek Memorial Park		2	2
perp	col.4	Hyner View State Park	1		
perp	col.5	Scotia Pond	1		
perp	wild	Bernel Road Park			2
perp	wild	Circleville Park		2	1
perp	wild	Coyler Lake			2
perp	wild	Penn State (UP)		1	
perp	wild	Pine Grove Mills	1	1	1
perp	wild	Rock Springs			3
perp	wild	Weiser State Forest			1
v/s	col.2	Black Moshannon State Park		5	
v/s	col.5	Hyner View State Park	1		
v/s	col.6	Hyner View State Park	1		
v/s	col*	wild	1		1
v/s	wild	Benezette Elk Center			1
v/s	wild	Bernel Road Park		1	
v/s	wild	Pine Grove Mills		1	2
v/s	wild	Rock Springs		9	2
v/s	wild	Weiser State Forest			3

Supplemental table 2. Number of B. impatiens workers and queens assayed for each THS analysis. Age-Q = queen age trials, Age-W = worker age trials, CO_2 Trials = CO_2 narcosis, ovary activation, and presence of brood analyses for queens, RH1 = initial humidity trial with air incubators with workers, RH2-Q = second humidity trial with queens, RH2-W = second humidity trial with workers, Col. = colony effect analysis among lab-reared workers, Sz. = body size analysis among wild and lad-reared workers. Source: BM = Bear Meadows Natural Area, CV = Circleville Park, HV = Hyner View State Park, PS = Penn State – University Park Campus, TP = Tom Tudek Memorial Park. *Indicates that the natal colony for these bees is unknown.

Type	Source	Age-Q				Age-W		CO_2 Trials				RH1			RH2-Q			RH2-W			Col.	Sz.
		4	7	10	12+	7	14	2C	2T	7C	7T	20	50	70	20	70	90	20	70	90		
wild	BM																					4
wild	CV																					1
wild	HV																					4
wild	PS																					6
wild	TP																					2
col.5	wild																1					
col.2	wild					7	3													16	16	16
col.9	wild					5	4													14	14	13
col.10	wild						2					3	3	3						12	12	12
col.*	wild						4													4	4	4
col.1	comm											3	3	3								
col.2	comm											3	3	3						5	5	5
col.3	comm											3	3	3						5	5	5
col.20	comm																	10	10			
col.22	comm	4		3	7			3	4	4	4						7			3	3	
col.23	comm	5	4	6	10			5	4	5	5				10	10	10			5	5	
col.25	comm	8																		4	4	

Chapter 3 Author contributions

Cody Feuerborn - Conceptualization (supporting), data curation (lead), formal analysis (lead), methodology (equal), investigation (lead), project administration (supporting), software (lead), writing - original draft preparation (lead), writing – review and editing (supporting). **Gabriela Quinlan** - Formal analysis (supporting), software (supporting), writing – review and editing (supporting). **Rachael Shippee** - Methodology (supporting), data curation (supporting), formal analysis (supporting), investigation (supporting), writing – review and editing (supporting). **Tori Strausser** - Methodology (supporting), data curation (supporting), investigation (supporting), writing – review and editing (supporting). **Tatiana Terranova** - Investigation (supporting), writing – review and editing (supporting). **Christina M. Grozinger** - Conceptualization (supporting), funding acquisition (supporting), project administration (supporting), methodology (supporting), writing – review and editing (supporting). **Heather M. Hines** - Conceptualization (lead); funding acquisition (lead), methodology (equal), project administration (lead), visualization (supporting), writing – original draft preparation (supporting), writing – review and editing (lead).

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